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# Dexamethasone induces dysferlin in myoblasts and enhances their myogenic differentiation

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## Abstract

Glucocorticoids are beneficial in many muscular dystrophies but they are ineffective in treating dysferlinopathy, a rare muscular dystrophy caused by loss of dysferlin. We sought to understand the molecular basis for this disparity by studying the effects of a glucocorticoid on differentiation of the myoblast cell line, C2C12, and dysferlin-deficient C2C12s. We found that pharmacologic doses of dexamethasone enhanced the myogenic fusion efficiency of C2C12s and increased the induction of dysferlin, along with specific myogenic transcription factors, sarcolemmal and structural proteins. In contrast, the dysferlin-deficient C2C12 cell line demonstrated a reduction in long myotubes and early induction of particular muscle differentiation proteins, most notably, myosin heavy chain. Dexamethasone partially reversed the defect in myogenic fusion in the dysferlin-deficient C2C12 cells. We hypothesize that a key therapeutic benefit of glucocorticoids may be the up-regulation of dysferlin as an important component of glucocorticoid-enhanced myogenic differentiation.

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## 1. Introduction

Loss of function mutations in the dysferlin gene, DYSF, cause Limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi Myopathy (MM) and distal anterior compartment myopathy (DACM) collectively known as dysferlinopathy [1-3]. The two main clinical features of these diseases are progressive loss of skeletal muscle and prominent muscle inflammation [4-10]. Age of onset is typically in early adulthood but there is considerable variability in the age of onset and severity of symptoms [11,12]. There is currently no treatment or cure for LGMD2B/MM [5].

Dysferlin is a 237kD, calcium binding, C2 domain-containing, transmembrane protein that is highly expressed in skeletal muscle and localized primarily in the sarcolemma [13,14]. Dysferlin-deficient mouse muscle fibers were defective in resealing laser-induced membrane disruption, therefore, it is thought to play a role in mediating the fusion of membrane vesicles to the sarcolemma at sites of membrane damage in muscle cells [15]. Dysferlin was also required for repairing scrape wounding-induced muscle membrane damage [16]. Interestingly, cultured dysferlin-deficient muscle cells from dysferlinopathy patients or normal muscle cells in which dysferlin was knocked down via dysferlin-specific siRNA oligonucleotides demonstrated impaired fusion and differentiation [17]. This and other studies in vitro and in vivo indicated that dysferlin has a role in myogenesis as well as in membrane repair [17-21].

Glucocorticoid treatment is highly effective for inflammatory myopathies and many types of muscular dystrophy such as Duchenne's muscular dystrophy (DMD) and can sometimes cure myositis [22,23]. Therapeutic effects of glucocorticoids are often attributed to their potent anti-inflammatory activity [22]. Studies in DMD patients indicated that glucocorticoid therapy reduced muscle inflammation and muscle proteolysis, while increasing myogenic repair and myoblast proliferation [22,23]. Surprisingly, even though there is significant muscle inflammation in LGMD2B/MM, glucocorticoid treatment is not effective in reducing myopathy and in some cases may have resulted in non-recoverable loss of strength [4-10].

Consistent with effects in DMD patients, prednisolone or deflazacort treatment increased muscle strength in *mdx* mice, a dystrophin-null mouse model for DMD [24,25]. The positive effects of prednisone on muscle function were shown to be equivalent in *mdx* compared to the *mdx;Rag2*-/- knock out in which B and T lymphocyte development is blocked resulting in immunodeficiency [25]. This indicated that a primary beneficial effect of glucocorticoids may be on the muscle itself and not on suppressing the immune system and inflammation [25].

Glucocorticoids have a broad range of effects in the body that are mediated at the molecular level by direct binding to the glucocorticoid receptor (GR) which activates it as a transcription factor [26-28]. GR is expressed in virtually all tissues, yet it has highly specific dose and context-dependent effects [26-29]. While glucocorticoids are beneficial for treating muscle diseases they also have well-documented catabolic effects on muscle [30]. The mechanisms underlying the positive versus negative effects of glucocorticoids on muscle are not understood but have been shown to be dose and context-specific both *in vitro* and *in vivo* [29,30]. For example, the catabolic effects *in vivo* were most evident under conditions of fasting and were greatly ameliorated by feeding [30]. Previous studies of glucocorticoid effects *in vitro* on C2C12 myoblasts showed that high doses of the glucocorticoids, dexamethasone or prednisolone, induced cell death and MyoD degradation via the ubiquitin-proteasome pathway [31,32]. On the other hand, treatment with lower doses of dexamethasone or prednisolone resulted in an increase in mRNA levels of the myogenic factors: MyoD, Myf-5, and MRF4 and decreased proliferation, but not death [31]. Treatment

of C2C12 cells with IGF-I in combination with dexamethasone resulted in synergistic myogenic differentiation that produced hypertrophic myotubes [33,34]; therefore, glucocorticoids can have direct positive and negative effects on muscle cells.

We sought to understand the molecular basis for why glucocorticoids are ineffective in treating dysferlinopathy patients even though they are beneficial therapy for many other muscular dystrophies and muscle inflammatory diseases [4-10,22,23]. We investigated the effects of glucocorticoid treatment at clinically equivalent doses on the muscle differentiation program in the C2C12 myoblast cell line and in dysferlin-deficient C2C12s in which dysferlin has been knocked down using shRNA (Maxwell, MM and Brown, RH, manuscript in preparation). We hypothesized that glucocorticoids may exert part of their therapeutic benefit in muscle diseases by increasing dysferlin levels thereby enhancing myogenic differentiation and muscle repair.

## 2. Materials and Methods

### 2.1 Cells and Reagents

The C2C12-1 murine myoblast cell line was a gift from Drs. A. Miyamoto and G. Weinmaster, Dept. of Biological Chemistry, UCLA [35]. We named this clone C2C12-1 to distinguish it from the C2C12 P9 clone, the parental C2C12 clone of the dysferlin-deficient C2C12s. Dysferlin-deficient C2C12s were generated using custom designed dysferlin shRNA stable constructs and generously given to us by Michele M. Maxwell, MGH, MA and Robert H. Brown, Dept. of Neurology, UMass. (Maxwell, MM and Brown, RH, manuscript in preparation). C2C12s were maintained in proliferation (growth or PM media: DMEM, 10% Fetal Bovine Serum (FBS) (Omega Scientific), 5% Cosmic Calf Serum (HyClone), 100 units/mL Penicillin-Streptomycin (Cellgro). Differentiation media (DM) consisted of DMEM, 2% Horse Serum (Sigma), 100 units/mL Penicillin-Streptomycin [35]. For dysferlin-deficient C2C12s, 1.5 μg/mL puromycin (Sigma) was added to PM. Dexamethasone (Sigma) was prepared as 10mM stock in 100% ethanol and diluted in respective media. An equal volume of ethanol was diluted in the respective media as the vehicle control.

### 2.2 Time Course Experiment Cultures

C2C12 time courses were performed based on the protocol in Doherty *et al.* [19]. Briefly, cells were plated 1:10 in PM (Day -1), media was changed the following day to PM plus 100nM dexamethasone or 0.001% ethanol as vehicle control (Day 0). Cells were switched to DM on Day 2 for cells harvested on Days 3 – 7, with subsequent media changes occurring on Days 4 and 6. Media changing and cell harvesting were at the same time each day.

