Absence of Dysferlin Alters Myogenin Expression and Delays Human Muscle Differentiation “in Vitro”*

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Mutations in dysferlin cause a type of muscular dystrophy known as dysferlinopathy. Dysferlin may be involved in muscle repair and differentiation. We compared normal human skeletal muscle cultures expressing dysferlin with muscle cultures from dysferlinopathy patients. We quantified the fusion index of myoblasts as a measure of muscle development and conducted optic and electronic microscopy, immunofluorescence, Western blot, flow cytometry, and real-time PCR at different developmental stages. Short interference RNA was used to corroborate the results obtained in dysferlin-deficient cultures. A luciferase reporter assay was performed to study myogenin activity in dysferlin-deficient cultures. Myoblast fusion was consistently delayed as compared with controls whereas the proliferation rate did not change. Electron microscopy showed that control cultured cells at 10 days were fusiform, whereas dysferlin-deficient cells were star-shaped and large. After 15 days the normal multinucleated appearance and structured myofibrils were not present in dysferlin-deficient cells. Strikingly, myogenin was not detected in myotubes from dysferlin-deficient cultures using Western blot, and mRNA analysis showed low levels (p < 0.05) compared with controls. Flow cytometry and immunofluorescence also showed reduced levels of myogenin in dysferlin-deficient cultures. When the dysferlin gene was knocked down (~80%), myogenin mRNA leveled down to ~70%. MyoD and desmin mRNA levels in controls and dysferlin-deficient cultures were similar. The reporter luciferase assay demonstrated a low myogenin activity in dysferlin-deficient cultures. These results point to a functional link between dysferlin and myogenin, and both proteins may share a new signaling pathway involved in differentiation of skeletal muscle in vitro.

Dysferlin is a 230-kDa protein located at the cell membrane of muscle cells. Mutations in the dysferlin gene cause a heterogeneous group of muscular dystrophies named dysferlinopathies (1–4).

The homology of dysferlin with fer-1 of Caenorhabditis elegans suggests a role for this protein in the fusion of intracellular vesicles with the sarcolemma (5). Dysferlin has recently been reported to be implicated in skeletal muscle membrane repair (6, 7). Studies performed using the mouse cell line C2C12 showed that dysferlin was expressed at low levels in myoblasts and at high levels in mature myotubes (8). In a previous study, in normal primary human muscle cultures, we reported that both dysferlin mRNA and protein expression were higher in multinucleated myotubes than at the myoblast stage, suggesting dysferlin plays a role in muscle differentiation (9).

The myogenic regulatory factors (MRFs) contain a conserved sequence-specific DNA binding domain and a helix-loop-helix motif required for heterodimerization. Two groups of MRFs are expressed in a temporally distinct pattern. Primary MRFs (MyoD and Myf-5) are required for myogenic determination, whereas the secondary MRFs (myogenin and Myf-4) are needed downstream of MyoD and Myf-5 as differentiation factors (10). Null mutations in the myogenin gene cause a severe reduction of skeletal muscle mass, showing that myogenin is essential for muscle development in vivo (11). Mutations in MyoD, Myf-5, and MRF-4 do not lead to morphological abnormalities of skeletal muscle (12–14).

In this study, we analyzed human skeletal muscle cultures from dysferlinopathy patients and normal controls at different developmental stages. Morphological changes and differences in the expression of the MRFs, MyoD as a proliferation muscle marker and myogenin as a differentiation marker, were studied using different methods.

EXPERIMENTAL PROCEDURES

Primary Human Skeletal Muscle Cultures—Muscle biopsies from three normal controls (Dysf<sup>-/-</sup>) and from three sex and age-matched dysferlinopathy patients (Dysf<sup>+/−</sup>) with total absence of the protein in Western blot (W-B) and immunohistochemical analysis were used for this study. All muscle biopsies were taken from triceps muscle.

Human muscle biopsies were minced and cultured in a multilayer according to the method described by Dr. Askanas (15). Briefly, the culture medium for the myoblast stage contains 75% Dulbecco’s modified Eagle’s medium and 25% FBS, 10 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, 10 ng/ml epidermal growth factor, and 25 ng/ml fibroblast growth factor.

