1α,25(OH)$_2$-Vitamin D3 Increases Dysferlin Expression *in vitro* and in a Human Clinical Trial

Noemi De Luna[1,2], Jordi Díaz-Manera[2,3], Carmen Paradas[2,4], Cristina Iturriaga[2,3], Ricardo Rojas-García[2,3], Josefa Araque[1,2], Mireia Genebriera[1,2], Ignasi Gich[5], Isabel Illa[2,3] and Eduard Gallardo[1,2]

[1]Laboratori de Malalties Neuromusculars, Institut de Recerca Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Spain; [2]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED); [3]Servei de Neurologia Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona, Spain; [4]Servicio de Neurología, Hospital Universitario Virgen del Rocío, Sevilla, Spain; [5]Servei de Epidemiologia Clínica, Hospital de la Santa Creu i Sant Pau i Institut de Recerca de HSCSP, Universitat Autònoma de Barcelona, Spain

Dysferlinopathies are a heterogenous group of autosomal recessive inherited muscular dystrophies caused by mutations in *DYSF* gene. Dysferlin is expressed mainly in skeletal muscle and in monocytes and patients display a severe reduction or absence of protein in both tissues. Vitamin D3 promotes differentiation of the promyelocytic leukemia HL60 cells. We analyzed the effect of vitamin D3 on dysferlin expression *in vitro* using HL60 cells, monocytes and myotubes from controls and carriers of a single mutation in *DYSF*. We also performed an observational study with oral vitamin D3 in a cohort of 21 carriers. Fifteen subjects were treated for 1 year and dysferlin expression in monocytes was analysed before and after treatment. Treatment with vitamin D3 increased expression of dysferlin *in vitro*. The effect of vitamin D3 was mediated by both a nongenomic pathway through MEK/ERK and a genomic pathway involving binding of vitamin D3 receptor to the dysferlin promoter. Carriers treated with vitamin D3 had significantly increased expression of dysferlin in monocytes compared with nontreated carriers ($P < 0.05$). These findings will have important therapeutic implications since a combination of different molecular strategies together with vitamin D3 uptake could increase dysferlin expression to nonpathological protein levels.

Received 3 July 2012; accepted 11 July 2012; advance online publication 21 August 2012. doi:10.1038/mt.2012.156

INTRODUCTION

Muscular dystrophies are a heterogeneous group of genetic disorders.[1] Among them dysferlinopathies, have autosomal recessive inheritance and can present high phenotypic variability[2–4] including the existence of symptomatic carriers.[5] The onset of the disease is in the late teens or early adulthood although late onset[6] and congenital forms[7] have been reported. Clinical status progresses to an inability to walk without support or confinement to a wheelchair. There are no effective treatments for dysferlinopathy. So far, the only report of a therapeutic strategy in humans consisted of the depletion of B cells with rituximab in two patients that increased muscle strength in both of them.[8] The remaining therapeutically approaches reported to date have been performed in animal models. Treatment with etanercept, an agent that blocks tumor necrosis factor, resulted in dose-dependent reductions in inflammation, necrosis, and fatty/fibrous tissue in *SJL* mice model.[9] In cell-based therapies the levels of dysferlin expression reached were variable depending on the cell type transplanted. When murine wild-type myoblasts were injected into muscles of *SJL* mice a variable percentage of dysferlin expression was obtained ranging from 20 to 31% compared with controls.[10] After intravenous injection of human umbilical cord cells in *SJL* mice, <1% of skeletal muscle fibers expressed dysferlin.[11] Using human adipose stromal cells, skeletal muscle dysferlin mRNA levels reached up to 26% expression compared with controls.[12] Finally, a single intramuscular injection of murine adult-derived mesoangioblasts resulted in dysferlin expression of up to 35% compared with controls.[13] Gene therapy strategies allowed higher levels of protein expression. Transfection of complete *DYSF* using adenovirus-associated vectors showed recovery of dysferlin expression up to 59% at 2 months and 30% after 1 year. In addition, the authors found an improvement of the histological features of the muscle, with a reduced number of necrotic fibres, restoration of membrane repair after injury and amelioration of some functional tests;[14] increased levels of dysferlin expression were also obtained when a minidysferlin construct was transfected.[15]

Dysferlin is expressed mainly in the sarcolemma and T-tubules of skeletal muscle cells[16,17] and in peripheral blood monocytes (PBMs).[18] The possibility to study dysferlin expression in PBM constitutes a less invasive method to diagnose dysferlinopathies and eases the molecular study of *DYSF*.[19]

The HL60 cell line is derived from a single patient with acute promyelocytic leukemia and these cells proliferate continuously in suspension culture. 1 α, 25-Dihydroxyvitamin D3 (D3) induces differentiation of human myeloid leukemia cells.[20,21] D3 is also important for the homeostasis of intracellular calcium in skeletal muscle and participates in myogenesis and muscle contractility.[22] The effect of D3 on cells can be meditated through two different pathways.[23]: (i) A genomic pathway that is activated upon D3 binding to the vitamin D receptor (VDR) at the cell membrane,

The first two authors contributed equally to this work.

Correspondence: *Eduard Gallardo, Department of Neurology, Institut de Recerca Hospital de la Santa Creu i Sant Pau. C/ Pare Claret, 167, 08025 Barcelona, Spain. E-mail: egallardo@santpau.cat*

that internalizes and activates gene expression by direct binding to the promoter of different genes.[24] (ii) A nongenomic pathway through MEK/ERK.[25]

The VDR is a member of the nuclear receptor superfamily. VDR forms a heterodimer with retinoid X receptor and binds to the vitamin D response element (VDRE), which is present in the regulatory regions of some genes.[26,27] A previous study in which VDREs were mapped in the human genome, showed that the dysferlin promoter contains a consensus DR3-type VDRE.[28]

Our present study shows that cells exposed to D3 increase dysferlin expression. We have also studied dysferlin levels in PBM in patients with dysferlinopathy, carriers of one mutation in *DYSF* and healthy controls to determine the range of dysferlin expression in each group. Finally, we performed an observational study to determine if the administration of D3 had a biological effect and was able to increase dysferlin levels *in vivo*. In an asymptomatic group of single mutation *DYSF* carriers, chosen because their baseline levels of dysferlin were lower than normal, we demonstrated a significant increase in dysferlin levels in PBM after 1 year of oral administration of D3.

RESULTS

Vitamin D3 treatment increases dysferlin expression in HL60 cells that is dose and time dependent

D3 was added to the HL60 culture media at different concentrations, 10, 25, 50, and 100 nmol/l, and dysferlin expression was assessed at 24, 48, and 72 h. By real time PCR, at 24 h dysferlin mRNA expression increased in parallel with increasing concentrations of D3. The highest dysferlin mRNA levels were reached at 48 h, in HL60 cultures treated with 50 nmol/l and 100 nmol/l of D3, no significant differences were found between these two concentrations of D3 (**Figure 1a**).