### 2.3 Myogenic Fusion Efficiency Quantification

Myogenic fusion efficiency was determined as previously described with minor modifications [19]. Briefly, C2C12s were grown on sterile glass coverslips. After four days of differentiation (Day 6 of time course), cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton® X-100 (Sigma) in PBS. Cells were blocked in 0.1% Triton® X-100 in PBS, 10% FBS and stained with mouse monoclonal anti-desmin antibody (1:300, Sigma), followed by goat anti-mouse FITC-conjugated secondary antibody (1:500, Jackson Immunoresearch) and mounted with Vectashield plus DAPI (Vector Labs). Ten fields at 10× magnification per treatment from three separate cultures were captured and analyzed. Using ImageJ, nuclei were counted and classified as being in cells containing one nucleus, two or three nuclei, or four or more nuclei. FISH microscope images were captured with Quips mFISH software (Vysis) on a

Leica DMR fluorescent microscope. Differences greater than $p < 0.05$ were assumed to be significant.

### 2.4 Real Time Quantitative PCR

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. RNA concentrations were measured using the NanoDrop (Thermo Scientific). cDNA was synthesized with qScript (Quanta BioSciences) using 1μg of *Turbo* DNase (Applied Biosystems) treated total RNA. Real time quantitative PCR (qPCR) was carried out using *Power* SYBR Green (Applied Biosystems) on an iCycler iQ5 (Bio-Rad) in triplicate 20μL reaction volumes using the following procedure: One cycle of 95°C for 10 minutes, 40 cycles of 95°C for 30 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. Melting curve data confirmed primer specificity and single product amplification. Dysferlin and myoferlin primers were designed using Vector NTI (Invitrogen) and spanned at least two exons. Primers used were: mouse dysferlin forward 5′-TAT GTG AAA GGC TGG ATG GTG GGA-3′ and reverse 5′-TTG CTC AGC AGG CAG ATA GTC GAA-3′, mouse myoferlin forward 5′-CCC TAC AAA CAG ACC TCC CTG CT-3′ and reverse 5′-CTG AAG GTG GGG TAT AGC CG-3′, mouse MyoD forward 5′-GGA CAG CCG GTG TGC ATT-3′ and reverse 5′-CAC TCC GGA ACC CCA ACA G-3′ (36), mouse myogenin forward 5′-GGA GAA GCG CAG GCT CAA G-3′ and reverse 5′-TTG AGC AGG GTG CTC CTC TT-3′ (36), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-TGA CGT GCC GCC TGG AGA AA-3′ and reverse 5′-AGT GTA GCC CAA GAT GCC CTT CAG-3′ (37,38), and mouse eukaryotic translation elongation factor 1 epsilon 1 (eEF1ε1) forward 5′-GCG GAG TTG AGG CTG CTG GAG A-3′ and reverse 5′-AGA CTC GGG CCA TTG TTT GTC TG-3′ (38). Based on geNorm analysis of six candidate reference genes, GAPDH and eEF1ε1 were chosen for normalization using the $2^{-\Delta\Delta Ct}$ method as previously described [39-41]. Effects of experimental treatments were assessed by paired comparisons within experiments and reported as the mean ± standard error (SE) normalized to untreated Day 1. Differences greater than $p < 0.05$ were assumed to be significant.

### 2.5 Cell Lysates and Western Immunoblotting

Protein extracts were made according to Doherty *et al.* [19]. Briefly, each $10cm^2$ plate of C2C12s was washed in cold PBS, lifted using PBS/1mM EDTA, transferred to 1.5ml tubes and pellets lysed for forty minutes on ice in 500μL of lysis buffer (25mM Tris pH 7.4, 300mM NaCl, 1mM $CaCl_2$, 1% Triton® X-100) plus protease and phosphatase inhibitor cocktails (Roche). Sixty micrograms of total protein per sample was run on 8% SDS-PAGE and transferred to Immobilon (Millipore). Immunoblots were probed for dysferlin (NCL-Hamlet, 1:1000, Leica Microsystems,), sarcomeric myosin heavy chain (MF-20, 1:1000, kind gift from Dr. G. Weinmaster, UCLA,), dystrophin (MANDYS1, 1:10), α-sarcoglycan (Ad1/20A6, 1:100), β-dystroglycan (MANDAG2, 1:250, all three DGC antibodies were kind gifts from Dr. R. Crosbie, UCLA), myogenin (F5D, 1:1000, kind gifts from Drs. M. Spencer and G. Weinmaster, UCLA,), desmin (DE-U-10, Sigma, 1:2000), and GAPDH as an internal loading control [42] (ab9484, AbCam, 1:2000). Visualization was carried out with goat anti-mouse horse radish peroxidase-conjugated secondary antibody (GE Healthcare) with all primary antibodies with SuperSignal West Dura ECL Substrate (Pierce) and HyBlot CL Autoradiography Film (Denville). Western quantification is detailed in Supplementary Table 1.

### 2.6 Immunofluorescence Microscopy

C2C12 cells were grown on sterile glass coverslips as described in Diaz-Perez *et al.* [43]. At each time point, the coverslips were fixed with 2% paraformaldehyde in KCM (120mM KCl, 20mM NaCl, 10mM Tris-HCl pH 8.0, 0.5mM EDTA, 0.1% Triton® X-100) and

permeabilized with 0.5% Triton® X-100 in KCM. The coverslips were blocked in 10% FBS/KCM. Rabbit polyclonal anti-dysferlin antibody (AbCam) was used at 1:50 in blocking solution followed by a goat anti-rabbit Texas Red-conjugated secondary antibody (1:100, Jackson ImmunoResearch) in blocking solution. Vectashield with DAPI was used to mount the coverslips on microscope slides. Images were captured with Quips mFISH software as above. Confocal images were captured using Leica Confocal Software on a Leica DM IRE2 confocal microscope. All confocal images shown are maximum-intensity z projections captured using the same gain and offset settings.

## 3. Results

### 3.1 Dexamethasone increased C2C12 myogenic fusion efficiency

We analyzed the effects of low dose dexamethasone treatment (100nM Dex) on C2C12 differentiation and dysferlin expression at the mRNA and protein level. Dex is a synthetic, stable, non-metabolized glucocorticoid, and thus, gives highly reproducible, glucocorticoid receptor (GR)-mediated effects [26-28,44]. Three time course experiments of C2C12-1 cell differentiation were performed with vehicle control (0.001% ethanol) or 100nM Dex. Cultures were visually inspected on each day for morphologic changes. Brightfield microscope images of the cells in culture are shown in Supplemental Figure 1. On Day 2 of the time course the C2C12-1 cells consisted of singly nucleated myoblasts with no visually discernible difference between the ethanol vehicle control compared to the Dex-treated cultures (Supplemental Figure 1A). Myotubes were present in both the vehicle control and Dex-treated C2C12-1 cultures on Days 5 – 7 of the time course when the cells had been in differentiation media for three to five days. However, there was a noticeable increase in the number and size of myotubes in the Dex-treated cultures compared to vehicle control-treated cultures (Supplemental Figure 1B – F). Interestingly, the Dex-treated myotubes appeared hypertrophic: they were broader and appeared to contain more myonuclei than the vehicle control-treated cultures.

Myogenic fusion efficiency was measured by counting the number of nuclei per myotube. Myoblasts and myotubes were visualized using a desmin-specific antibody and counterstained with DAPI for nuclear visualization [19]. Nuclei were counted and categorized as being present within singly nucleated cells, cells with two or three nuclei, or cells with four or more nuclei. The percentage of nuclei present within cells with four or more nuclei was significantly greater in the Dex-treated C2C12-1 cultures than in vehicle control-treated cultures indicating enhanced myoblast and myotube fusion (Figure 1).