To obtain highly purified myoblasts, primary cultures were sorted for the early surface marker CD56 by immunomagnetic selection. Each 10<sup>7</sup> cells were mixed with 20 μl of CD56-coated microbeads (Milteny Biotec, Bergisch Gladbach, Germany) and incubated at 6–12 °C for 15 min. Unbound microbeads were removed by washing cells in excess PBS buffer followed by centrifugation at 300 × g for 10 min. The cell pellet was resuspended in PBS buffer to a concentration of 2 × 10<sup>6</sup> cells/ml before separation on a midMACS cell separator (Milteny Biotec, Bergisch Gladbach, Germany).

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2 The abbreviations used are: MRF, myogenic regulatory factor; FBS, fetal bovine serum; PBS, phosphate-buffered saline; FI, fusion index; EM, electron microscopy; W-B, Western blot; M.F.I., mean fluorescence index; siRNA, short interfering RNA; TK, thymidine kinase; RER, rough endoplasmic reticulum.

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CD56-positive cells were seeded at 2000 cells/cm² using the culture medium for the myoblast stage containing 15% of FBS and without growth factors to avoid interference with the results. At day 10, when the myoblasts started to fuse, the medium was substituted with one containing 2% of FBS. The medium was changed twice a week, and the muscle cultures were examined to confirm confluent growth of myoblasts or myotubes. Each condition was performed in duplicate.

We measured the fusion index (FI) at each stage (5, 10, and 15 days) by calculating the mean percentage of nuclei in myotubes in respect to the total number of nuclei (myoblasts + myotubes) found in five randomly chosen areas. A cell number count was conducted to analyze culture growth rate at each stage.

**Electron Microscopy (EM)**—Dysf+/+ and Dysf−/− myoblasts were seeded at 2000 cells/cm² in a Lab-Tek chamber slides of 4 wells (Nalge Nunc International, Naperville, IL) and were fixed in 3.5% glutaraldehyde for 30 min at 37 °C. Cells were postfixed in 1% OsO₄ for 30 min at Nunc International, Naperville, IL) and were fixed in 3.5% glutaraldehyde. Serial semi-thin (2 μm) sections were cut with a diamond knife and mounted onto slides and stained with 1% toluidine blue. Semi-thin sections were glued (Super Glue) to Araldite blocks and detached from the glass slide by repeated freezing (in liquid nitrogen) and thawing. Ultra-thin (0.05 μm) sections were prepared with an Ultracut UC-6 (Leica, Heidelberg, Germany), stained with lead citrate, and photographed on a Jeol 1010 CX electron microscope.

**Immunofluorescence**—Cells cultured on glass coverslips were fixed in acetone:methanol (1:1), and immunofluorescence of human skeletal muscle culture was performed as described previously (16). Briefly, after 30 min in blocking solution, the samples were incubated overnight with a monoclonal antibody anti-desmin, anti-dysferlin (Novocastra, Newcastle upon Tyne, UK), and anti-myogenin (Santa Cruz Biotechnology Inc., Santa Cruz, CA), followed by 45 min with biotinylated horse anti-mouse IgG (5 μg/ml) (Vector Laboratories Inc., Burlingame, CA). The samples were rinsed with phosphate-buffered saline and incubated with avidin-fluorescein (1 μg/ml) (Vector Laboratories Inc.). Coverslips were then mounted in a glycerol medium containing polyvinyl alcohol (Fluoprep, BioMerieux, Marcy L’Etoile, France). As a negative control, sections were processed using either an isotype-matched monoclonal antibody to an unrelated antigen (monoclonal mouse anti-cytomegalovirus) or without a primary antibody but using the same secondary antibody. The nuclei were dyed with ethidium bromide. Finally, the samples were examined using an Axioskop 2 plus photomicroscope equipped with epifluorescence optics (Carl Zeiss Microscope, Munich, Germany).

