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ORIGINAL ARTICLE

Muscle satellite cells and fibro-adipogenic progenitors from muscle contractures of children with cerebral palsy have impaired regenerative capacity

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Abstract

Aim: To evaluate the mechanosensitivity of muscle satellite cells (MuSCs) and fibro-adipogenic progenitors (FAPs) in cerebral palsy (CP) and the efficacy of the drug verteporfin in restoring cells' regenerative capacity.

Method: Muscle biopsies were collected from six children with CP and six typically developing children. MuSCs and FAPs were isolated and plated on collagen-coated polyacrylamide gels at stiffnesses of 0.2 kPa, 8 kPa, and 25 kPa. Cells were treated with verteporfin to block mechanosensing or with dimethyl sulfoxide as a negative control. MuSC differentiation and FAP activation into myofibroblasts were measured using immunofluorescence staining.

Results: Surprisingly, MuSC differentiation was not affected by stiffness; however, stiff substrates resulted in large myonuclear clustering. Across all stiffnesses, MuSCs from children with CP had less differentiation than those of their typically developing counterparts. FAP activation into myofibroblasts was significantly higher in children with CP than their typically developing peers, but was not affected by stiffness. Verteporfin did not affect differentiation or activation in either cell population, but slightly decreased myonuclear clustering on stiff substrates.

Interpretation: Cells from children with CP were less regenerative and more fibrotic compared to those of their typically developing counterparts, with MuSCs being sensitive to increases in stiffness. Therefore, the mechanosensitivity of MuSCs and FAPs may represent a new target to improve differentiation and activation in CP muscle.

Cerebral palsy (CP) describes a group of permanent disorders of the development of movement and posture, which has been attributed to non-progressive disturbances that occur in the fetal or infant brain.¹ Disturbances to motor neurons impair signaling to skeletal muscle, resulting in pathological development.² Muscles in children with CP are often spastic and form contractures, limiting range of motion and mobility.² A defining characteristic of contractures is an excessively high stiffness.^{2, 3} While the influence of substrate stiffness on stem cell migration, proliferation, and

differentiation was reported previously,^{4, 5} to the best of our knowledge, the impact of high stiffness on stem cells in the context of contractures is unknown. Stem cells are responsible for creating muscle tissue structures during development. Understanding the effect of stiffness on their development provides insights into the development of contractures.

Muscle satellite cells (MuSCs) and fibro-adipogenic progenitors (FAPs) are two key cell populations that work together to form muscle during development and regeneration, and maintain homeostatic muscle mass.^{6, 7}

This original article is commented by Domenighetti on pages 9–10 of this issue.

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Abbreviations: αSMA, alpha smooth muscle actin; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; FAP, fibro-adipogenic progenitor; MuSC, muscle satellite cell; YAP, Yes-associated protein.

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MuSCs are the cells that differentiate into muscle fibers. They activate after injury and during muscle development to form muscle fibers, which combine together to form whole muscles.^{7,8} FAPs are cells that reside in the muscle, but do not directly form muscle. Rather, they deposit extracellular matrix to provide structure and support to the muscle. FAPs also signal to MuSCs to promote muscle formation.^{6,9,10} In disorders such as CP, the signaling to these cells is disrupted and leads to pathological development. The stem cell pool is depleted and cells have lower myogenic capacity in contractured CP muscle compared to the typically developing MuSC population.^{11–13} MuSCs from the early development of contractures in CP show an enhanced fusion index, indicating an overall alteration of the MuSC phenotype in CP.¹⁴ In diseased conditions, FAPs are chronically activated into myofibroblasts, leading to excessive extracellular matrix deposition known as fibrosis.^{9,15,16} FAP population levels are similar in children with CP and typically developing peers; however, little is known regarding the activation of FAPs in the contractures of children with CP.¹⁷

Previous reports established that MuSCs and FAPs isolated from mice are mechanosensitive.^{15,18–20} MuSCs differentiate optimally on stiffness mimicking healthy muscle, while FAPs activate into myofibroblasts more frequently on stiff substrates.^{15,20} Given that excessive stiffness is a defining feature of fixed muscle contractures, the mechanosensitivity of stem cells in the stiff environment has profound implications for muscle development. An increase in stiffness as seen in fibrotic conditions impaired murine MuSC differentiation and increased FAP activation into myofibroblasts.^{15,18} Yet, to the best of our knowledge, the mechanosensitivity of these cells in the context of muscle contractures in humans is not known.

If mechanosensitivity has a key role in impairing muscle development in contracture, then blocking mechanosensing could be a viable therapeutic target. Verteporfin is a U.S. Food and Drug Administration-approved drug used in photodynamic therapy that effectively blocks cellular mechanosensing. Verteporfin works by preventing Yes-associated protein 1 (YAP1) from translocating to the nucleus. When a cell senses a stiff substrate, YAP translocates to the nucleus to induce transcriptional changes, including increasing myofibroblast activation in fibroblast cells like FAPs and activating MuSCs.^{21–24} Verteporfin reduces myofibroblast activation in murine FAPs and other fibroblast-like cells on fibrotic-like stiffnesses, although its use in CP has yet to be investigated.^{15,21} To address this knowledge gap, we assessed the mechanosensitivity of stem cells from children with CP and typically developing peers and the ability of verteporfin to rescue cells in children with CP.