### 3.2 Dex induced early expression of dysferlin, myoferlin, MyoD and myogenin mRNA

The mRNA levels of mouse dysferlin during myogenesis have not been previously quantified. We established a real time quantitative PCR (qPCR) assay to determine the level of dysferlin mRNA during growth and differentiation of C2C12-1 myoblasts. As shown in Figure 2A, the level of dysferlin mRNA was initially low in the vehicle control-treated cells (blue bars) when the cells were in proliferation media (Days 1 and 2) then increased significantly after the switch to differentiation media (Days 3 – 7) as would be anticipated from previous analyses of dysferlin protein levels [19]. In contrast, there was a significant increase in dysferlin mRNA in the Dex-treated cultures (red bars), over that of vehicle control-treated cells (blue bars) at early time points (Days 1 – 5). The maximum fold increase of dysferlin mRNA in Dex-treated over vehicle control-treated cells was on Day 2 (10.71 ± 0.61) prior to the switch to differentiation media. The maximum mRNA dysferlin levels occurred in the Dex-treated cells on Day 5. Therefore, Dex treatment induced an early increase in dysferlin mRNA levels in proliferating and differentiating C2C12-1 cells (Figure 2A).

Myoferlin is a ferlin family member closely related to dysferlin that is expressed in myoblasts, myotubes, and mature muscle fibers and is also involved in myoblast fusion and myogenesis [19,45]. As seen in Figure 2B myoferlin mRNA was strongly expressed throughout the C2C12-1 time course. There was a two-fold increase in myoferlin in the Dex-treated cultures on Days 1 and 2, however, myoferlin expression levels were relatively unchanged by Dex treatment during differentiation on Days 3-7 (Figure 2B).

The mRNA levels of the myogenic transcription factors: MyoD and myogenin were determined in the Dex-treated and vehicle control-treated C2C12 time courses. MyoD upregulates expression of myogenin which activates genes required for terminal muscle differentiation [31]. As shown in Figure 2C, MyoD mRNA increased during differentiation in both the Dex and vehicle control-treated cultures, however, MyoD mRNA expression was induced earlier (Day 2) and peaked earlier and higher in the Dex-treated cells (Day 3) compared to the vehicle control-treated cells (Days 5 and 6). On the following days (Days 4 – 7), MyoD expression levels were not significantly different between the Dex-treated and untreated cells (Figure 2C). Therefore, Dex treatment not only increased the maximum MyoD mRNA expression but also shifted its expression peak two to three days earlier in Dex-treated (Day 3) compared to vehicle control-treated cells (Day 5 and 6).

Myogenin mRNA levels were strongly upregulated during differentiation with a maximum peak of ~3000 fold on Day 4 in both the Dex and vehicle control-treated cells (Figure 2D). Myogenin mRNA levels also showed an earlier increase in Dex-treated than in vehicle control-treated cells (Days 2 and 3). Interestingly, the peak of maximum myogenin expression level, which occurred on Day 4, was the same in the Dex and vehicle control-treated cells (Figure 2D). Toward the end of the time course (Days 6 and 7), myogenin mRNA levels were significantly suppressed in Dex-treated cells compared to vehicle control-treated cells (Figure 2D). This is consistent with previous reports of myogenin mRNA levels in Dex-treated C2C12 cells [31].

In summary, Dex treatment induced earlier, higher mRNA expression of dysferlin, myoferlin, MyoD, and myogenin in the proliferating, or growth, phase and in the early differentiating phase of the C2C12 time course (Figure 2). Thus, Dex treatment accelerated the timing of induction of myogenic differentiation genes. Dex treatment also increased the maximum expression levels of dysferlin, myoferlin, and MyoD, whereas, the maximum level of myogenin mRNA was the same in the Dex and vehicle control-treated C2C12s. We also noted that the levels of MyoD and myogenin demonstrated reproducible, seemingly oscillatory behavior; their levels rose and fell then rose again. This may be related to previous observations of waves of gene expression during differentiation and that glucocorticoids are well known to drive synchronized oscillations and circadian rhythms in gene expression [7,30,46,47].

### 3.3 Dex induced early increase in dysferlin and other myogenic proteins

The steady-state level of dysferlin protein was determined at each time point using Western blot analysis. Equal protein was loaded for each sample as demonstrated by the comparable GAPDH levels in all the samples [42]. Normalized ratios of dysferlin to GAPDH were determined for each sample from triplicate immunoblots (Supplementary Table 1). Dysferlin protein levels were consistently higher at early time points in the Dex-treated C2C12 cells than the vehicle control-treated cells (Figure 3). The largest difference was seen on Day 1 (2.57 ± 0.26 fold Dex / No Dex) with approximately the same increase (1.5 – 2 fold) in dysferlin from Days 2 – 4 (Supplementary Table 1). The steady-state levels of dysferlin protein continued to increase from Days 5 – 7, but were equivalent in the Dex and vehicle control-treated cells. Therefore, Dex treatment induced earlier, higher levels of dysferlin protein as well as mRNA during proliferation and early times in C2C12-1 differentiation.

Myogenin protein levels increased in both Dex-treated and vehicle control-treated cultures indicating that the cells were differentiating into myotubes (Figure 3). One day after the switch to differentiation media (Day 3), myogenin protein was higher in Dex-treated C2C12-1 cells. As the time course progressed, however, myogenin levels climbed higher in untreated cells than in Dex-treated cells in contrast with the other myogenic proteins (Figure 3). In general, myogenin protein levels mirrored their mRNA levels (Figure 2D).

Western blots were performed on the same C2C12-1 lysates for other proteins expressed in differentiating muscle: myogenin, myosin heavy chain (MyHC), desmin, and three dystrophin glycoprotein complex (DGC) members: dystrophin, α-sarcoglycan, and β-dystroglycan [48]. In the vehicle control-treated C2C12-1 cells, the expected increases were detected in the levels of MyHC, desmin and the DGC proteins dystrophin, α-sarcoglycan, and β-dystroglycan showing that the cells were reaching late stages of myogenic differentiation (Figure 3). Dex-treatment, however, induced earlier and higher expression levels of all these proteins (Figure 3). Most notably, MyHC levels were significantly higher in the Day 4 Dex-treated cultures compared to vehicle control-treated cells (Figure 3).

### 3.4 Dex induced dysferlin in myoblasts as well as myotubes

Immunofluorescence staining of dysferlin was performed in the C2C12-1 differentiation time courses in order to determine the dysferlin levels in individual cells and its sub-cellular localization. As expected, in vehicle control-treated C2C12-1s, dysferlin was not detected in myoblasts on Day 2 and was expressed in myotubes on Day 6 after four days of differentiation media (Figure 4 A and B, No Dex) [19]. Interestingly, in Dex-treated C2C12-1s, dysferlin protein was expressed in some myoblasts on Day 2 consistent with the Western immunoblots (Figures 3 and 4A). On Day 6, stronger dysferlin staining was observed in the Dex-treated than in vehicle control-treated myotubes (Figure 4B, Supplemental Figure 2B). The Dex-treated myotubes had a distinct, hypertrophic morphology compared to the vehicle control-treated myotubes: they were broader and contained more myonuclei which were often clustered rather than distributed (Figure 4B and Supplemental Figure 2A). In fact, Dex-treatment of C2C12-1 consistently resulted in more adherence and longer survival on glass coverslips or in standard tissue culture dishes than vehicle control-treated C2C12-1 cells. Confocal microscopy images showed punctuate, primarily cytoplasmic dysferlin staining in myotubes (Figure 4B and in Supplemental Figure 2B) that was similar to that seen in Klinge *et al.* [20].