**Western Blot**—Cell samples corresponding to each day analyzed (days 5, 10, and 15) were scraped from the human muscle culture dish and quickly weighed and homogenized twice for 15 s in 19 volumes of treatment buffer containing 0.125 mol/liter Tris/HCl buffer, pH 6.4, 10% glycerol, 4% SDS, 4 mol/liter urea, 10% mercaptopetoehanol, and 0.001% bromphenol blue (final pH of treatment buffer was 6.8) as described previously (17). The samples were placed in boiling water for 3 min and centrifuged at 9500 × g for another 3 min before 30 μl aliquots of the supernatants (equivalent to ~200 μg of non-collagen protein) were applied to each lane. Unspecific binding sites on the blots were blocked by incubation in 5% low fat dried milk powder in a phosphate buffer saline. The primary mouse monoclonal antibody was then added. Peroxidase-conjugated anti-mouse secondary antibody (Dako P260, Glostrup, Denmark) was applied at a 1:300 dilution when using anti-dysferlin antibody (Novocastra) and at a 1:10,000 dilution when using anti-β-actin (Sigma, Steinheim, Germany) as a primary antibody. When anti-myogenin antibody (BD Biosciences) was used as a primary antibody, the secondary antibody was a horse anti-mouse labeled to biotin (Vector Laboratories, Inc.). Immunoreactive bands were visualized by an enhanced chemiluminescence system (Pierce). Since myogenin and β-actin have a similar molecular mass (34 and 42 kDa, respectively), the membrane was first incubated with myogenin. After development, membranes were briefly washed with PBS and incubated for 15 min with Restore Western blot stripping buffer (Pierce) to eliminate bound myogenin antibody. Membranes were then incubated with the antibody anti- β-actin, as indicated above.

**Flow Cytometry**—One-color flow cytometry was performed for the detection of human myogenin. Monoclonal primary antibody anti-human myogenin (clone D-10, mouse IgG3, Santa Cruz Biotechnology Inc.) and an IgG isotype control were used to stain myotubes from both normal controls and dysferlinopathy patients. Cells were trypsinized, fixed with cold 4% formaldehyde in PBS for 1 h at 4 °C, and permeabilized with 0.4% Triton-X-100 for 15 min at 37 °C. Positive signals were detected by adding fluorescein isothiocyanate-labeled goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK). Cells were finally acquired and analyzed in a FACSscan cytomter (BD Biosciences). Expression levels of myogenin were referred to as mean fluorescence index (M.F.I.).

**RNA Extraction and Real-time PCR**—RNA from muscle cultures at the three stages (days 5, 10, and 15) was extracted using Ultraspec (Bio-tech Laboratories, Inc., Houston, TX) following the manufacturer’s instructions. Total RNA (0.2 μg) of muscle cultures at each stage was reverse transcribed into cDNA using TaqMan reverse transcriptase (Applied Biosystems, Inc.). Quantification of the mRNA coding for dysferlin, desmin, MyoD, myogenin, and 18S as internal standard was performed using TaqMan Universal Master Mix technology (Applied Biosystems). Quantitative PCR was performed in a total reaction volume of 20 μl per well. The primers used for real time PCR were designed by Applied Biosystems: dysferlin, Hs 00243339_ml; MyoD, Hs 00159528_ml, myogenin Hs 00231167_ml, desmin Hs 00157258_ml, and 18S Hs 99999901_s1 (Applied Biosystems). The comparative Ct method (ΔΔCt) for relative quantification of gene expression was used. As long as the target and normalizer (18S) have similar dynamic ranges, this method is the most practical. The calculations for the quantification start with differentiating (ΔCt) between the target Ct values and the normalizer: 

\[ \Delta C_t = C_t - C_n \]

where C_t is the Ct value for each sample. A pool of myoblast Dysf+/+ was used as a reference (base line). The comparative ΔΔCt calculates the difference between sample ΔCt value and the base-line ΔCt. Finally, the comparative expression level was obtained transforming the logarithmic values to absolute values using 2−ΔΔCt. The *t* test was used for statistical analysis.