METHOD

Muscle collection

Ethical approval for this study was obtained through Shriners Children's and the WCG institutional review board (www.wcgirb.com). Samples were coded before receipt and given an identifier to protect patient identities.

What this paper adds

- Muscle satellite cells (MuSCs) showed impaired differentiation in children with cerebral palsy (CP).
- MuSCs formed large nuclear clusters on contracture-like stiffnesses.
- Fibro-adipogenic progenitors (FAPs) from children with CP exhibited higher myofibroblast activation.
- Verteporfin may reduce nuclear clustering but not FAP activation.

Informed written consent was obtained from parents and age-appropriate assent was obtained from participants. Participants were children with spastic bilateral (quadriplegic) CP undergoing hamstring or hip adductor longus lengthening surgery (Table 1). Typically developing children, children with no known neurological disorders, undergoing elective anterior cruciate ligament reconstruction with hamstring tendon autograft were recruited as controls, providing access to distal gracilis muscle tissue that was routinely discarded during surgery. Inclusion criteria included spasticity for patients with CP and an age range of 4 to 18 years for both cohorts. Biopsies (200–400 mg) were collected from the surgical sites and stored in phosphate buffered saline on ice until processing.

Tissue digestion

Biopsy tissue was digested using an enzyme mix (Miltenyi Biotec, Bergisch Gladbach, Germany) at 37°C for 1 hour under continuous rotation. Samples were mechanically digested using a tissue homogenizer for 30 seconds every 30 minutes. The digested solution was processed through a 70- μ m filter and hemolysis was induced using a red blood cell lysis solution (Miltenyi Biotec). Cell debris was removed using a debris removal solution (Miltenyi Biotec). Cells were frozen in 900 μ L of fetal bovine serum with 100 μ L of dimethyl sulfoxide (DMSO) in liquid nitrogen until it was time for cell isolation.

Cell isolation and culture

Individual cell types were isolated using magnetic-activated cell sorting. The cell solution was incubated with a blocking reagent; then, cells were labeled with CD140a (PDGFR α) magnetic beads (Miltenyi Biotec) to label the FAPs. The labeled cell solution was added to a magnetic-activated cell sorting column and separator (Miltenyi Biotec); FAPs were isolated using positive selection. The remaining cells were labeled with a satellite cell isolation kit (Miltenyi Biotec)

TABLE 1 Patient demographics.

Group	Muscle collected	Age, years	Ethnicity	Sex	GMFCS level
Typically developing	Gracilis	16	White	Male	–
Typically developing	Gracilis	15	Other/Hispanic	Female	–
Typically developing	Gracilis	12	White	Female	–
Typically developing	Gracilis	15	White	Female	–
Typically developing	Gracilis	14	White/Hispanic	Male	–
Typically developing	Gracilis	15	White/Hispanic	Female	–
With cerebral palsy	Adductor longus	4	White/Hispanic	Male	V
With cerebral palsy	Adductor longus	4	White/Hispanic	Male	V
With cerebral palsy	Gracilis	8	Asian	Male	V
With cerebral palsy	Gracilis	8	White	Female	IV
With cerebral palsy	Gracilis	11	White	Female	IV
With cerebral palsy	Gracilis	16	White	Male	IV

The average age (SD) of the typically developing children was 14 years 6 months (1 year 3 months) and for the children with CP was 8 years 6 months (4 years 2 months); $p < 0.05$ was used for average age. All patients with CP had spastic bilateral (quadriplegic) CP. Abbreviations: CP, cerebral palsy; GMFCS, Gross Motor Function Classification System; N/A, not applicable.

and added to the magnetic-activated cell sorting column and separator. MuSCs were collected using negative selection through the column. FAPs and MuSCs were then plated on tissue culture plastic in FAP medium (F-10, catalog no. 11550043, Thermo Fisher Scientific, Waltham, MA, USA), 20% fetal bovine serum (catalog no. S1620, Biowest, Bradenton, FL, USA), 1% penicillin–streptomycin (catalog no. 15140122, Thermo Fisher Scientific), 10 ng/mL fibroblast growth factor 2 (catalog no. PHG6015, Thermo Fisher Scientific), and 0.2 µg/mL dexamethasone (catalog no. 1126100, Thermo Fisher Scientific), or myoblast growth medium (Dulbecco's Modified Eagle Medium [DMEM], catalog no. 11965092, Thermo Fisher Scientific), 15% medium 199 (catalog no. 11150067, Thermo Fisher Scientific), 20% fetal bovine serum, 1% penicillin–streptomycin, 25 µg/mL fetuin (catalog no. 3415061GM, Sigma-Aldrich, St. Louis, MO, USA), 5 ng/mL human epidermal growth factor (catalog no. PHG0311, Thermo Fisher Scientific), 0.5 ng/mL fibroblast growth factor 2, 5 µg/mL insulin (catalog no. MP219390025, Thermo Fisher Scientific), and 0.2 µg/mL dexamethasone respectively. FAPs were cultured in either adipogenic (DMEM, 10% fetal bovine serum, 1% penicillin–streptomycin, 0.25 µM dexamethasone, 0.5 mM IBMX [catalog no. 102516252, VWR, Radnor, PA, USA], 5 µM troglitazone [catalog no. 501150786, Thermo Fisher Scientific], and 1 µg/mL insulin) or fibrogenic (FAP medium, 5 ng/mL transforming growth factor-β [catalog no. T7039-2UG, Sigma-Aldrich]) medium to validate the multipotency of FAPs. Cells were fed fresh medium every 2 to 3 days while undergoing proliferation, and were passaged when 60% to 80% confluent for two to three passages before the experiments.