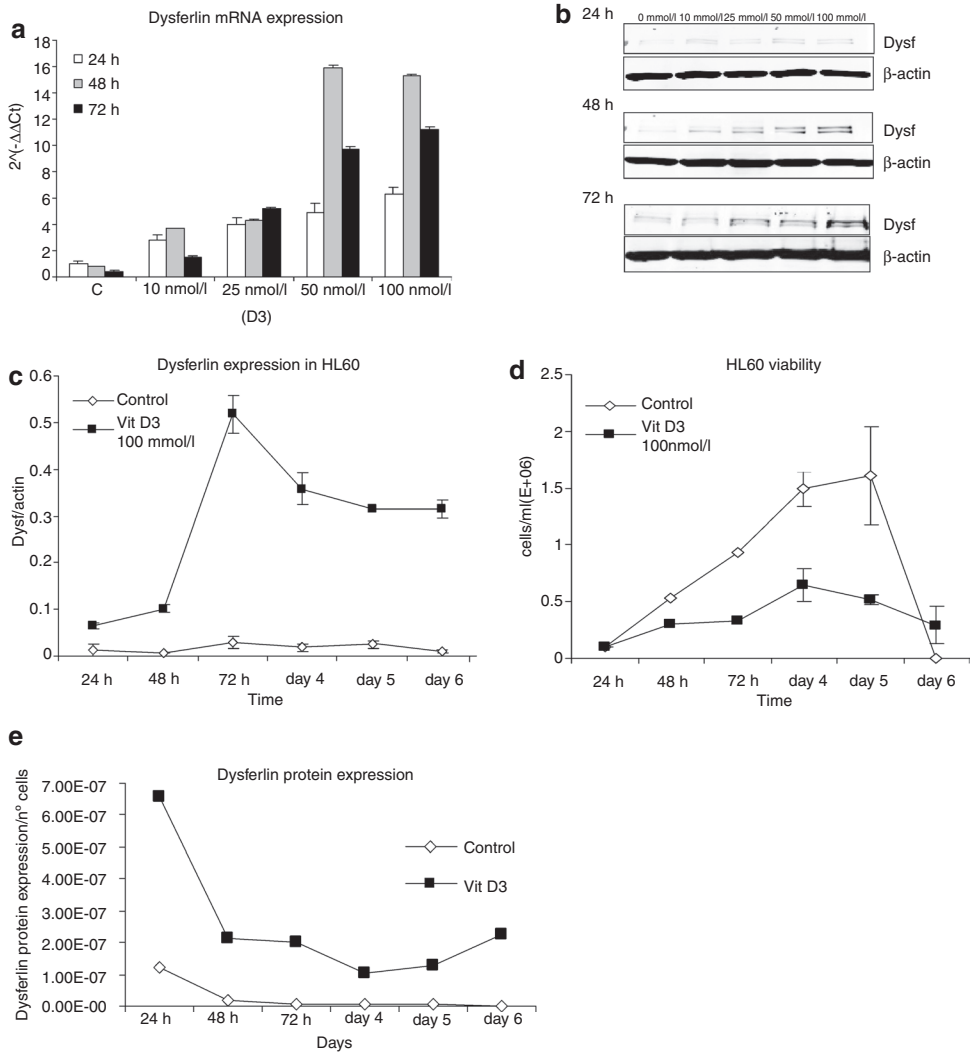

Figure 1 Dysferlin expression increased in HL60 cells after the addition of D3 to the culture. (**a**) Dysferlin mRNA analysis of HL60 cells treated with different concentrations of D3 showed that the highest levels were achieved when 50 nmol/l and 100 nmol/l were used during 48 h. (**b**) WB of HL60 D3-treated cells, showed that dysferlin expression leveled up in parallel with increasing concentrations of D3. (**c**) HL60 cells treated with 100 nmol/l of D3, reached a peak of dysferlin expression at 72 h and after that decreased until day 4 and remained at the same levels until day 6. (**d**) D3-treated HL60 cells stopped proliferating and their number remained constant until day 6. Nontreated cells proliferated exponentially until day 5 when cells started dying. (**e**) Dysferlin protein expression normalized with number of cells. Each experimental condition was independently performed three times. WB, western blot.

We also studied the expression of dysferlin by western blot (WB) (**Figure 1b**). At 24 h, no differences were observed using HL60 cells treated with 0, 10, 25, 50, and 100 nmol/l of D3. At 48 h, the higher levels of dysferlin were observed after treatment with 100 nmol/l D3 compared with controls. Maximum dysferlin expression was observed after 72 h of incubation with 100 nmol/l of D3. At this concentration of D3, we studied changes in dysferlin expression after a longer period of time. Dysferlin expression decreased significantly in HL60 cells treated with 100 nmol/l of D3 from day 3 to day 4 and remained unchanged until day 6 (**Figure 1c**). In nontreated cells, dysferlin expression remained stable (**Figure 1c**). As the maximum dysferlin protein concentration was achieved at 100 nmol/l of D3, we studied the effect of this concentration on HL60 cells proliferation. Cell number remained stable during the 6 days after the addition of 100 nmol/l of D3. In nontreated cultures, cell number increased at day 5 after which cell death started to occur (**Figure 1d**).

The increase of dysferlin expression in HL60 cells after vitamin D3 treatment is not dependent on cell differentiation

To elucidate if the increase of dysferlin expression after treatment with D3 is mediated through the nongenomic MAPK (mitogen activated protein kinase) kinase/MEK/ERK-signaling pathway, we treated HL60 cells with 10 and 20 µmol/l of 4-diamino-2, 3 dicyano-1, 4 bis (2-aminophenylthio) butadiene (UO126), which is a selective inhibitor of MAP kinase kinase MEK.[29] We observed reduced levels of phospho-ERK in cultures treated with UO126 (**Supplementary Figure S1** online), indicating the effective inhibition of the MAPK kinase signaling pathway 30 min after treatment with UO126. The inhibitor was added to the HL60 cultures alone or together with 100 nmol/l of D3. We analyzed dysferlin expression at 48 and 72 h and observed decreased expression of dysferlin mRNA when 10 µmol/l of UO126 were added together with D3 to the culture ($P < 0.01$) (**Figure 2a**). Addition of 20 µmol/l of UO126 blocked expression of dysferlin mRNA both at 48 and 72 h (**Figure 2a**). Dysferlin protein analysis showed basal expression in cultures treated with UO126 (10 and 20 µmol/l). Cells treated with D3 and UO126 displayed a decrease of dysferlin protein expression in a UO126 dose-dependent manner (**Figure 2b**). Dysferlin expression was still detectable after UO126 treatment, suggesting the involvement of an alternative pathway of *DYSF* activation.