### 3.5 Dex partially reversed impairment in myogenic fusion of dysferlin-deficient C2C12s

Differentiation time course experiments were performed with dysferlin-deficient C2C12 cells that stably expressed dysferlin shRNAs in parallel with the matching parental cell line, C2C12 P9 (Maxwell MM and Brown, RH, manuscript in preparation). Interestingly, the C2C12 P9 cells demonstrated more robust myotube formation and higher myogenic fusion efficiency in the vehicle control-treated cultures than the C2C12-1 clone used in the first time courses (compare Figures 1 and 5A). Dex treatment enhanced myogenic fusion efficiency in C2C12 P9 cells although to a lesser extent than in C2C12-1 (compare Figure 2 and Figure 5A). When compared to the matched parental C2C12 P9 cells, the dysferlin-deficient C2C12 cells demonstrated a reduction in myogenic fusion efficiency of long myotubes (4 or more nuclei per myotube) (Figure 5A). The dysferlin-deficient C2C12 cells showed a concomitant increase in short myotubes (2 or 3 nuclei per myotube). Dex treatment partially reversed the reduced myogenic fusion efficiency of dysferlin-deficient C2C12s compared to Dex-treated C2C12 P9s (Figure 5A). Dysferlin-deficient C2C12 myotubes were thinner and shorter in both vehicle control and Dex-treated cells compared to parental C2C12 P9 cells (Figure 5B).

Western immunoblot analysis confirmed that dysferlin protein was almost completely abolished in the dysferlin-deficient C2C12 cells yet strongly induced in the parental C2C12 P9 cells, as expected (Supplemental Figure 3). Myogenin levels were the same in the dysferlin-deficient C2C12 cells compared to the parental C2C12 P9 cells throughout the differentiation time course (Supplemental Figure 3).

Western immunoblot analysis showed that Dex treatment induced dysferlin in the parental C2C12 P9 cells as it did in C2C12-1, albeit, more modestly (Figure 6). There was no detectable dysferlin protein expression in the dysferlin-deficient C2C12 cells in Dex-treated or untreated cells at early time points (Figure 6). Upon very long exposures of Western immunoblots, exceedingly low levels of dysferlin were detected in the dysferlin-deficient C2C12 cells on Days 4 to 7 of the time course compared to dysferlin expression in the C2C12 P9 cells (Supplemental Figure 3).

Dex treatment modestly increased myogenin expression in the C2C12 P9 cells on Day 5 (Figure 6A) whereas Dex treatment of dysferlin-deficient C2C12 cells resulted in a small increase in myogenin protein levels on Days 6 and 7 late in the time course (Figure 6B). Desmin was expressed in the parental C2C12 P9 and dysferlin-deficient C2C12 myoblasts and its levels increased during differentiation as expected. A modest increase in desmin in the Dex-treated cells compared to vehicle control was seen in the C2C12 P9 cells only on early days of the time course. Dex treatment significantly increased myosin heavy chain (MyHC) levels in the C2C12 P9 cells on Days 3 and 4 during differentiation (Figure 6A and B), thus, reproducing the same effect and timing of MyHC induction in the C2C12 P9 cells that we demonstrated in the independently obtained sub-clone, C2C12-1 (Figure 3). Intriguingly, the dysferlin-deficient C2C12 cells showed strong up-regulation of MyHC levels on Day 3 even without Dex treatment just one day after switching to differentiation media (Figure 6A and B) [21].

The protein expression levels of the DGC members: dystrophin, α-sarcoglycan, and β-dystroglycan were compared in the parental C2C12 P9 cells and the dysferlin-deficient C2C12 cells. Unusually early expression of the late myogenic differentiation protein, α-sarcoglycan was detected in dysferlin-deficient C2C12 cells without Dex treatment (Figure 6). Dystrophin and β-dystroglycan levels were only modestly higher in the dysferlin-deficient C2C12 cells as compared to the parental C2C12 P9 cells. Dex further increased the levels of all three DGC proteins in both cell lines but did not further accelerate the time of their induction. Strikingly, Dex treatment markedly increased dystrophin levels at later times in the differentiation of dysferlin-deficient C2C12 cells (Figure 6).

## 4. Discussion

The primary therapeutic action of glucocorticoids in muscle diseases has been attributed to their potent anti-inflammatory action [22,23]; thus, the lack of benefit in dysferlinopathy, which often presents with prominent inflammation, has been paradoxical [4-10]. We show for the first time that low, clinically equivalent doses of the glucocorticoid, dexamethasone (Dex), not only enhanced the myogenic fusion efficiency of C2C12 cells but also hastened and amplified the expression of dysferlin along with several other muscle differentiation proteins. At these physiological-equivalent levels we found that glucocorticoids had two effects on C2C12 differentiation: 1. Acceleration, or “fast-forward button”, and 2. Augmentation, or “turbo-booster” of myotube fusion and the terminal muscle differentiation program. Dex treatment accelerated and augmented the levels of the membrane fusion mediators: dysferlin and myoferlin, the transcription factors: MyoD and myogenin, and the muscle structural and sarcolemmal proteins: MyHC, dystrophin, α-sarcoglycan, and β-

dystroglycan. Therefore, low-dose Dex treatment accelerated and enhanced the myogenic differentiation program of C2C12 myoblasts.

We wished to determine whether Dex induction of dysferlin was necessary for Dex-enhanced myogenic differentiation. The approach we chose was to use dysferlin-deficient C2C12 cells generously provided to us by Drs. M. M. Maxwell and R.H. Brown in which dysferlin had been knocked down via shRNA. We report here for the first time the systematic characterization and comparison of the myogenic fusion efficiency and expression of myogenic differentiation proteins in the dysferlin-deficient C2C12s and their parental C2C12 P9 cells in the presence and absence of Dex in time course experiments, as we did for the C2C12-1 cells. We confirmed that dysferlin protein was essentially eliminated in the dysferlin-deficient C2C12s in differentiation time course experiments. While performing these experiments we determined that the C2C12 P9 cells, the parental cells used to generate the dysferlin-deficient C2C12 cells, were significantly more robust in myotube formation than the C2C12-1 cells used in the first part of our work [35]. Clone-to-clone differences in established cell lines are common and we have carefully quantified the effect of Dex on the different C2C12 sub-clones. The C2C12 sub-clone differences may reflect differences in passage number or possible selection for C2C12 sub-clones that have upregulated myotube fusion mechanisms. Importantly, Dex treatment enhanced the myogenic fusion efficiency in C2C12 P9 (4 or more nuclei per myotube, Figures 2 and 6B) which was consistent with the enhancing Dex effects in the C2C12-1 clone. Moreover, the timing and pattern of Dex-induced expression of myogenic proteins such as MyHC was remarkably consistent between these C2C12 sub-clones. Therefore, we demonstrated the Dex-enhancement of fusion and myogenic differentiation in two different sub-clones of C2C12 cells.

Being mindful of the difference in myogenic fusion efficiency between the C2C12-1 and P9 sub-clones we compared the myogenic fusion efficiency of the dysferlin-deficient C2C12s to their parental C2C12 P9 clone and found it was decreased in the dysferlin-deficient C2C12s. The dysferlin-deficient C2C12 cells showed a reduction in the number of long myotubes and a concomitant increase in short myotubes compared to the parental C2C12 P9 cells. Notably, the dysferlin-deficient C2C12 cells expressed high levels of MyHC and α-sarcoglycan at unusually early times in the differentiation time course compared to either the C2C12 P9 or C2C12-1 sub-clones. These proteins are typically used as markers of terminal differentiation and it is possible that in the absence of dysferlin the myotube fusion stops early and terminal differentiation is activated early. Thus, our systematic, independent characterization of this dysferlin-deficient C2C12 cell line has uncovered a possible signaling feedback function for dysferlin in myotube elongation that may forestall terminal differentiation. It is important to keep in mind the caveat, however, that this effect may be limited to this one dysferlin-deficient C2C12 line. This intriguing yet preliminary finding needs to be reproduced in additional dysferlin-deficient cells. Experiments to independently derive additional dysferlin-deficient C2C12 P9 and C2C12-1 cells for comprehensive quantification of myotube fusion and differentiation are currently underway and also planned for dysferlin-deficient primary mouse and human myoblasts in order to confirm whether impaired myotube elongation, that is, a decreased number of myotubes with 4 or more nuclei, is in fact due to the absence of dysferlin.