Mechanosensitivity and verteporfin assay

Polyacrylamide gels were obtained from Matrigen Life Technologies (Irvine, CA, USA) as stiffnesses of 0.2 kPa,

8 kPa, and 25 kPa, to represent physiologically relevant stiffnesses. Gels and tissue-cultured plastic (approximately 10000 kPa) were coated with a 0.1% collagen-I (catalog no. 5226, Advanced BioMatrix, Carlsbad, CA, USA) solution for 1 hour at 37°C before seeding. Cells were plated at a concentration of 1.5×10^5 cells per cm^2 . MuSCs were switched to serum-free differentiation medium (DMEM) (50 µg/mL gentamicin [catalog no. 15710064, Thermo Fisher Scientific] and 10 µg/mL insulin). All cells were fixed with 4% paraformaldehyde on day 5 (catalog no. AA433689M, Thermo Fisher Scientific).

For the verteporfin assay, cells were treated with verteporfin (catalog no. SML0634, Sigma-Aldrich) at a concentration of 0.5 µM, solubilized in DMSO, or DMSO alone as a control. Medium with verteporfin or DMSO was replaced every other day and cells were fixed in 4% paraformaldehyde on day 5.

Immunostaining

After fixation, cells were permeabilized with Triton X-100 (brand no. BP151500, Thermo Fisher Scientific) for 10 minutes, washed with 0.1% bovine serum albumin (BSA) for 5 minutes, and blocked with 5% BSA for 30 minutes. Primary antibodies were added to the cells in 5% BSA and left overnight. FAPs were stained with alpha smooth muscle actin (αSMA) primary antibody (1:800 dilution, catalog no. MS113P1, Thermo Fisher Scientific) and perilipin (1:200 dilution, catalog no. 9349S, Cell Signaling Technology, Danvers, MA, USA); MuSCs were stained with myosin heavy chain (1:500 dilution, catalog no. 14650382, Thermo Fisher Scientific) primary antibody. Cells treated with verteporfin were stained with a YAP1 primary antibody (1:1000 dilution, catalog no. 14074, Cell Signaling Technology). After two washes with 0.1% BSA, a secondary fluorophore and acti-stain 555 phalloidin (1:250 dilution, catalog no. 50646254, Thermo Fisher Scientific) were added to 0.1% BSA

for 90 minutes. Hoechst 33342 stain (1:2000 dilution, catalog no. H3570, Thermo Fisher Scientific) in 0.1% BSA was added for 15 minutes; cells were washed in 0.1% BSA twice and kept in phosphate buffered saline at 4°C.

Imaging and image analysis

Cells were imaged using an inverted DMi8 microscope (Leica Microsystems, Durham, NC, USA) at 20× dry objective. Images were captured using a DFC9000 GTC camera (Leica Microsystems) and LAS X software (Leica Microsystems). Images were analysed using custom macros in Fiji ImageJ (National Institutes of Health, Bethesda, MD, USA) to determine FAP activation and MuSC differentiation. FAP activation into myofibroblasts was determined by the area of α SMA expression. The differentiation index was determined as the fraction of nuclei within a myosin heavy chain⁺ myotube. Myotube Analyzer in MATLAB (MathWorks, Natick, MA, USA) was used to assess myonuclear clustering. A nuclear cluster was defined as four or more myonuclei that were all within 4 μ m of each other.²⁵

Statistical analysis

Statistical analysis was completed using Prism v10.1.2 for Windows (GraphPad Software, Boston, MA, USA). A two-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test for pairwise comparisons was used to analyse the differences between stiffness and condition (CP vs typically developing). A three-way ANOVA followed by a Tukey's post-hoc test for pairwise comparisons was used to analyse differences between stiffness, condition, and treatment (verteporfin vs DMSO). Normality was tested using a Shapiro–Wilk test for all ANOVAs and was normal.

Homoscedasticity was tested using linear regression and visual analysis of plots. MuSC differentiation and myonuclear clustering data were homoscedastic. FAP differentiation data required a Box–Cox transformation to obtain homoscedasticity. A $p < 0.05$ was deemed significant.