**RNA Interference Dysferlin Knockdown**—The oligonucleotides for the construction of a short interfering RNA (siRNA) were designed following the manufacturer’s instructions for the Silencer siRNA construction kit (Ambion, Austin, TX). The sense and antisense siRNA oligonucleotide templates for dysferlin were 5′-AAGAAAGAGAAC-CCAAGTCTATCCCGTTC-3′ and 5′-AGATGACTTTGGTTCTTCTTCCCTGTTC-3′. The luciferase gene was used as a negative control, and the oligonucleotide templates used to create the double-stranded
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RNA were: 5′-AACCTGGAAGTATACGGTGTGCCTGTCTC-3′ and 5′-ACACGGTATACGGTGAACGCTGTCTC-3′. The siRNA triggers degradation of homologous mRNA by the formation of double-stranded RNA. 25,000 cells/cm² were transfected with siRNA/siPORT Amine (Ambion) at a concentration of 20 nM/6 μL. The appropriate transfection agent was determined previously, demonstrating that the amine siPORT transfection agent (Ambion) provided a stronger silencing effect as compared with the lipid siPORT transfection agent (Ambion) in these cells. Muscle cultures were incubated with siRNAs mix and the transfection agent alone and analyzed at 24, 48, and 72 h.

Construction of Luciferase Reporter Gene Plasmids—We used RNA from control myotubes as a template to amplify a 329-bp fragment containing an E-box (CAGCTG), which is recognized by myogenin. An retrotranscriptase PCR reaction using previously described primer pair (18) that appended unique BglII and NheI sites on the 5′ and 3′-ends was performed. The amplified sequence was digested using BglII and NheI restriction enzymes (Promega, Madison, WI) opened with the same set of enzymes. The ligation reaction was performed using T4 DNA ligase (Promega). The correct open reading frame and the orientation of the cloned fragment were confirmed by DNA sequencing. The plasmid pGL3-basic containing the E-box recognized by myogenin was purified using endotoxin-free Maxiprep (Qiagen, Valencia, CA).

Transient Transfection—Myoblasts from Dysf+/+ and Dysf−/− were seeded at 5000 cells/cm² in 24-well plates 1 day before the transfection. Cells were transfected with 0.5 μg of RNA using FuGENE 6 (Roche Diagnostics) at a ratio of 1:6. As internal control for each transfection, cells were co-transfected with 10 ng of the plasmid RL-TK (Promega) at a ratio of 1:6. As internal control for each transfection, cells were co-transfected with 10 ng of the plasmid RL-TK (Promega) expressing Renilla under the herpes simplex virus promoter.

Luciferase Assay—The culture medium was discarded after 48 h, and cells were washed twice with PBS. For the luciferase assay, we used the dual-luciferase reporter assay system (Promega). Transient transfectants were harvested with lysis buffer, and the luciferase activity was measured with a luminometer (VICTOR 3, PerkinElmer Life Sciences). The values obtained were normalized with Renilla activity. We used the pGL3-basic vector without insert as a negative control.

RESULTS

Myotube Formation Is Slower in Dysf−/− than in Dysf+/+ Muscle Cultures—The analysis of cellular growth of Dysf−/− human muscle cultures compared with Dysf+/+ cultures showed no significant differences in the proliferation rate (Fig. 1A). However, at days 10 and 15, while the FI increased considerably in Dysf−/− cells, the number of lysosomes and vacuoles was higher than in Dysf+/+ muscle cultures, myoblasts started to fuse later than in those of Dysf+/+ (p < 0.05). Myoblasts in Dysf−/− cultures started to fuse at a very early stage in muscle development, while those in the Dysf+/+ cultures started to fuse at day 15.

Electron Microscopy Revealed Abnormal Differentiation of Dysf−/− Primary Cultures—Morphological differences were appreciated in semi-thin sections and EM at 10 and 15 days of culture. At day 10, while Dysf+/+ cultures were mostly homoromp, with a diameter of 10–20 μm and often multinucleated, Dysf−/− myoblasts were in general star-shaped and had a diameter of 30–50 μm. At EM, Dysf−/− myoblasts showed enlarged rough endoplasmic reticulum (RER) cisterns, large Golgi complexes, and a heterogeneous population of lysosomes. Smooth endoplasmic reticulum was greatly developed. In contrast, in Dysf+/+ cells, RER cisterns were not enlarged or only slightly enlarged. Lysosomes were not abundant, and the heterogenous shapes found in Dysf−/− were not observed.