Previously, de Luna et al. reported impaired fusion and differentiation in dysferlin-deficient muscle cells either from dysferlinopathy patients or with siRNA knock down of dysferlin in normal muscle cells [17]. Here we showed that myogenic fusion efficiency was modestly impaired in a dysferlin-deficient C2C12 mouse myoblast cell line and that the dysferlin-deficient myotubes were thinner and shorter yet mature. The impaired myogenic fusion in dysferlin-deficient C2C12 cells in our study was not nearly as profound as in the primary

human myoblasts [17]. In addition, myogenin levels were equivalent in the dysferlin-deficient C2C12 cells and the parental C2C12 P9 cells whereas myogenin was significantly decreased during in vitro differentiation of primary human myoblasts from dysferlinopathy patients [17]. The reasons for these differences are not known but may be due to differences in the behavior of mouse versus human myoblasts, primary versus immortalized cell line, or a cell line specific effect as discussed above. It is possible that the compensatory mechanisms for myotube fusion may be more effective in the C2C12 cell line than in primary myoblast cultures. For example, myoferlin was strongly expressed in the C2C12s and may be largely responsible for the ability of the dysferlin-deficient myotubes to fuse [19,45,48,49]. Impaired myogenic fusion efficiency was also demonstrated in myoferlin-deficient mouse myoblasts [19]. Other fusion mediating proteins such as M-cadherin/beta-catenin may also partly compensate for dysferlin-deficiency [52]. Nevertheless, in both *in vitro* differentiation systems the myogenic fusion efficiency of dysferlin-deficient myoblasts was impaired.

Dexamethasone treatment partially reversed the impairment in myogenic fusion in the dysferlin-deficient C2C12 cells and still augmented the levels of some late differentiation proteins such as dystrophin and β-dystroglycan. However, Dex did not further augment the already accelerated and increased expression of MyHC and α-sarcoglycan in this dysferlin-deficient C2C12 cell line in contrast to C2C12-1 and C2C12 P9. The Dex induced increase in a sub-set of the myogenic proteins including dystrophin may be responsible for the partial rescue of myogenic fusion efficiency seen in the Dex-treated dysferlin-deficient C2C12 cells [19,45,48,49]. Our evidence supports the model that dysferlin is part of a multi-component myogenic program that is more robustly induced by Dex treatment. Thus, our studies of Dex enhancement of myotube formation uncovered a potentially novel contribution of dysferlin function to the beneficial effects of low dose glucocorticoid therapy [50,51].

Previous studies of dysferlin expression during differentiation in C2C12 cells showed that the onset of dysferlin expression during differentiation was in C2C12 myotubes with two to three or more nuclei per myotube [19]. In the present immunofluorescence and immunoblotting analysis of dysferlin, we found that Dex induced the expression of dysferlin in some singly nucleated C2C12s as well as myotubes. Whether these dysferlin positive mononuclear C2C12 cells are still proliferating myoblasts or have differentiated into myocytes is currently under investigation. The morphology and myonuclear distribution were also strikingly different in the Dex-treated myotubes and consistent with the qualitative as well as quantitative differences in myogenesis we demonstrated due to Dex treatment.

Glucocorticoid action is mediated by the glucocorticoid receptor (GR), a transcription factor [26,27]. Is dysferlin a primary GR transcriptional target? Transcription regulation of dysferlin expression is not well-understood although Foxton and co-workers defined a human dysferlin promoter region that regulated luciferase reporter expression in differentiated C2C12 cells containing several potential MyoD sites [53]. In agreement with a previous study from tePas *et al.* [31], we demonstrated that low-dose Dex treatment upregulated MyoD which may in turn induce dysferlin transcription. Using comparative genomics and transcription factor binding site prediction programs we identified several conserved GR/androgen receptor (AR)/progesterone receptor (PR) binding elements on predicted dysferlin and myoferlin promoter regions from human, dog, rat, and mouse as well as a striking conservation of an overlapping MyoD and NFAT binding sites analogous to those shown for the myogenin promoter [54] (Belanto and Jamieson, manuscript in preparation). Thus, dysferlin may be a direct target of GR, MyoD and NFAT [26,27,31,54,55].

Our study showing that low dose Dex treatment accelerated and amplified expression of dysferlin along with other muscle differentiation proteins and enhanced myotube formation is consistent with previous studies showing that glucocorticoids can promote myogenesis and identifies novel molecular mechanisms that may be important for its beneficial effects in muscular dystrophy. Our findings suggest that dysferlinopathy patients may fail to respond to glucocorticoid therapy because glucocorticoid-enhanced muscle differentiation may be partially dysferlin-dependent. Treatments that are aimed at the myogenic targets of the Dex-enhancement that we uncovered here may lead to therapies in dysferlinopathy patients that avoid the use of glucocorticoids and its deleterious side-effects [56,57]. Deeper understanding of the molecular mechanisms for improving myogenesis and muscle repair may also be beneficial for treating muscle atrophy in aging, cancer and AIDS [58].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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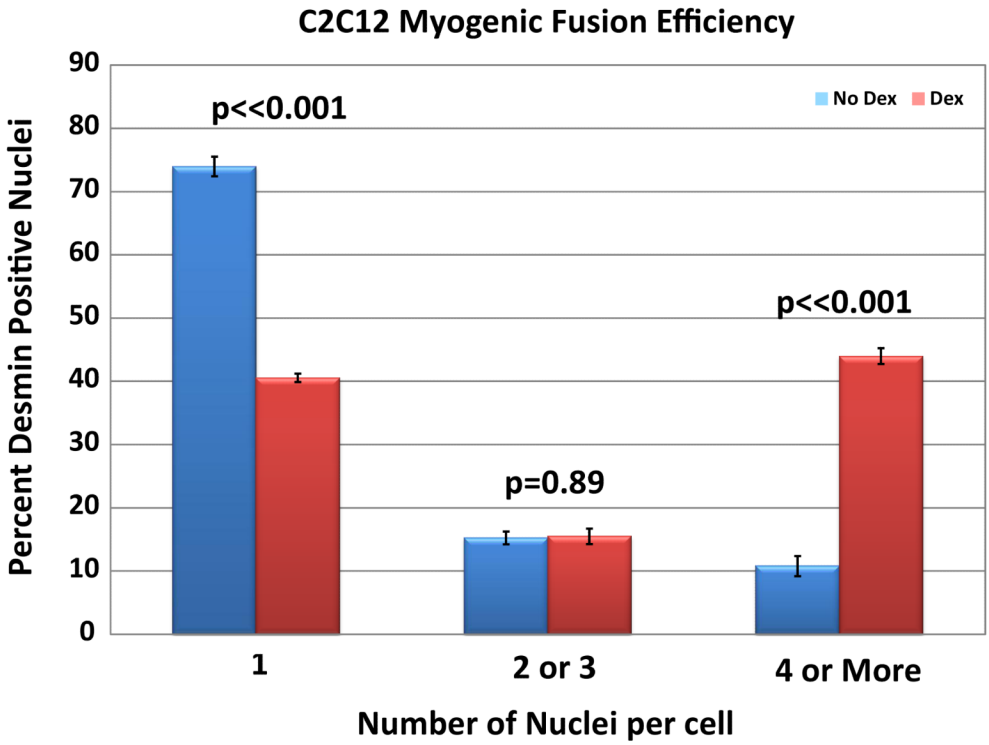


**Figure 1.**

Myogenic fusion efficiency showing percentage of nuclei present within singly nucleated cells, those within cells containing two or three nuclei, and those within cells with four or more nuclei. Dex-treated cells showed a significant increase in myotube formation as evidenced by the marked increase in nuclei present within cells with four or more nuclei over that of untreated cells. A total of 1930 nuclei were counted from untreated cells and 2116 nuclei were counted from Dex-treated C2C12-1 cells three days after switching to differentiation media. Error bars represent standard error. P-values greater that $p<0.05$ were considered significant.