RESULTS

MuSCs from children with CP have impaired differentiation

MuSCs differentiate best on stiffnesses that are physiologically relevant to healthy skeletal muscle.²⁰ Therefore, we assessed if MuSCs would differentiate better on substrates possessing a physiologically relevant stiffness of 8 kPa and whether MuSCs cultured on substrates with a fibrotic stiffness of 25 kPa exhibit impaired differentiation. We did not detect any significant effect of stiffness on the differentiation index (Figure 1a,b and Figure S1). Across stiffnesses, MuSCs from children with CP had significantly lower differentiation than those from typically developing peers (Figure 1b and Figure S1), which agrees with previous work.²⁶ Overall, MuSCs from children with CP had impaired differentiation and formed myotubes less readily.

Increased stiffness causes myonuclear clustering

While the differentiation index of MuSCs was not sensitive to changes in stiffness, we observed a noticeable change in the morphology of myotubes as stiffness increased, most notably in the cells from children with CP. MuSCs from children with CP formed thicker myotubes with more branching and high nuclear clustering on stiff substrates of 25 kPa and tissue culture plastic (Figure 1a,c). The MuSCs of typically developing

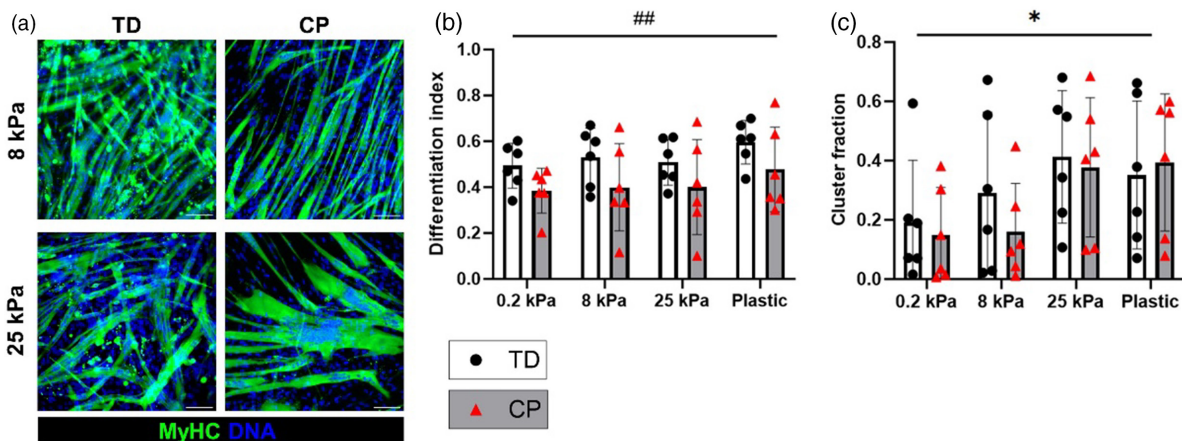


FIGURE 1 Muscle satellite cell (MuSC) differentiation is lower in children with cerebral palsy (CP) and myonuclear clustering increases on stiffer substrates. (a) MuSC differentiation on 8 kPa and 25 kPa collagen-coated polyacrylamide gels demonstrated using myosin heavy chain (MyHC) staining (green, nuclei in blue). (b) Quantification of the percentage of nuclei within myotubes. (c) Quantification of the number of myonuclei with clusters. * $p < 0.05$ for the main effect of stiffness in a two-way ANOVA, ## $p < 0.01$ for the main effect between CP and typically developing (TD) cells on substrates of different stiffnesses. Scale bar, 100 μ m. Data are shown as the mean (SD) ($n = 6$).

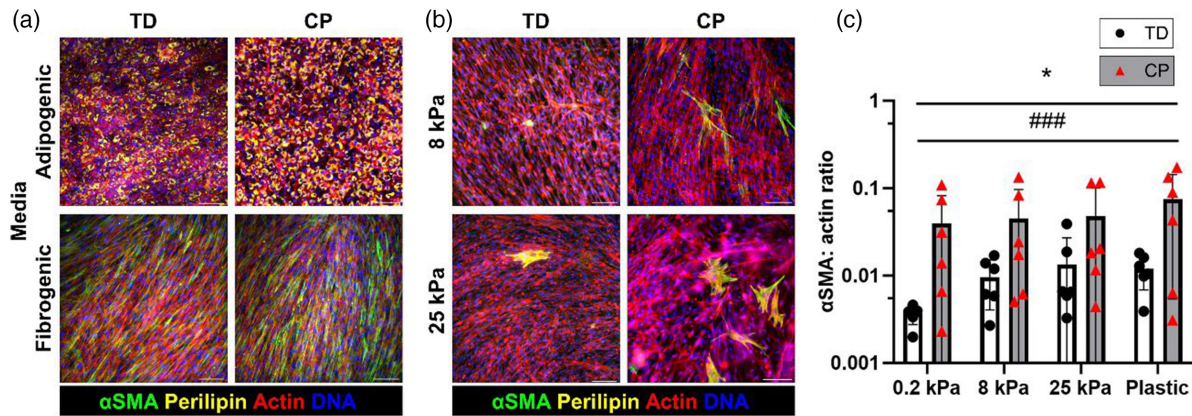


FIGURE 2 Fibro-adipogenic progenitor (FAP) activation into myofibroblasts is higher in cells from children with cerebral palsy (CP). (a) Expression of perilipin and alpha smooth muscle actin in FAPs after 5 days in adipogenic or fibrogenic medium on tissue-cultured plastic. (b) Myofibroblast activation in FAPs on 8 kPa and 25 kPa collagen-coated polyacrylamide gels. (c) Quantification of myofibroblast activation. Data are shown as the mean (SD) ($n=6$). * $p < 0.05$ main effect of stiffness. Statistics were carried out on transformed data to obtain homoscedasticity for the ANOVA; ### $p < 0.001$ for the main effect between children with CP and typically developing (TD) children in a two-way ANOVA; the raw data are shown in the graphs. Scale bar, 100 μm .