To clarify if the effect of UO126 on dysferlin expression in HL60 cells treated with D3 was due to a direct disruption of MAPK signaling pathway or was secondary to a blockade of cell differentiation, we analyzed the expression of the monocytic differentiation marker CD14 using flow cytometry. Untreated

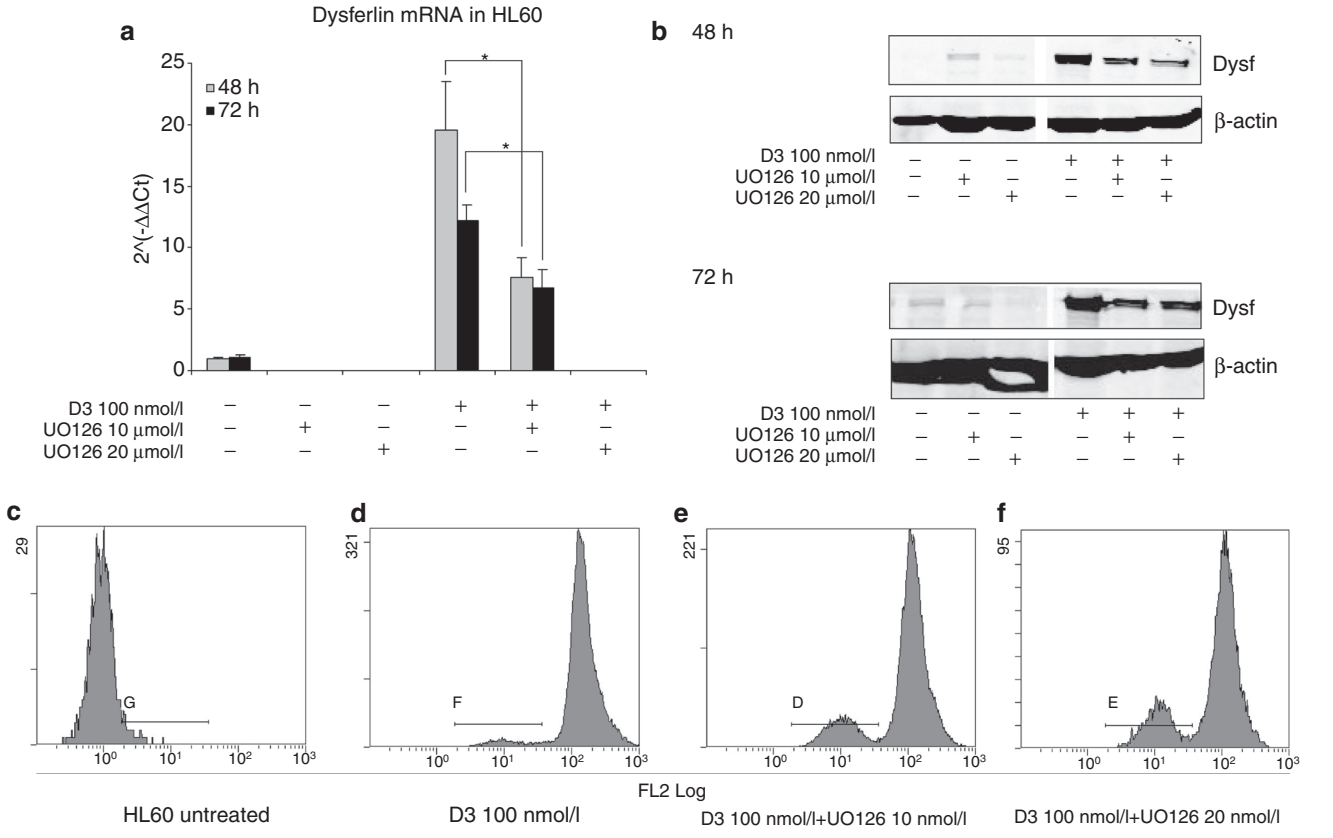

Figure 2 HL60 cells differentiated to CD14+ monocytes after treatment with D3 and dysferlin expression leveled down after addition of UO126. (**a**) Dysferlin mRNA diminished significantly when 10 µmol/l of UO126 was added to the culture, and no expression was observed with 20 µmol/l of UO126 (*$P < 0.01$). (**b**) WB of HL60 cells treated with 100 nmol/l of D3 showed that after the addition of UO126, dysferlin expression decreased. (**c**) Nontreated HL60 cells did not express the monocytic marker CD14. (**d**) After stimulation with D3 for 72 h, all HL60 cells expressed CD14. (**e** and **f**) Treatment with UO126 did not block HL60 cells differentiation measured as MFI with a monoclonal antibody to CD14. Each experimental condition was independently performed three times.

HL60 cells showed no expression of CD14 (mean fluorescence index, MFI = 2.1) (**Figure 2c**), whereas after 72 h of the addition of 100 nmol/l of D3 all cells expressed the CD14 marker (MFI = 168) (**Figure 2d**). When UO126 was added together with D3 to the HL60 cell cultures we observed that all cells expressed CD14 (M.F.I = 116 and 101) (**Figures 2e** and **2f**). These results indicated that the degree of cell differentiation was not affected by treatment with UO126. The proportion of dead cells was higher when a concentration of 20 µmol/l of UO126 was used (**Figure 2e**; D = 24.6%) compared with that observed when 10 µmol/l of UO126 were added to the cultures (**Figure 2f**; E = 18.9%).

The fact that dysferlin expression was still detectable, although considerably reduced, after UO126 treatment, suggested the involvement of an alternative pathway of *DYSF* activation.

Luciferase reporter activity containing a VDRE DR3-type activated by VDR was higher in HEK-293 D3-treated cells

Since UO126 did not block dysferlin expression completely, we analyzed a possible role of a genomic pathway involving binding of D3-VDR to the *DYSF* promoter. We performed a functional study using a pGL3-basic luciferase reporter plasmid containing a 252-bp fragment from dysferlin promoter included between −985 and −733, containing a specific DR3 element at −855 (TGACCCtaaTGACCT).[28] DR3-type VDRE located in the dysferlin promoter. The luciferase assay demonstrated significant differences between D3-treated and nontreated HEK-293 cultures ($P < 0.01$) transfected with DR3-type VDRE. The reporter gene was highly expressed in D3-treated cultures, indicating that VDR bound the VDRE located in the dysferlin promoter (**Figure 3**).

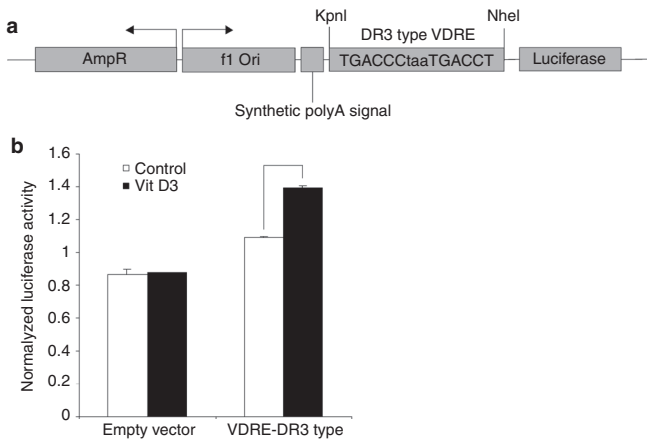

Figure 3 D3 treatment increased luciferase activity of a plasmid containing a VDRE consensus sequence that is present in the dysferlin promoter. (**a**) Representation of the D3 responsive element (DR3-type VDRE) present in the dysferlin promoter and cloned in the luciferase pGL3 vector. (**b**) Bars on the left show basal levels of luciferase activity in HEK-293 cells transfected with the empty vector, bars on the right show HEK-293 cells transfected with the pGL3b vector containing the DR3-type VDRE. After the addition of 100 nmol/l of D3 (black bars) to HEK-293 cells transfected with the pGL3b vector containing the VDRE, luciferase activity increased significantly ($P < 0.01$) compared to non-treated cells (white bars). Each experimental condition was independently performed four times. VDRE, vitamin D response element.