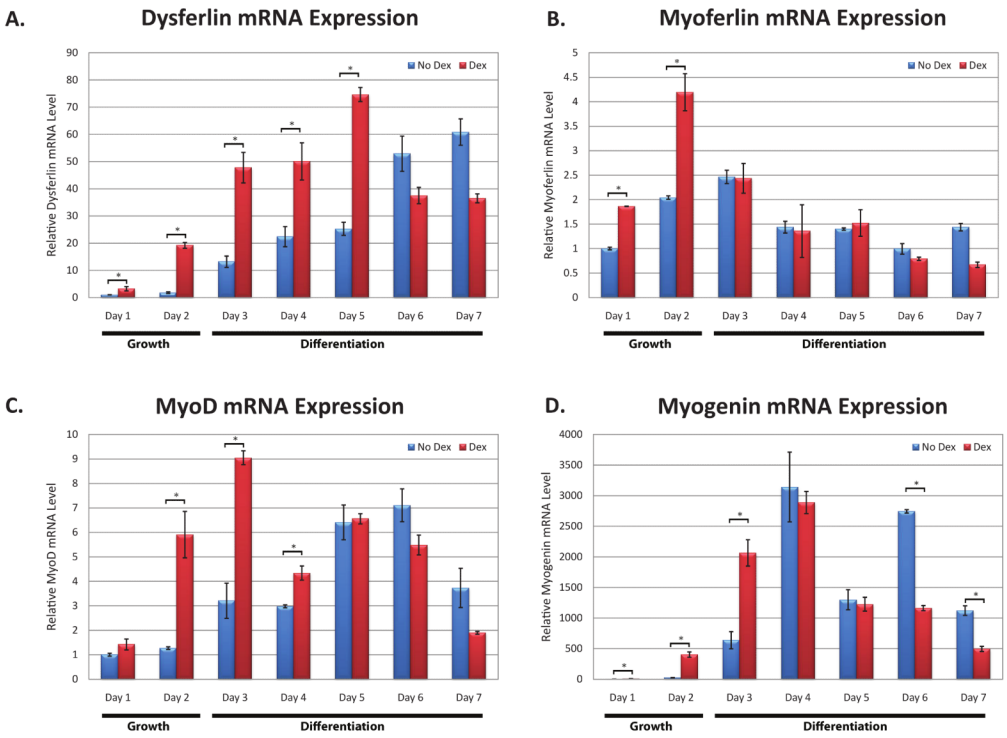


**Figure 2.**

Real time PCR analysis of mRNA levels in C2C12-1 cells growing in the presence or absence of 100nM Dex. A. Dex treatment significantly increased dysferlin mRNA levels in C2C12-1 cells over that of vehicle control treatment on Days 1 – 5 of the time course of differentiation. Differences in mRNA levels were not significant on Days 6 and 7. B. Dex treatment significantly increased myoferlin mRNA levels during proliferation. After the switch to differentiation media, differences in mRNA levels leveled out and were not significantly different between the cultures. C. Relative MyoD mRNA levels. D. Relative myogenin mRNA levels. Based on geNorm analysis of six candidate reference genes, mRNA levels were normalized to the reference genes GAPDH and eEF1ε1. qPCR data are graphed as fold induction relative to Day 1, vehicle control-treated C2C12-1 cells from three assays each run in triplicate. Error bars represent standard error. Differences greater than $p < 0.05$ are indicated by the * and were considered significant.