At 15 days of culture, analysis by light microscopy demonstrated some myotube Dysf+/+ containing up to eight nuclei (Fig. 2A). However, in Dysf−/− muscle cultures the majority of myotubes were binucleated (Fig. 2B). Interestingly, some of the images observed resembled fusion phenomena by phagocytosis. Indeed, many picnotic nuclei were found, in agreement with the scarce number of cells bearing more than one nucleus in Dysf−/− cultures. At this point, Dysf+/+ cells had a more prominent fusiform shape, whereas Dysf−/− cells remained star-shaped. The myofibril structure was clearly observed in Dysf+/+ cells (Fig. 2, C and E) but was not visible in Dysf−/− cells. Myofibril structure was disorganized in Dysf−/− cells (Fig. 2D). RER remained enlarged in Dysf−/− muscle cells, and the number of lysosomes and vacuoles was higher than in Dysf+/+ (Fig. 2F). Mitochondrial morphology was also very irregular in Dysf−/− cultures at 15 days, compared with that of Dysf+/+ cells.

Dysferlin Expression Is Absent in Myotube Dysf−/−—Immunofluorescence results showed that there were no dysferlin expression in myoblasts in control cultures at days 5 and 10. When myoblasts started to fuse to form myotubes, dysferlin expression increased, reaching its maximum levels at day 15 following the pattern previously described (9). Interestingly, dysferlin accumulated at the growing bud (end of the myotube) from day 15 (Fig. 3, A–C). In Dysf−/− cultures, we did not detect dysferlin expression at any of the three stages (days 5, 10, and 15) (Fig. 3, D–F).

Dysferlin expression in human primary muscle cultures from control biopsies was detectable from day 10 using W-B analysis. Protein expression remained at similar levels until day 15. W-B of dysferlin in Dysf−/− muscle cultures showed no bands corresponding to the molecular mass
of dysferlin or lower at any of the stages analyzed either in myoblasts or in myotubes (data not shown).

**MyoD Expression Is Normal, but Myogenin Is Reduced in Dysf−/− Muscle Cultures**—MyoD and desmin mRNA levels were no different from those of control cultures. MyoD expression in control cultures was higher in the proliferation than in the differentiation stages, reaching its maximum levels at day 5. MyoD expression showed the same pattern in Dysf−/− cultures at all the days analyzed, with slightly reduced levels as compared with controls. However, these differences were not statistically significant.

Myogenin expression was tested at days 5, 10, and 15 in Dysf−/− and Dysf−/− myoblasts or in Dysf−/− myotubes. Dysferlin antibody was incubated with a secondary antibody labeled with fluorescein isothiocyanate, and the nuclei were dyed with ethidium bromide. A–C, dysferlin immunofluorescences from control skeletal muscle cultures (Dysf+/+). Dysferlin staining was present in myotube stages of Dysf+/+ muscle cultures (day 15). An accumulation of dysferlin was observed in the growing pole of myotube. This region was involved in fusion phenomena of myoblasts (arrowheads). D–F, dysferlin immunofluorescence from dysferlin-deficient skeletal muscle cultures (Dysf−/−). Dysferlin was absent in all stages of development of skeletal muscle cultures. Scale bar = 5 μm.
tubes using an anti-myogenin antibody showed that the nuclei staining were weaker than in control myotubes (Fig. 4, B and C).

Using flow cytometry, we then examined whether myogenin expression in control myotubes was higher than in Dysf<sup>−/−</sup>. A flow cytometry assay with an anti-human myogenin antibody showed a decreased expression of myogenin in the Dysf<sup>−/−</sup> myotubes (M.F.I.: 12.4) compared with Dysf<sup>+/+</sup> myotubes (M.F.I.: 20.3) (Fig. 4D).

Similar results were obtained when mRNA levels were studied, showing that a reduced expression of myogenin paralleled a reduction of dysferlin mRNA in Dysf<sup>−/−</sup> muscle cultures (p < 0.05) (Fig. 5, A and B).