Vitamin D3 treatment increases dysferlin expression in human PBMs and in human primary skeletal muscle cultures

It has been reported that carriers of one mutation in *DYSF* gene have decreased dysferlin expression in their skeletal muscle[5] and monocytes.[7] To test if dysferlin expression also increased in monocytes/macrophages after the addition of D3, we stimulated PBM from healthy donors and from carriers of one mutation in *DYSF* gene with 100 nmol/l of D3 during 72 h. D3 treatment increased dysferlin protein expression in human PBM both in healthy donors and carriers (**Figure 4a**). The increase of dysferlin expression was more evident in carriers of one mutation in *DYSF* since after the addition of D3 dysferlin expression values reached normal levels.

No significant differences of dysferlin mRNA expression were found between control myotubes and myotubes from carriers of one mutation in *DYSF*. After treatment with 100 nmol/l of D3 during 72 h no increase in dysferlin mRNA expression was observed in healthy myotubes. In contrast, a significant increase of dysferlin mRNA was observed in myotubes from carriers with one mutation in *DYSF* (*$P < 0.05$). In myotubes from dysferlinopathy patients a slight increase of dysferlin mRNA was observed (**Figure 4b**).

Dysferlin protein expression in myotubes from carriers was reduced compared to controls (**Figure 4c**). When D3 was added to the cultures, dysferlin expression increased, reaching the highest levels at a concentration of 100 nmol/l at 72 h. In myotubes from dysferlinopathy patients no protein expression was observed after the addition of D3 (**Figure 4c**).

Dysferlin expression in human monocytes remained stable over time

In order to assess if dysferlin expression remains stable over time we studied monocytes of seven healthy donors at different time points. The results did not reveal significant variation in any of the samples studied (**Figure 5a**). Moreover, there were no significant differences in dysferlin expression between HL60 treated with 100 nmol/l of D3 during 72 h and human monocytes (**Figure 5b**).

Threshold of dysferlin expression in PBM

To establish a threshold of dysferlin expression in human PBM it was necessary to evaluate dysferlin expression changes after treatment with oral D3. Dysferlin expression in monocytes was quantified in 20 dysferlinopathy patients, 53 carriers of one mutation in *DYSF* and 80 healthy controls. The statistical analysis comparing dysferlinopathy patients (with a range of expression from 0 to 11.2% (mean 1.1 ± 2.6) with 133 healthy individuals (80 controls and 53 carriers with a mean expression of 51.2 ± 15.4% (range 24.5–78.2%) showed a significant difference in mean expression between these two groups ($P < 0.001$). The levels of dysferlin expression to distinguish between patients and healthy individuals with 96.4% sensitivity (CI 89.9–99.3) and 92% specificity (CI 87.2–95.3) were between 24.5 and 23.5%. Thus, we established that values below 23% of dysferlin expression in PBM indicated the diagnosis of dysferlinopathy (**Figure 6a**).

The statistical analysis comparing healthy controls (excluding carriers) and patients showed that values of dysferlin expression under 23% indicate a dysferlinopathy with a sensibility of 98% and a specificity of 100%.

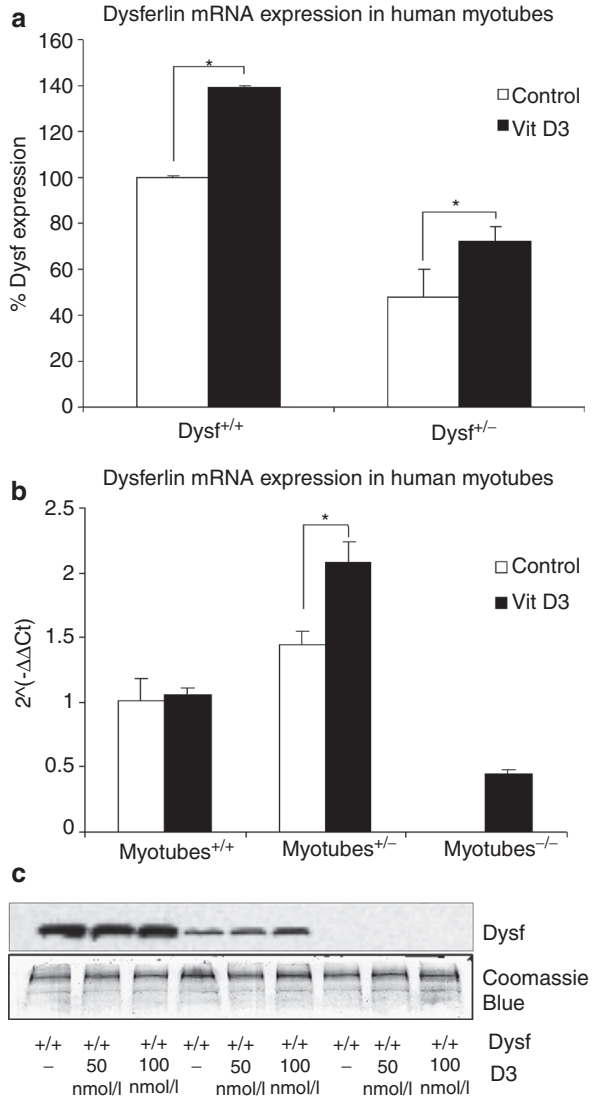

Figure 4 Human monocytes and myotubes treated with D3 showed increased dysferlin levels. (**a**) After treatment of human monocytes with 100 nmol/l of D3, we observed an increase of dysferlin mRNA expression in both healthy subjects (Dysf$^{+/+}$) and in carriers of one mutation in *DYSF* (Dysf$^{+/-}$). (**b**) Dysferlin mRNA from myotubes from healthy subjects, carriers of one mutation in *DYSF* and in dysferlinopathy patients showed an increased mRNA levels in carriers and patients. (**c**) WB of myotubes showed a dose-dependent increase of dysferlin expression in carriers of one mutation in *DYSF*. Each experimental condition was independently performed three times. WB, western blot.

The statistical analysis comparing healthy controls and carriers of one mutation in *DYSF* showed that values of dysferlin expression under 75% indicate a carrier condition with a sensibility of 97.8% and a specificity of 97.1% (**Figure 6a**). Therefore, values of dysferlin expression over 75% can be considered as normal.