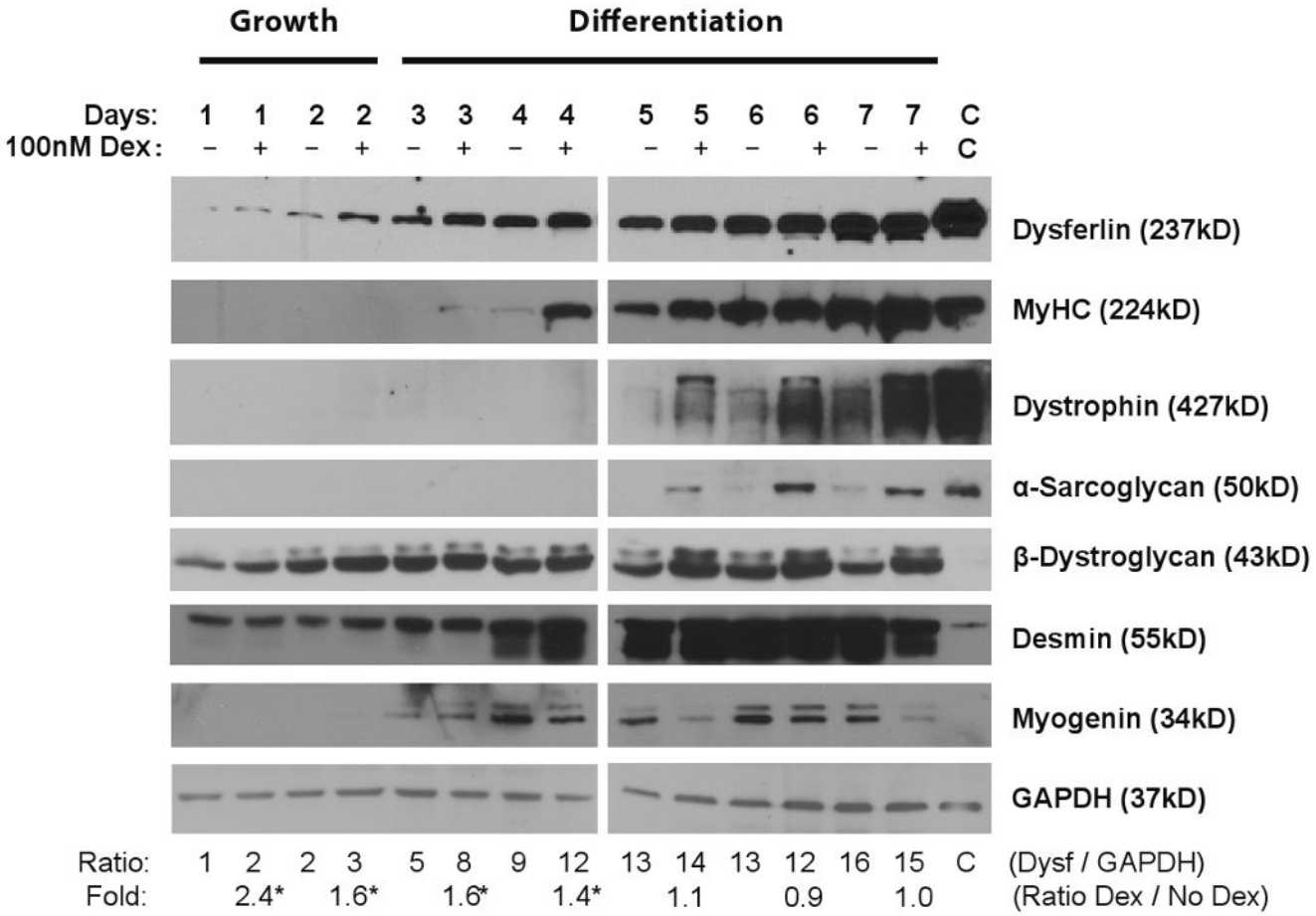


**Figure 3.**

Western blot analysis of dysferlin and myogenic proteins from the C2C12 time course in the presence or absence of 100nM Dex. Dex treatment increased dysferlin protein levels in C2C12 cells earlier in the time course of differentiation than vehicle control treatment. C2C12-1 cells were cultured with vehicle control (-) or 100nM Dex (+) and protein extracts were prepared from each day of the time course (Days 1 – 7). After harvesting the Day 2 time point, the rest of the cultures were switched from proliferation media to differentiation media for Days 3 – 7. Sixty micrograms of protein were loaded for each time point and the samples were loaded pairwise: minus and plus Dex for each time point. “C” represents mouse muscle control extract. GAPDH protein levels are shown as the loading control. Ratios of pixel intensities in each dysferlin band normalized to the corresponding internal control GAPDH band are shown below the immunoblots.

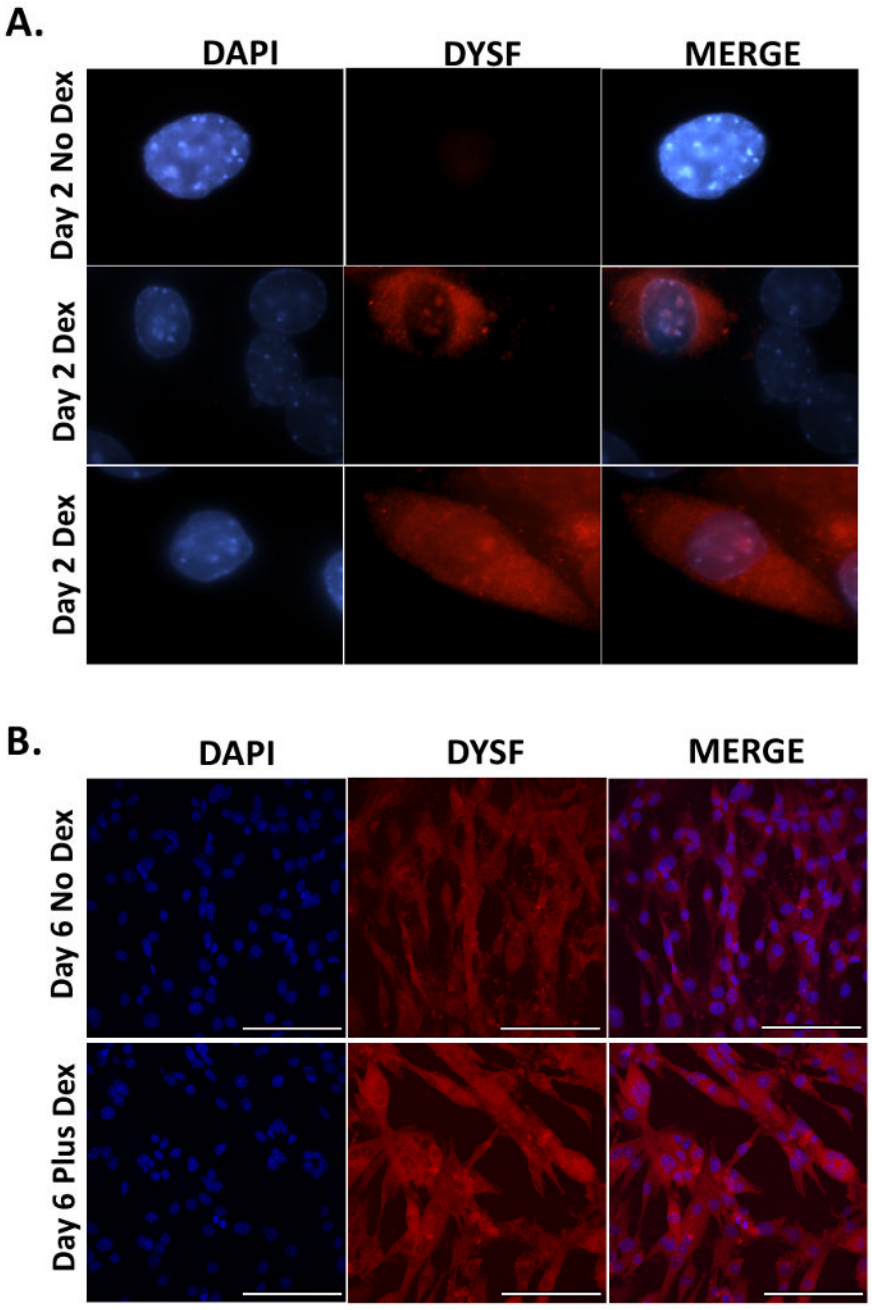


**Figure 4.**

Immunofluorescence and confocal microscopy images of dysferlin in C2C12-1 cells treated with vehicle control or 100nM Dex. Dex treatment increased dysferlin levels and enhanced the size of C2C12-1 myotubes compared to vehicle control-treated cells. A. Immunofluorescence microscope images of C2C12-1 on Day 2 of time course. C2C12-1 cells growing in proliferation media with vehicle control (top panels) or 100nM Dex (middle and bottom panels), 100× magnification. B. Confocal microscope images of C2C12-1 cells on Day 6 of time course: vehicle control (top panels), and 100nM Dex (bottom panels), 40×, bar represents 75 μm. Rabbit polyclonal anti-dysferlin antibody was used with Texas Red-

conjugated secondary antibody. All samples were counterstained with the nuclear stain, DAPI.