children also exhibited increased nuclear clustering on stiffer substrates but less branching and thickening of the myotubes compared to MuSCs from children with CP. Cluster fraction increased with increasing stiffness (Figure 1c) for both cell populations. MuSCs were sensitive to fibrotic stiffnesses, which altered their differentiation into myotubes.

FAP activation into myofibroblasts is higher in cells from children with CP

Myofibroblast activation increases on stiffer substrates. Therefore, we assessed if FAPs would more readily activate into myofibroblasts on fibrotic-like stiffnesses and whether myofibroblast activation would be higher in children with CP. The multipotency of FAPs was confirmed by culturing FAPs in both adipogenic and fibrogenic media to assess adipogenic and myofibroblast activation (Figure 2a). The FAPs from typically developing children exhibited relatively low αSMA expression across stiffnesses, with an increase in expression on stiffer substrates (Figure 2b,c and Figure S2). The FAPs from children with CP more readily activated into myofibroblasts, with αSMA expression being 5 to 10 times higher than that of FAPs from typically developing peers (Figure 2c). Myofibroblasts were more spread than non-activated FAPs, although the cell area was not affected by stiffness (Figure S3). The FAPs from children with CP were less sensitive to changes in stiffness, but were more primed overall to activate into a fibrotic state than the FAPs from typically developing children.

YAP nuclear localization is lower in cells from children with CP and may affect myonuclear clustering

Because of the increased clustering seen in myotubes on stiffer substrates, we investigated the effect of blocking

cellular mechanosensing as a potential method to restore the regenerative capacity of cells from children with CP. FAPs and MuSCs were treated with 0.5 μM verteporfin to reduce myofibroblast activation and nuclear clustering. Overall, both MuSCs and FAPs had lower YAP nuclear localization in children with CP compared to typically developing peers (Figures 3a and 4a). On stiff substrates, verteporfin did not affect MuSC differentiation but reduced YAP nuclear localization and slightly reduced nuclear clustering, although not significantly (Figure 3a–d and Figure S4).

YAP nuclear localization in FAPs was sensitive to changes in stiffness, with higher nuclear localization on stiffer substrates. The FAPs of typically developing children were more mechanosensitive than the FAPs from children with CP, with a more significant increase in YAP nuclear localization with increasing stiffness (Figure 4a,b). The FAPs from children with CP were resistant to verteporfin, with little change in YAP nuclear localization or myofibroblast activation (Figure 4a,c).

DISCUSSION

The aim of this study was to determine the mechanosensitivity of human MuSCs in the context of fibrosis and CP. The focus was on MuSC differentiation into muscle, a necessary part of regeneration, and FAP activation into myofibroblasts, an instigator of fibrosis. We used an established culture substrate, collagen-coated polyacrylamide gels, across a range of stiffnesses that were physiologically relevant to healthy and fibrotic muscle.^{18, 20} Surprisingly, neither MuSC differentiation nor FAP myofibroblast activation was particularly sensitive to changes in stiffness. The lack of mechanosensitivity in the cells from children with CP is attributable to the overall lower YAP expression compared to the cells of typically developing peers, indicating that the signaling pathways have been disrupted