Oral D3 Treatment Increased Dysferlin Expression in Carriers of a Single Mutation in the DYSF Gene

In order to evaluate if oral D3 treatment increased the expression of dysferlin by PBM *in vivo*, we designed an open-label randomized clinical trial in carriers of a single mutation in *DYSF*. Treatment with D3 significantly increased the expression of dysferlin in PBM after 1 year of follow-up (**Figure 6b** and **Table 1**). The D3-treated group (n = 15) had a mean increase of 43.1% in dysferlin expression, whereas the nontreated group (n = 6) had a mean increase of 9.8% after 1 year of follow-up (Greenhouse-Geisser test, P = 0.037).

In the treated group, mean dysferlin expression before treatment was 48.5% whereas after treatment it was 91.6% compared with controls (**Figure 6b**). Increases in dysferlin levels were observed in 12/15 participants treated (range from 12.9 to 96.9% of increase) (**Table 1**). In contrast, in the nontreated group mean dysferlin expression before treatment was 48.3%, whereas after the study it was 58.1% compared with controls. Increase of dysferlin levels was observed in all nontreated participants (range from 1.7 to 22.4% of increase).

The increase of dysferlin expression in subjects with some mutations, such as the p.Ala927LeufsX21, p.Gly519Arg, was not related to age or sex. Other mutations such as p.Gly1628Arg showed age variability and others such as p.Pro1452Thr showed sex variability although the low number of subjects with these mutations is too low to draw any conclusions. Time to response to therapy was variable depending on the subject; in some participants we observed a significant increase 1 month after the beginning of therapy whereas others needed 12 months to raise dysferlin expression levels (**Figure 6c**).

Treatment was well tolerated and none of the participants experienced adverse-events during the study. Moreover, blood calcium, creatinine, and urea were within normal levels during follow-up. No changes were detected in CK (creative kinase) levels or in muscle MRI that remained in the normal range after the study for all asymptomatic participants treated or not with D3. Included in the study was a previously reported symptomatic carrier (participant 11).[5,30] In this subject, dysferlin expression in PBM normalized after treatment with D3 with dysferlin expression changing from 39.9% to 114% compared to controls. Despite this increase, CK levels and muscle MRI were unchanged at the end of the study.

DISCUSSION

In the present work, we demonstrate that D3 enhances dysferlin expression both *in vitro* and in a clinical trial. *In vitro* studies with human myotubes from control samples and from one carrier of a mutation in the gene *DYSF* showed that D3 is able to increase dysferlin expression in a dose-dependent manner. Similar results were obtained using human PBM. These results lead us to perform an observational study in carriers of one mutation in *DYSF* gene and controls treated with oral D3·that confirmed an effect on dysferlin expression levels in PBM.

We observed that dysferlin expression increases with D3 and that this process is mediated by two different pathways, a MAPK cascade and VDR binding to dysferlin promoter. We demonstrate that the expression of dysferlin in promyelocytic HL60 cells after treatment with D3 was not a mere consequence of the differentiation process since treatment of the cells with UO126, a MAPK inhibitor, impaired dysferlin expression but did not block differentiation into CD14^{+} cells. However, dysferlin expression was not completely abolished after treatment with UO126. D3 promotes proliferation of myoblasts and their differentiation into myotubes

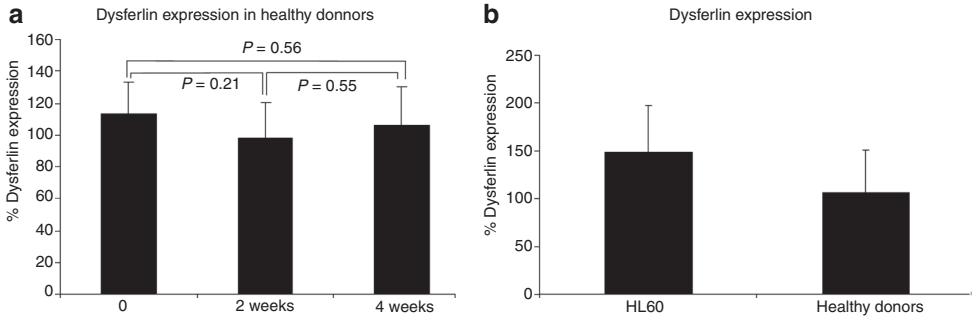

Figure 5 Dysferlin expression in human healthy controls. (**a**) Dysferlin expression remained at similar levels at consecutive time points in seven healthy subjects analyzed. (**b**) No significant differences were observed in dysferlin expression between HL60 treated cells with D3 (seven independent measures) during 72 h and those observed in seven nontreated human monocytes.

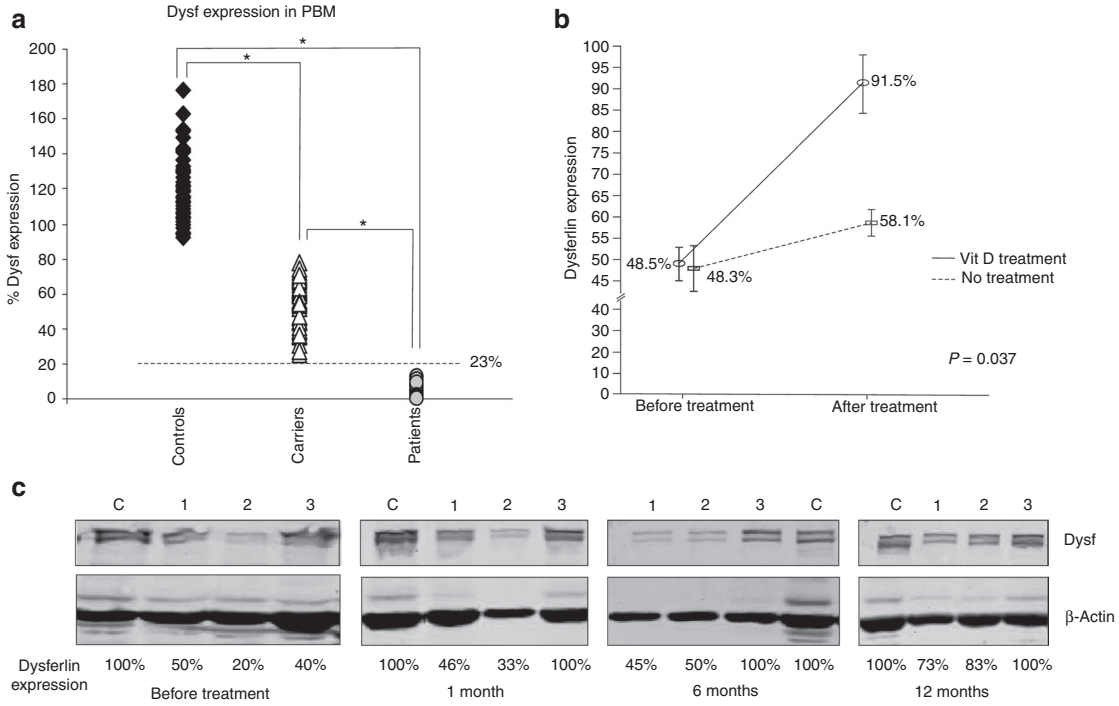

Figure 6 Dysferlin protein expression in human monocytes from healthy donors, carriers of one mutation in DYSF and in dysferlinopathy patients before and after treatment with D3. (**a**) Dysferlin protein expression levels in PBM of patients affected by dysferlinopathy, carriers of one mutation in the *DYSF* gene and controls. (*$P < 0.001$) Differences reached statistical significance. (**b**) Average dysferlin protein expression in PBM from the carriers of one mutation in the *DYSF* gene included in the D3 trial. Dysferlin expression was set as a percentage compared with controls. Values represent average expression before and after treatment. (**c**) WB of dysferlin expression in PBM from three members of the same family treated with D3. Even though a significant increase was achieved in all of them, the time point at which dysferlin expression was maximal was different in each case. PBM, peripheral blood monocyte.