**Dysferlin siRNA Effectively Reduces Dysferlin mRNA Levels**—To further demonstrate the link between dysferlin and myogenin mRNA expression, we performed real-time PCR on cultures treated with dysferlin siRNA. Dysferlin mRNA was not reduced in the first 24 h. However, after 48 h of treatment with dysferlin siRNA, cultures showed a significant reduction of dysferlin mRNA (~80%) compared with those treated with luciferase siRNA (p < 0.01). This observation paralleled with a reduction of myogenin of ~70% (p < 0.01) observed in cultures treated with dysferlin siRNA (Fig. 6). At 72 h, both dysferlin and myogenin mRNA reached normal levels of expression. As

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**FIGURE 4.** Western blot, immunofluorescence, and flow cytometry of Dysf<sup>+/+</sup> and Dysf<sup>−/−</sup> muscle cultures shows reduced expression of myogenin in the absence of dysferlin. A, Cells corresponding to the three different stages were processed for W-B. 200 μg of non-collagen protein was added in each lane, and antibody anti-β-actin was used as a loading control. In Dysf<sup>+/+</sup> cultures, myogenin was absent in the early stages of myoblasts (day 5) and was detected when myotubes were abundant (day 15). Myogenin could not be detected in Dysf<sup>−/−</sup> extracts from any of the days analyzed and presented a severe reduction even at the myotube stage. B, Dysf<sup>+/+</sup> myotube nuclei showed positive staining for myogenin. C, myogenin expression was reduced in Dysf<sup>−/−</sup> myotubes. D, acquisition and analysis of Dysf<sup>+/+</sup> and Dysf<sup>−/−</sup> myotubes by flow cytometry showed a decrease in the expression of myogenin in myotube Dysf<sup>−/−</sup> (M.F.I.: 12.4) with regard to Dysf<sup>+/+</sup> myotubes (M.F.I.: 20.3). Scale bar: 50 μm.
a control, we analyzed desmin mRNA expression and no differences were found between cultures treated with dysferlin siRNA or luciferase siRNA. At 24, 48, and 72 h we also analyzed the FI, and no differences were found between muscle cultures treated with dysferlin siRNA and the controls.

**Luciferase Reporter Activity Containing an E-box Activated by Myogenin**

**Was Lower in Dysf−/− Patients**—To demonstrate the reduction of myogenin levels observed in Dysf−/− cultures, we performed a functional study using a pGL3-basic luciferase reporter plasmid containing a specific E-box as a myogenin target. Luciferase assays demonstrated significant differences between Dysf+/+ and Dysf−/− cultures. The reporter gene was highly expressed in Dysf+/+ cultures, indicating that myogenin had a normal activity. In contrast, in Dysf−/− cultures the reporter expression of firefly Renilla activated by myogenin was significantly decreased (p < 0.001) (Fig. 7). This result suggests that myogenin down-regulation in Dysf−/− cultures may have functional implications.

**DISCUSSION**

Our study in human skeletal muscle primary cultures shows that the absence of dysferlin remarkably alters the process of muscle differentiation in vitro and points to a signaling pathway that would involve dysferlin and myogenin. The cultures from the three Dysf−/− muscles showed complete absence of dysferlin expression in the cellular membrane both in myoblasts and myotubes at all stages analyzed. In contrast, the three normal primary cultures showed that dysferlin was progressively up-regulated from the myoblast to the myotube stage, as described previously (9). The absence of dysferlin seen in vitro correlated with the absence of dysferlin in vivo in the muscle biopsy of the three patients studied both by immunofluorescence and W-B.

By means of immunohistochemistry, we also found an accumulation of dysferlin at the sites of the myotubes where myoblasts fuse (growing buds) in Dysf−/− muscle cultures, suggesting the involvement of dysferlin in fusion events. Semiquantitative real-time PCR showed a low expression of dysferlin mRNA in Dysf−/− muscle cultures at the myotube stage. This was probably due to an instability of dysferlin mRNA and may explain why we did not detect the protein product by W-B or immunohistochemistry.

In view of the lack of expression of dysferlin at the myoblast stage in normal muscle, we did not anticipate significant differences between Dysf−/− and Dysf+/+ cultures, and in fact, the proliferation rate in Dysf−/− myoblasts was similar to that of controls. Accordingly, the levels of the primary MRF MyoD did not differ from Dysf+/+ cultures.
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In contrast, in Dysf\(^{-/-}\) cultures, myoblasts showed a reduced FI as compared with control cultures. Strikingly, Dysf\(^{-/-}\) cultures displayed a reduction of myogenin mRNA levels by semiquantitative real-time PCR, and the protein was undetectable by W-B. These findings were confirmed at the protein level using immunohistochemistry and flow cytometry, which showed a severe reduction of myogenin in Dysf\(^{-/-}\) myotubes. These results suggest that myogenin could be involved in the defective muscle differentiation observed in Dysf\(^{-/-}\) cultures, measured as lower FI.