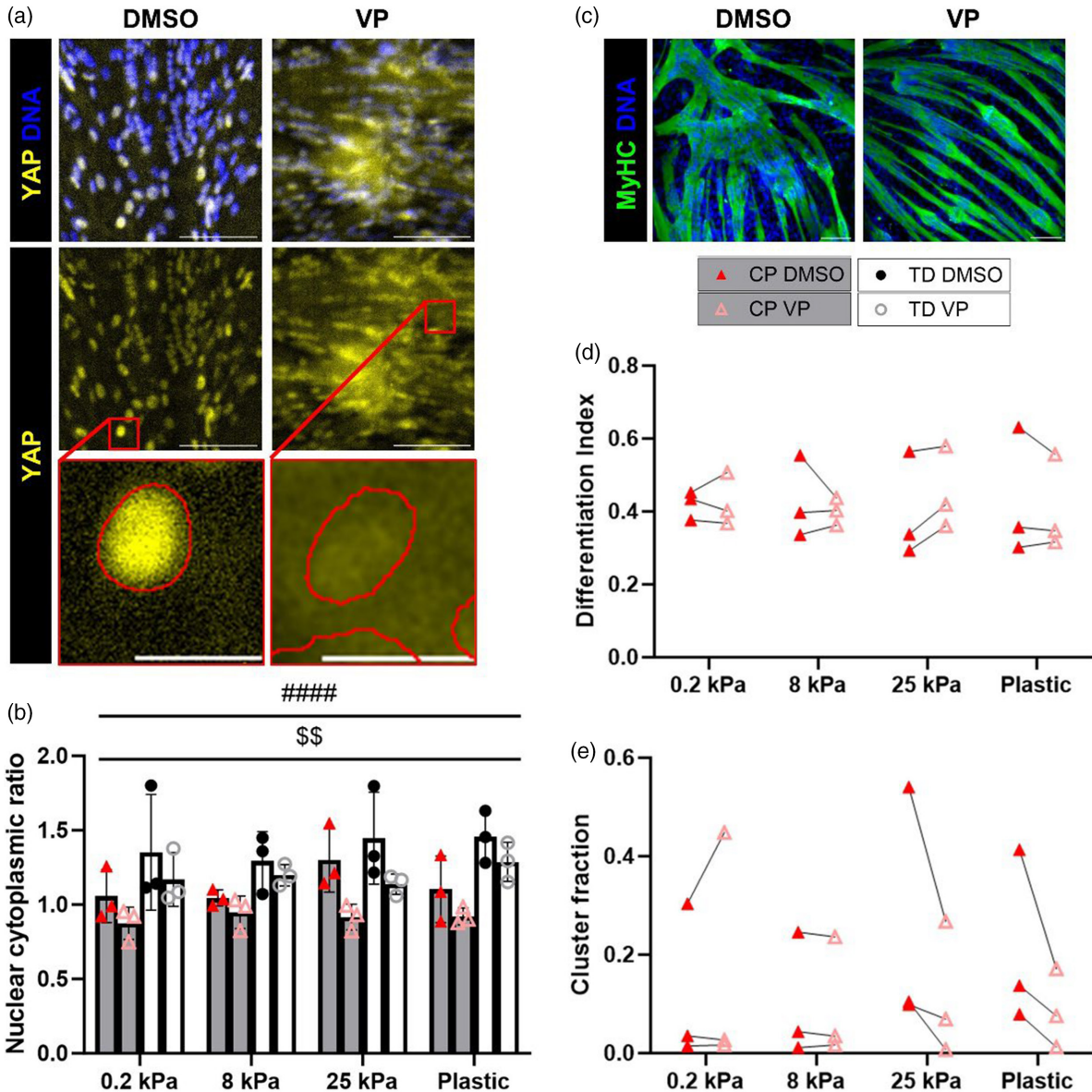


FIGURE 3 Verteporfin (VP) treatment in muscle satellite cells (MuSCs). (a) Yes-associated protein (YAP) nuclear expression in dimethyl sulfoxide (DMSO) control or verteporfin treatment on 25 kPa stiffness in the MuSCs from children with CP. The insets show individual nuclear expression; the red outline highlights the nuclei. (b) Quantification of YAP nuclear cytoplasmic ratio. (c) MuSC myotubes from children with CP at 25 kPa. (d) CP MuSC differentiation index with verteporfin treatment. (e) MuSC myonuclear clustering in children with CP after verteporfin treatment, $p=0.112$ for treatment. Data are shown as the mean (SD) ($n=3$). $^{***}p < 0.001$ main effect between children with CP and typically developing (TD) children. $^{SS}p < 0.01$ for the main effect between DMSO and verteporfin treatment in a three-way ANOVA. Scale bar, 100 μm ; scale bar for the insets, 20 μm .

or cells maintain a level of mechanical memory based on their in vivo environments.²⁷ Overall, the MuSCs from children with CP possessed lower differentiation capacity compared to those of typically developing controls; MuSCs in both conditions on fibrotic-like stiffnesses had altered phenotypes, with high myonuclear clustering. The FAPs from children with CP more readily activated into myofibroblasts compared to the FAPs from typically developing children. Verteporfin was used to restore the regenerative capacity of the cells from children with CP, but had little effect on MuSC differentiation or FAP myofibroblast activation, although myonuclear clustering was slightly

decreased. Overall, stem cells from the muscle of children with CP adapted to a more fibrotic and less regenerative phenotype compared to typically developing controls, who were less dependent on YAP signaling.

While CP is a non-progressive brain disorder, skeletal muscles demonstrate progressive contracture. The proper functioning of MuSCs is necessary to maintain muscle mass and recover from injury. The impaired differentiation index in the MuSCs from children with CP compared to their typically developing counterparts, as revealed in this article, suggests that these cells do not regenerate as well, leading to loss in muscle function. Previous studies showed