through a MAPK pathway that activates ERK-1/2.[31] These data are in agreement with our previous work showing that dysferlin plays a role in human muscle differentiation and levels of myogenin, a myogenic transcription factor, are reduced in dysferlin-deficient muscle.[32] Also, it has been reported that D3 enhances grafting of human muscle precursor cells (hMPCs) into muscle after transplantation into SCID mice.[33] The authors suggested that D3 increase proliferation and differentiation of hMPCs and that this would explain the transplantation improvement. Our present results suggest that the improvement could be due to an increase of dysferlin expression after treatment of hMPCs with D3.

The MAPK/ERK cascade is upregulated by Ca^{2+} and calmodulin-dependent protein kinase II at the level of c-Src.[22] Also, dysferlin is involved in a membrane repair pathway that is highly dependent on Ca^{2+}.[34,35] Together, these data suggest that after membrane injury, leakage of Ca^{2+} would promote, not only the activation of membrane vesicle trafficking to reseal the membrane, but also upregulation of dysferlin, which is part of the protein complex present in those vesicles, through the nongenomic pathway.

We also analysed the genomic pathway that activates gene expression through binding of VDR to the *DYSF* gene. We demonstrate that the specific DR3-type VDRE consensus sequence present in the *DYSF* promoter is activated by D3 as shown in our luciferase assay. This result indicates that the transcriptional activity of *DYSF* can be modulated by D3. However, it is known that there are different co-regulatory factors, not fully characterized,

Table 1 Data corresponding to the carriers of one mutation in DYSF included in this study

Participants	Treatment	Sex	Age	Nucleotide change DYSF gene	Protein change	Dysferlin expression before treatment (%)	Dysferlin expression after treatment (%)
1	Yes	M	65	c.2779 delG	p.Ala927LeufsX21	32	104.9
2	Yes	M	40	c.2779 delG	p.Ala927LeufsX21	47.7	112.9
3	Yes	M	39	c.2779 delG	p.Ala927LeufsX21	48.9	61.8
4	Yes	W	38	c.2779 delG	p.Ala927LeufsX21	55.7	89.4
5	Yes	W	30	c.2779 delG	p.Ala927LeufsX21	59.5	88.5
6	Yes	M	75	c.1555 G>A	p.Gly519Arg	38.6	82.5
7	Yes	W	75	c.1555 G>A	p.Gly519Arg	50.9	139.1
8	Yes	M	35	c.1555 G>A	p.Gly519Arg	26.3	83.8
9	Yes	M	24	c.4882 G>A	p.Gly1628Arg	37.9	66.3
10	Yes	M	54	c.4882 G>A	p.Gly1628Arg	70.2	69.8
11	Yes	W	59	c.1873 G>T	p.Asp625Tyr	39.9	114
12	Yes	M	49	c.3112C>T	p.Arg1038X	43.5	140.4
13	Yes	W	46	c.3191_3196dup	p.Ala1064_ Glu1065dup	40.6	116.5
14	Yes	M	30	c.4354C>A	p.Pro1452Thr	66.8	65.3
15	Yes	W	31	c.4354C>A	p.Pro1452Thr	70	49.2
16	No	M	68	c.5999G>A	p.Arg2000Gln	55.6	61
17	No	W	64	c.5194G>T	p.Glu1732X	53.6	62.4
18	No	W	80	c.6124C>T	p.Arg2042Cys	68.3	70
19	No	M	20	c.6124C>T	p.Arg2042Cys	44	58.4
20	No	W	26	c.5159delG	p.Arg1720LeufsX2	30.8	53.2
21	No	M	32	c.5159delG	p.Arg1720LeufsX2	38.6	44

Accession number used NM_003494.2.
M, man; W, woman.

that modulate changes in gene expression driven by D3.[36] Further studies are needed to reveal the co-regulatory factors involved in *DYSF* expression mediated by D3.

Here we have demonstrated that D3-treated HL60 cells differentiate and start expressing dysferlin. Therefore, this cell line constitutes a powerful *in vitro* model to gain further insight into the role of dysferlin in the physiology of human monocytes. Dysferlin-deficient monocytes display increased phagocytic activity that may perpetuate the pathology triggered by the absence of dysferlin in skeletal muscle.[37] In fact, the muscle biopsy of patients with dysferlinopathy is characterized by the presence of inflammatory infiltrates constituted mainly by macrophages.[38,39]

Our *in vitro* results together with observations *in vivo*[7] prompted us to perform a study with D3 in carriers of one mutation in *DYSF*. Interestingly, this observational study showed that treatment with D3 for a year can increase dysferlin expression in PBM *in vivo*. Levels of expression of dysferlin in PBM in asymptomatic carriers of one mutation in *DYSF* can be as low as 24% (present data and ref. 40). We found a mean upregulation of dysferlin expression of 42%. Collectively, these results may have important implications in therapies aimed to repair mutations in one allele of the gene *DYSF* in patients with dysferlinopathy (i.e., heterozygous cases). For example, a combination of a pharmacological therapy (e.g., PTC124[41] and treatment with D3 could restore dysferlin expression to curative levels. Our observational study is a proof-of-principle that oral D3 can increase expression levels of dysferlin in PBM. In addition, experiments performed in our lab[30] showed that there is a positive correlation between the expression of dysferlin in PBM and skeletal muscle. This latter finding together with the results obtained in this study using human muscle *in vitro* treated with D3 suggest that the increased expression observed in PBM after oral D3 also occurs in skeletal muscle. There is evidence that oral D3 improves muscle function[42] even at doses lower than those used in our study. However, even with the positive effect on dysferlin expression, one year of treatment with D3 did not result in changes in blood CK levels or muscle MRI of the single symptomatic carrier included in the study.[5] These results suggest that one year of treatment with D3 is not sufficient to improve the muscle function. However, the fact that the patient did not worsen clinically and that the MRI had not changed significantly indicates that the disease had not progressed after one year and this is clinically relevant. Our group is enrolled in an three-years international project to study the natural history of the disease, including the MRI, that will clarify the progression of the disease. Regarding the dosage, it has been reported that treatment with 20.000IU of D3 weekly improved gait disturbances in a patient with proximal myopathy (Fabricciani J Clin Rheumatol 2010). Excessive D3 intake has been established as above 50.000 IU[43] and is associated with additional significant clinical adverse effects, mainly hypercalcemia, renal stones, pain, conjunctivitis, fever, chills, thirst, vomiting, and weight loss.[44]

It should be noted that treatment with D3 increases mRNA expression in patient cells (**Figure 4b**). Our experience after testing many patients is that they normally show 0% expression of dysferlin.[30] However, there are some cases in whom some dysferlin is expressed. In fact, it has been recently reported that the observation that patients with dysferlinopathy always show very low levels of dysferlin may be related to the ubiquitin-proteasome pathway.[45] The cases in whom the mutation produces a functional dysferlin, a combination of both therapies (proteasome inhibition plus treatment with D3) might be therapeutic.