When we analyzed myogenin mRNA levels at early stages (days 5 and 10), we found that it was low both in Dysf\(^{-/+}\) and Dysf\(^{-/-}\) muscle cultures, in agreement with the function of this secondary MRF, which is expressed with the differentiation toward the myotube stage.

In addition, using a luciferase activity-based assay, we demonstrated a diminished activity of myogenin only in Dysf\(^{-/-}\) myotubes. This potential link between dyserlin and myogenin was further confirmed by siRNA assays, since the knockdown of dyserlin gene in vitro conveyed a myogenin mRNA reduction. All together, these findings suggest that dyserlin is somehow involved in the muscle differentiation pathway conducted by myogenin.

As described previously, myogenin expression in Dysf\(^{-/-}\) cultures was up-regulated during myoblast differentiation both in culture and in vivo (19), in a similar pattern to dysferlin expression in vitro (9). Continuous cell lines of myoblasts isolated from myogenin mutant embryos from null mice deficient in myogenin normally differentiate in culture (20), indicating that various myoblast differentiation pathways must co-exist in vitro. In vivo, homozygous mutant myogenin null mice die perinatally due to a severe defect in the diaphragm. These mice present a significant reduction in skeletal muscle mass throughout the body (11, 20). However, in the present study, the number of myoblasts appeared to be normal, confirming that myogenin is not required for lineage commitment but is needed for terminal differentiation. In agreement with these observations, the ultrastructure of Dysf\(^{-/-}\) myoblasts/myotubes differed from that of control cultures. Our findings in Dysf\(^{-/-}\) muscle cultures correlated with the original description of muscle biopsies from Miyoshi myopathy patients in which abnormalities of Z-disk, loss of myofilaments, dilation of RER, and sarcocellular vacuoles were observed (21). Myofiber disorganization and absence of Z-lines is also observed in homozygous mutant myogenin null mice (11, 20). Together with other ultrastructural disturbances, the absence of properly organized myofilibrils at day 15 confirms the impairment in muscle differentiation observed in skeletal muscle cultures from dysferlinopathy patients.

Myogenin is a member of the basic helix-loop-helix gene family, which is essential for muscle development. In adult skeletal muscle myogenin expression is coincidental with satellite cell differentiation and fusion (22), and we have reported that dyserlin is expressed in activated satellite cells in adult skeletal muscle (9) indicating its role in muscle regeneration. In addition, dyserlin is involved in sarcocellular repair mechanisms (6). The interaction of dyserlin with annexins A1 and A2 indicates that they may play a role in the aggregation and fusion of intracellular vesicles in response to membrane injury (7). Caveolin-3, which has been shown to interact with dyserlin (23), could also participate in vesicle fusion to the sarcolemma. Dyserlin also interacts with affixin, an intracytoplasmic protein that accumulates in the disruption site of the membrane and plays an important role in wound healing (24). All these data point to the hypothesis that dyserlin could act as a calcium sensor that regulates membrane repair machinery (25). Indeed, the calcium-sensitive interaction of the C2A domain of dyserlin with phospholipids supports its role in membrane fusion events. However, since dyserlin is a large protein, and the remaining five C2 domains do not necessarily bind Ca\(^{2+}\) and phospholipids, it is reasonable to hypothesize that they may participate in protein-protein interactions (8) and signal transduction pathways (26). The absence of dyserlin would impair these other functions and, among other effects, disrupt myogenin expression. Further studies are needed to elucidate which proteins/factors interact with C2 domains of dyserlin to positively regulate myogenesis by increasing myogenin gene transcription.

In conclusion, this study supports the role of dyserlin in human muscle fusion and differentiation in vitro. Furthermore, it provides evidence of a link between dyserlin and myogenin and suggests they share a signaling pathway involved in differentiation of skeletal muscle in vitro.

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