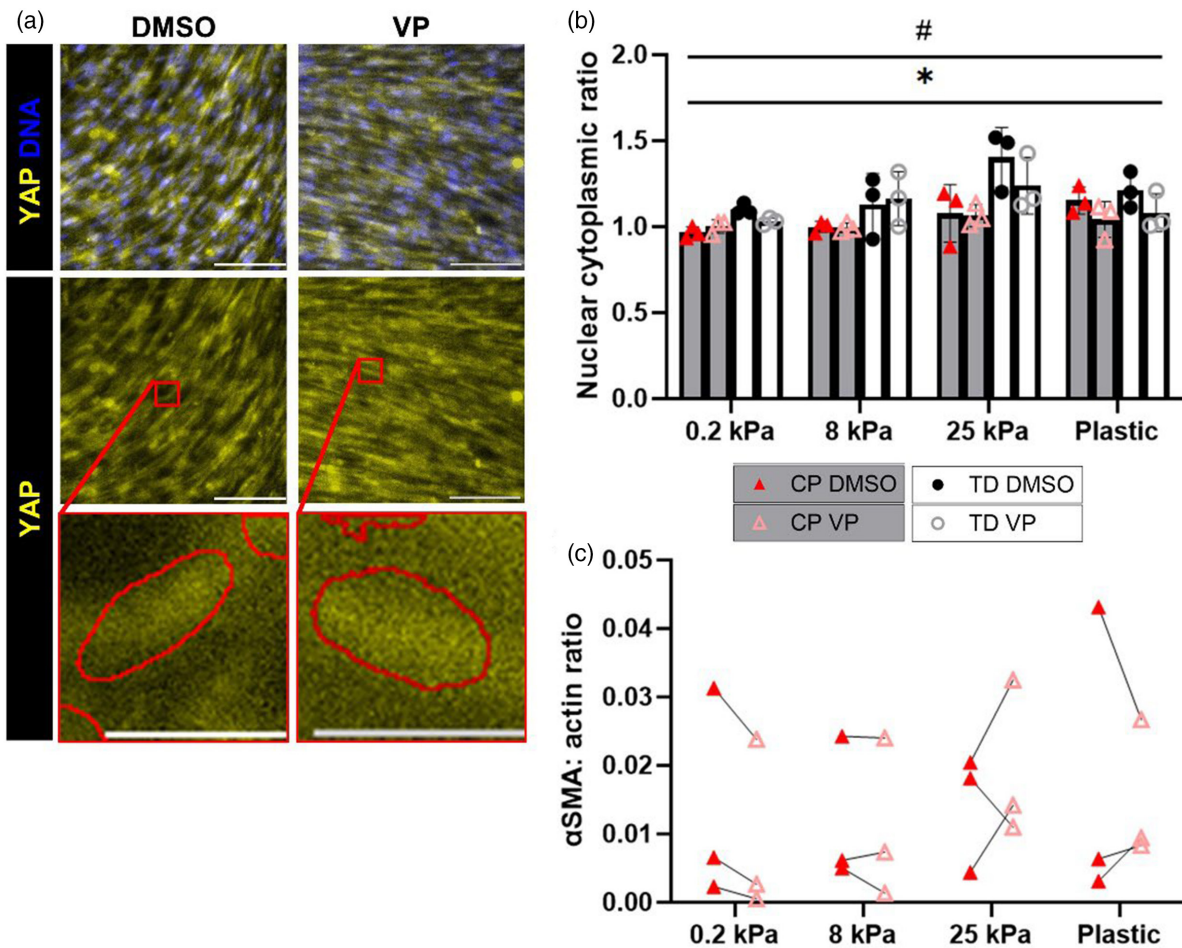


FIGURE 4 Verteporfin (VP) treatment in fibro-adipogenic progenitors (FAPs). (a) Yes-associated protein (YAP) nuclear expression at 25 kPa in the FAPs from children with CP and quantification of the nuclear cytoplasmic ratio. The red boxes indicate the insets; the red outline indicates nuclei within the inset. (b) Quantification of the YAP nuclear cytoplasmic ratio. (c) Myofibroblast activation in the FAPs of children with CP after verteporfin or dimethyl sulfoxide (DMSO) treatment. Data are shown as the mean (SD) ($n=3$). $^{\#}p < 0.05$ for the main effect between cells from children with CP and cells from typically developing (TD) children; $^*p < 0.05$ for the main effect of stiffness in a three-way ANOVA. Scale bars, 100 μm ; scale bars for the insets, 20 μm .

similar loss in differentiation potential, linking it to changes in DNA methylation.^{26, 28} Changes in methylation patterns have been associated with changes in matrix stiffness.²⁹ The MuSCs from children with CP may have altered methylation patterns because of increased stiffness in contracture and may maintain a level of mechanical memory, thus impairing differentiation in vitro.²⁷ Further studies could elucidate the mechanisms and potential therapeutic targets to induce regeneration. CP muscle pathology is largely heterogeneous. In this study, gracilis and adductor longus muscle biopsies were obtained from non-ambulatory children with CP classified in Gross Motor Function Classification System (GMFCS) levels IV and V. A previous study in ambulatory patients with CP classified in GMFCS levels I to III found an increased fusion index in MuSCs from children with CP from the medial gastrocnemius, highlighting the vast heterogeneity in CP.¹⁴ Disparities in findings can be attributed to ambulatory status, age, and heterogeneity within individual muscles.¹²