In conclusion, our study demonstrates that D3 promotes an increase of dysferlin expression in human skeletal muscle and in PBMs *in vitro* and in a clinical trial. These findings may have important implications for future therapies combining D3 and different molecular strategies.

MATERIAL AND METHODS

Cell lines. HL60 were obtained from ATCC (CCL-240). Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere, in Iscove's modified Dulbecco's medium (Lonza group, Basel, Switzerland) supplemented with 20% of FBS (Lonza), 2 mmol/l of Glutamine (Lonza) and 100 U/ml penicillin (Lonza) and 100 µg/ml streptomicin (Lonza). HL60 cells were seeded at 10^5 cells/ml supplemented with 100 nmol/l 1α-25-dihydroxy vitamin D3 (Sigma-Aldrich, St. Louis, MO) and 10 µmol/l, 20 µmol/l of UO126 and incubated during 24, 48, and 72 h. Dysferlin expression was analyzed by WB.

PBMC cultures. PBMC from carriers of one mutation in *DYSF* and healthy controls were isolated from human blood using Ficoll density gradient with Lymphoprep (Axis-Shield PoC As, Oslo, Norway). PBM were isolated by adhesion to polystyrene tissue culture flasks for 1 h, and incubated with 100 nmol/l D3 during 72 h. Dysferlin expression was assessed by WB.

Primary human skeletal muscle cultures. Human muscle biopsies were minced and cultured as previously described.[32] Myoblast were seeded a 2,500 cells/cm^2, in the proliferation media containing 75% Dulbecco's Modified Eagle's Medium and 25% M199, supplemented with 15% FBS (Lonza), 10 µg/ml insulin (Sigma-Aldrich), 2 mmol/l glutamine (Lonza) and penicillin-streptomycin (Lonza). When muscle cells started to fuse the medium was substituted with one containing 2% FBS. When myotubes were formed we added 100 nmol/l D3 to the cultures and incubated during 24, 48, and 72 h.

Participants. Subjects were recruited from January 2002 to April 2010 at the Neuromuscular Unit of Hospital de la Santa Creu i Sant Pau, a reference center for dysferlin myopathy in Spain. The diagnosis of dysferlinopathy and other muscular dystrophies included the neurological examination, muscle MRI and in most cases a muscle biopsy to guide the genetic analysis according to the defective protein using immunohistochemistry and WB. PBMC WB was carried out in 20 patients with two mutations in the *DYSF* gene, in 53 obligate carriers (i.e., relatives of genetically confirmed patients) with one recognized mutation and in 80 normal controls to study dysferlin levels in these groups. Informed consent was obtained from all patients for muscle biopsy, blood samples, and RNA/DNA analysis.

Isolation of PBM. PBM CD14 positive isolation was carried out in all subjects as previously described.[18] Briefly, PBMC were mixed with 20µl of CD14-coated microbeads (Milteny Biotec, Bergisch Gladbach, Germany) and incubated at 4 °C for 30 min. Unbound microbeads were removed by washing cells in PBS followed by centrifugation at 300g for 10 min. The cell pellet was resuspended in PBS buffer before separation on a MACS apparatus according to manufacturer's instructions (Milteny Biotec).

Flow cytometry. One-color flow cytometry was performed for the detection of human CD14. 500,000 cells were used to analyze CD14 expression. Unspecific binding sites were blocked with 10% normal human serum in PBS for 5 min. Monoclonal primary antibody anti-human CD14_PE (clone MEM-19, mouse IgG1) (Immunotools, Friesoythe, Germany) was used to stain HL60 stimulated with different induction agents and unstimulated cells. Cells were finally acquired and analyzed in a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ). Expression levels of CD14 were referred as MFI.

Real time PCR. RNA from HL60, PBM and primary skeletal muscle cultures was extracted using Ultraspec (Biotech Laboratories, Houston, TX). Total RNA (0.2 µg) of each condition was reverse transcribed into cDNA using MultiScribe reverse transcriptase (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ).

Quantification of the mRNA coding for DYSF, and 18S as internal standard was performed using TaqMan Universal Master Mix technology (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ). Quantitative PCR was performed in a total reaction volume of 12 µl per well. The primers used for real time PCR were designed by Applied Biosystems (Roche Molecular Systems, Branchburg, NJ) (DYSF Hs 00243339_m1 and 18S Hs 99999901_s1). The comparative CT method (ΔΔCT) for relative quantification of gene expression was used as previously reported.[32] As long as the target and normalizer (18S) have similar dynamic ranges, this method is the most used. The *t*-test was used for statistical analysis.

Phosphorylated protein isolation. Phosphorylated proteins were isolated from treated and nontreated HL60. Cells were lysed with a buffer containing 0.02 mol/l Tris/acetate, 0.27 mol/l of sucrose, 0.01 mmol/l of EDTA, 0.01 of EGTA, 1% Triton x-100, 1 mmol/l ortovanadate, 1 mmol/l glycerophosphate, 0.05 mol/l NaF, 0.5 mmol/l of sodium pyrophosphate, 1% of 2b mercaptoethanol, 1 mmol/l of benzamidine, 0.035 mg/ml of PMSF, and 0.005 mg/ml of leupeptin. After 15 min of incubation on ice and a centrifugation step, supernatants were quantified and 50 µg of protein were loaded into each lane.

Immunoblot analysis. WB from human monocyte samples was performed using a mouse monoclonal antibody to dysferlin (NCL-Hamlet, Novocastra, Newcastle, UK) and a mouse monoclonal antibody to β-actin (Sigma-Aldrich) as a loading control. IRDye conjugate goat anti-mouse (Li-Cor, Lincoln, Nebraska) was used as a secondary antibody. Fluorescent bands corresponding to dysferlin and β-actin were acquired and analyzed using an Odyssey Infrared Imaging System (Li-Cor). To determine dysferlin expression we quantified the double band observed in PBMC. The control samples were used as reference values and assigned 100% of dysferlin expression using β-actin as a normalizer. When more than one control was used in the same WB, 100% was randomly assigned to one of the controls. For this reason, results were over 100% in some cases and under 100% in others. This variability occurs in the normal population. Analysis was conducted by two readers but only one reading was performed for each sample since quantification was performed with the software Odyssey (Li-Cor). Dysferlin expression in all samples was quantified using the control values to obtain the percentages of dysferlin expression/reduction.