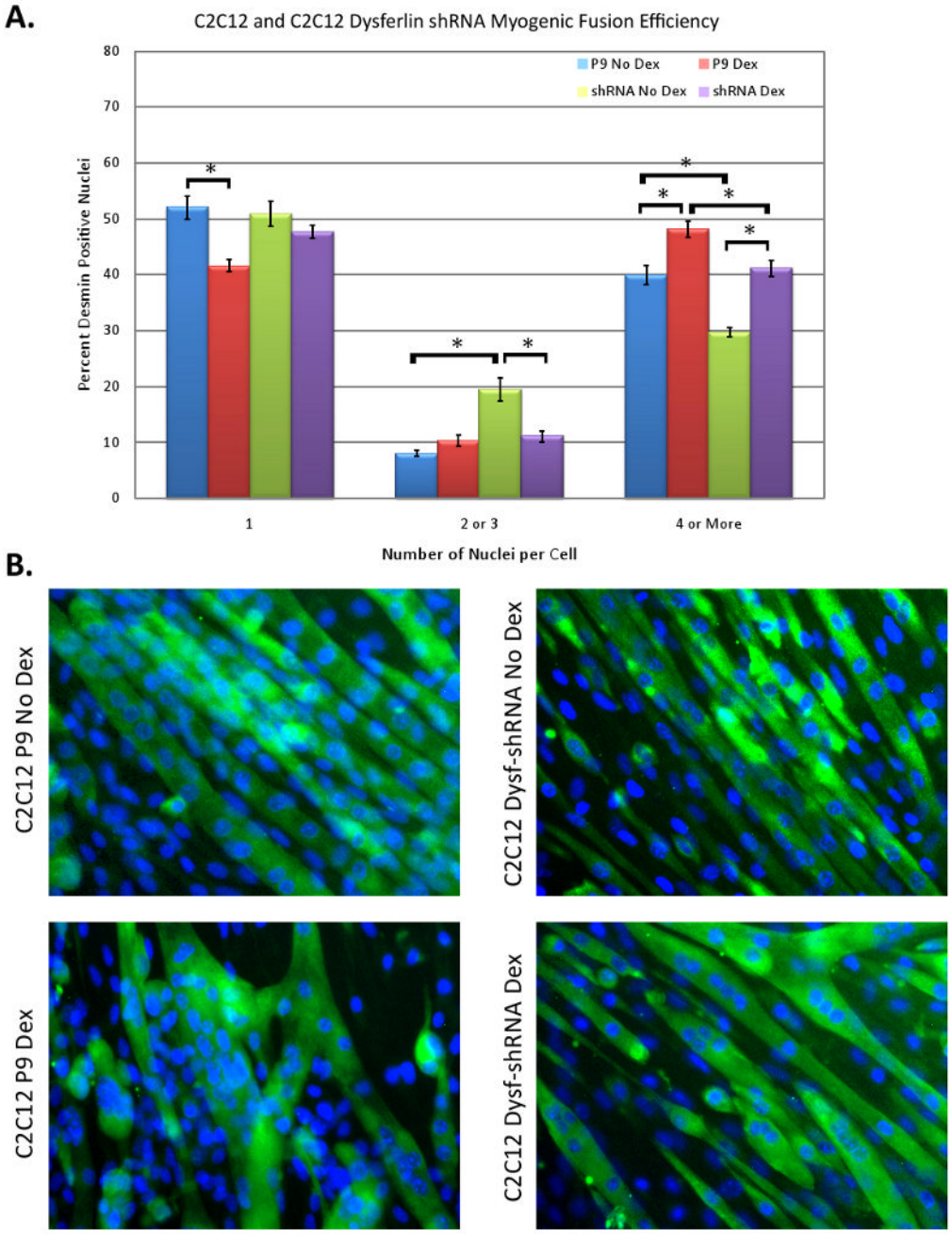


**Figure 5.**

Myogenic fusion efficiency was decreased in dysferlin-deficient C2C12 cells. Myogenic fusion efficiency was determined three days after the switch to differentiation media (Day 5 of time course). Percentage of DAPI-stained nuclei present within desmin-immunostained singly nucleated cells, those within cells containing two or three nuclei, and those within cells with four or more nuclei are shown in the graph. A. Dysferlin-deficient C2C12 cells (shRNA) showed decreased myogenic fusion of mature myotubes (4 or more nuclei) compared to parental C2C12 P9 cells even with Dex treatment. B. Representative immunofluorescent images from parental C2C12 P9 cells and dysferlin-deficient C2C12 cells in the presence or absence of 100nM Dex are shown in lower panels. Desmin

immunostaining appears green and DAPI is blue. A total of 1926 nuclei and 2672 nuclei were counted from vehicle control-treated parental C2C12 P9 and dysferlin-deficient C2C12s, respectively. A total of 1780 nuclei and 2178 nuclei were counted from Dex-treated parental C2C12 P9 and dysferlin-deficient C2C12s, respectively. Error bars represent standard error. P-values greater that $p<0.05$ were considered significant.

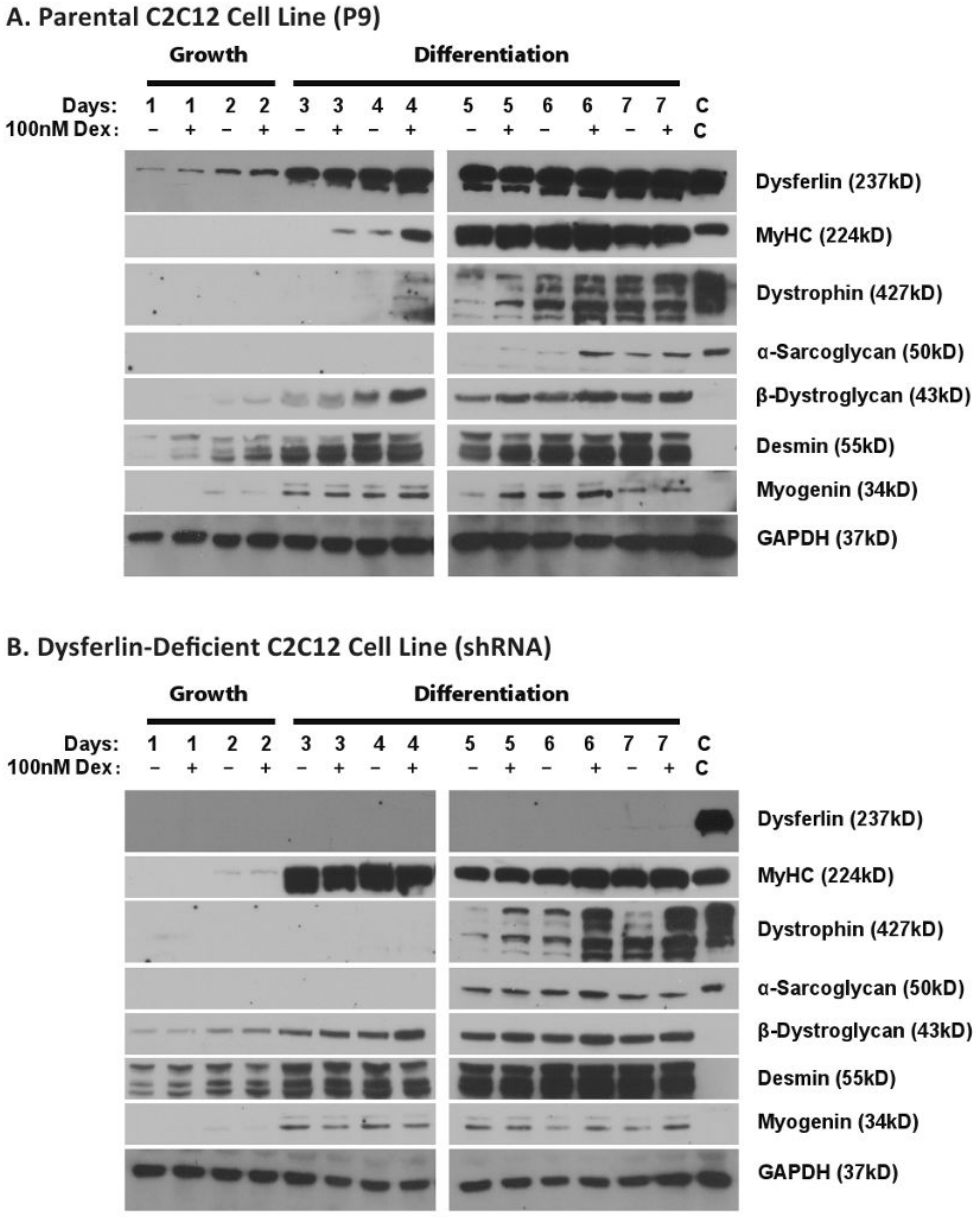


**Figure 6.**

Western blot analysis of dysferlin and myogenic differentiation proteins with GAPDH as a loading control during each day of the differentiation time course (Days 1 – 7) in parental C2C12 P9 cells and dysferlin-deficient C2C12 cells treated with vehicle control (-) or 100nM Dex (+). A. Parental C2C12 cells (P9). B. Dysferlin-deficient C2C12 cells (shRNA).