While the differentiation capacity of MuSCs in children with CP has been studied previously, to our knowledge,

the phenomenon of nuclear clustering in response to stiffness has not been reported. Previous reports of nuclear clustering did not find differences in the expression of genes involved in myoblast fusion, although other genes may be involved in myonuclear clustering.¹⁷ Myofibers consist of nuclei that should be evenly distributed, their positioning being driven by microtubules.^{30, 31} However, on stiff substrates, we observed myonuclear clustering in large groups, leaving large sections of the myofiber without a nucleus close by. This has implications for muscle function. The observation of nuclear clusters indicates that the myonuclear domain is disrupted. The even distribution of nuclei along a myofiber allows each nucleus to oversee the maintenance of a certain section of the myofiber. Altering the position of myonuclei results in reduced contractility of the myofibers.^{30, 32} The assembly of sarcomeres is dependent on close proximity to nuclei.³³ This suggests that large areas of the myofibers, apart from the nuclear clusters, are without properly formed sarcomeres, resulting in reduced contractility and strength. Coupled with impaired differentiation, MuSCs in patients with CP

form muscle at a slower rate than their typically developing counterparts;²⁶ the muscle that is formed is weaker and less contractile because of nuclear clustering. This motivates further research into nuclear clustering in CP, potential mechanisms, and methods to reduce clustering.

Both MuSCs from patients with CP and typically developing counterparts formed nuclear clusters on stiff, contracture-mimetic substrates, suggesting that mechanosensing pathways are involved. Therefore, cells were treated with verteporfin, a drug known to block cellular mechanosensing through the degradation of YAP.²¹ As expected, verteporfin did not influence MuSC differentiation because of the lack of change in differentiation across the different stiffnesses. We observed a slight decrease in nuclear clustering in cells from children with CP on stiff substrates, suggesting that some nuclear clustering is rescued through mechanosensing targets. However, the limited sample size because of patient availability, and heterogeneity between patients and muscle types, make it difficult to make definitive conclusions on the effectiveness of this method in restoring the regenerative capacity of MuSCs. Higher concentrations of verteporfin or pretreatment before differentiation may yield different results. Other mechanosensing activators, such as transcriptional coactivators with PDZ-binding motifs, may have a more significant role in MuSC differentiation. Future studies can further elucidate the signaling pathway that drives clustering.

MuSC differentiation is dependent on several myogenic signaling pathways, some of which are derived from FAPs. FAPs and MuSCs work together in healthy muscle to restore the extracellular matrix and muscle respectively after injury. FAPs release myogenic signals to MuSCs after injury to induce their migration to the injury site and differentiation into muscle.^{10, 34} However, in disease conditions, FAPs activate into myofibroblasts and can release soluble factors that impair rather than promote myogenesis, as well as depositing fibrotic extracellular matrix and leading to increased stiffness.³⁵ The increased activation into myofibroblasts seen in FAPs from children with CP compared to the FAPs of typically developing children suggests that these cells take on a more pathological rather than regenerative role in CP muscle and impair MuSC differentiation. Myofibroblast activation was high in the FAPs from children with CP regardless of stiffness, suggesting that activation is not mechanosensitive. However, the FAPs from typically developing children increased the markers of myofibroblast activation as substrate stiffness increased, although such indicators were relatively low compared to the FAPs from children with CP. Overall, myofibroblast activation was low in the FAPs of both children with CP and typically developing peers compared to studies in murine models, yet their activation into both myofibroblasts and adipocytes suggests that cells are indeed a relatively pure population of FAPs.^{15, 36} Myofibroblast activation is sensitive to increases in stiffness.¹³ The FAPs

from children with CP face other dysregulation *in vivo* that disrupts their mechanosensing pathways, making them less sensitive to changes in stiffness and explaining why verteporfin did not influence FAPs. Further research is needed to determine if FAPs are profibrotic in the contracture because of the low activation observed in this study. Identification of YAP-independent pathways for myofibroblast activation would better identify targets to reduce the activation of FAPs in children with CP.

The use of human cells in *in vitro* experiments offers an opportunity for more translational research compared to immortalized or other animal cell lines, but it comes with limitations. The limited number of biopsies and cells available limits the potential to have large sample groups to account for heterogeneity across muscle types, as both adductors and gracilis muscle were used, and variability in ages. The effectiveness of verteporfin as a treatment to restore the regenerative capacity of MuSCs is difficult to determine with the small sample size presented in this study, especially because of the inherent variability between patients.

The role of FAP activation into myofibroblasts is limited by the sample size because of the low activation rates and large variability between patients. An increase in sample size would allow for further investigation of the effectiveness of treatment and muscle heterogeneity alongside further understanding of potential interactions of treatment, disease state, and stiffness that may influence results.

This study shows the dysregulation of MuSCs in children with CP. MuSCs and FAPs from children with CP take on a less regenerative and more fibrotic phenotype than those of their typically developing counterparts. The identification of myonuclear clustering on stiff substrates provides insights into the loss of contractility seen in CP. Verteporfin offers a potential avenue to reduce this clustering, yet further research is needed to validate its use in the context of CP. The apparent lack of mechanosensitivity of the MuSCs and FAPs of children with CP in the context of differentiation and activation suggests that other therapeutic targets may yield improved results.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

The following additional material may be found online:

Figure S1: Myotube area per myonuclei.

Figure S2: Verteporfin treatment of FAPs from typically developing children.

Figure S3: The average nuclear and cellular area did not change across conditions.

Figure S4: Verteporfin treatment of MuSCs from typically developing children.

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