WB of phosphorylated proteins was performed using a rabbit anti phospho-ERK1/2 and a rabbit anti-total ERK1/2 (Cell Signaling, Life Technologies, Paisley, UK). IRDye conjugate goat anti-rabbit (Li-Cor, Lincoln, Nebraska) was used as a secondary antibody.

To determine if the monocyte blood test could be useful not only for the diagnosis but also for patient follow-up when therapy is available, we evaluated the stability of dysferlin expression in 10 controls at baseline and at two time points (2 weeks and 1 month).

Construction of luciferase reporter gene plasmids. We used genomic DNA from a healthy volunteer as a template to amplify a 252-bp fragment from dysferlin promoter included between −985 and −733, containing a DR3 element at −855 (TGACCCtaaTGACCT),[28] which is recognized by VDR. A PCR reaction was performed using the following primers: 5′ CTC TCTTCTTGGCTTGCAGA 3′ and 5′ GCTGAATTAGGTCCTCCGA 3′

that appended unique KpnI and NheI sites on the 5′ and 3′ ends. The amplified sequence was digested using KpnI and NheI restriction enzymes (New England Biolabs, Ipswich, MA) and cloned in frame into a pGL3-basic luciferase vector (Promega, Madison, WI) opened with the same set of enzymes. The ligation reaction was performed using T4 DNA ligase (New England Biolabs, Ipswich, MA). The correct open reading frame and the orientation of the cloned fragment were confirmed by DNA sequencing. The plasmid pGL3-basic containing the DR3 recognized by VDR (pGL3b_DR3) was purified using endotoxin-free Maxi-prep (Qiagen, Valencia, CA).

Transient transfection. HEK-293 (ATCC CRL-1573) cells were transfected using nucleofector system following manufacturer's instructions. 10^6 cells were transfected with 1.8 µg of pGL3b-DR3 DNA. As internal control for each transfection, cells were cotransfected with 90 ng of the plasmid RL-TK (Promega, Madison, WI) expressing Renilla under the HSV TK promoter. After 4 h of transfection, D3 (100nmol/l) was added to the cultures for 24 h.

Luciferase assay. Cells were washed twice with PBS. For the luciferase assay, we used the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) following manufacturer's instructions. Transient transfectants were harvested with lysis buffer and the luciferase activity was measured with a luminometer (Victor 3, Perkin Elmer, Wellesley, MA). The values obtained were normalized with Renilla activity. We used the pGL3-basic vector without insert as a negative control.

In vivo *studies.* In order to evaluate if oral D3 treatment increased the expression of dysferlin by PBM *in vivo*, we designed an open-label, prospective, randomized study (Eudra-CT: 2007-005808-41) that was performed in two different hospitals in Spain from October 2008 to April 2010. Our clinical research ethics committee (Comité Etic d'Investigació Clínica (CEIC) Sant Pau) approved this study and was performed in accordance with the Declaration of Helsinki for Human Research. Written informed consent was obtained from all participants in the study. The inclusion criteria were: age older than 18 years old and to be carrier of a single mutation in the *DYSF* gene producing almost a reduction of 75% expression of dysferlin by PBM. Exclusion criteria were: current treatment with D3 for other medical conditions, renal failure defined as blood creatinine levels higher than 100 mmol/l or urea levels higher than 8 mmol/l, blood calcium levels higher than 2.6 mmol/l, pregnancy and subjects unable to follow all the scheduled procedures.

Participants were recruited from the families of our dysferlinopathy patients. Twenty-three volunteers (9 women, 47.1 years median age) were included in the study. All participants were asymptomatic, except for one who had mild atrophy of medial gastrocnemius (this patient has been previously reported in ref. 5. General physical examination, muscle strength, blood CK levels, and muscle MRI were normal in all participants before treatment with D3 was started, except for the symptomatic subject. There were no significant differences between groups in sex, age, or basal dysferlin expression (**Table 1**).

The study was funded by the Spanish Health Department and the institutional review board at each center approved the protocol. All participants gave written informed consent to participate in the study.

Baseline assessments for all participants included medical history, general physical examination, standard blood laboratory analyses, and current medications.

Participants were randomized to receive oral D3 or no treatment following a three to one randomization protocol (treated vs. nontreated). Seventeen participants were treated with D3 whereas 6 were not treated. Two of the treated subjects left the study during the follow-up and their results were included in the final analysis. Treated participants received one single vial of D3 weekly (Hidroferol, FAES Farma, Spain) for 1 month (one vial contained 16.000 UI of D3), and then doubled the dose if hypercalcemia or renal failure were not detected in the blood analysis. The duration of the study was 12 months.

Follow-up visits were performed at day 15 and at months 1, 3, 6, and 12 after the beginning of the treatment. General physical examination and blood analysis to study CK, calcium levels, renal function, and dysferlin expression in PBM were performed at each visit. At month 12, a new muscle MRI was performed in all participants.

Statistical analysis. Expression values include mean expression and SD for each of the three groups. Mean expression values were compared using the one-way ANOVA. The post-hoc test (Scheffé's test) was also used to assess differences between groups. We evaluated the discrimination power by the corresponding receiver operating characteristic curve. The 95% confidence intervals (CI) were calculated to evaluate sensitivity and specificity indexes. The relationship between two quantitative variables was assessed with the Pearson correlation coefficient. To compare the response to D3 treatment between groups we performed ANOVA test, to avoid possible sphericity problems a Greenhouse-Geisser test was performed. The significance level was set at 5%. The analysis was done with SPSS (V18.0; Chicago, IL).

SUPPLEMENTARY MATERIAL

Figure S1. UO126 effectively inhibits MAPK kinase pathway. (**a**) Representative WB of phospho-ERK and total ERK, from HL60 cells nontreated, treated with D3 and/or UO126, at 30 min. (**b**) Quantitation of levels of Phospho-ERK and total ERK in treated and nontreated HL60 cells. WB, western blot.

ACKNOWLEDGMENTS

We thank Myrna Rosenfeld for editorial support and critical review of the manuscript. This work was supported by research grants from the Fondo Investigación Sanitaria Instituto de Salud Carlos III, PI06/0455, PI09/1944, and Grup de Recerca Consolidat de la Generalitat de Catalunya 2009 SGR 1004 to E.G., EC07/90601, ETES 08/90622, CIBERNED, and from the Fundación Isabel Gemio to I.I. N.L., J.D.M., C.P., C.I., R.R.G., J.A., M.G., I.G. have nothing to disclose. I.I. and E.G. are co-inventors of the patent: Blood-Based Assay for Dysferlinopathies, Number: US 7172858 Status issued 2007(US), 2008 (Canada), 2009 (Japan).

REFERENCES

1. Bushby, KM (1999). Making sense of the limb-girdle muscular dystrophies. *Brain* **122** (Pt 8): 1403–1420.
2. Bashir, R, Britton, S, Strachan, T, Keers, S, Vafiadaki, E, Lako, M *et al.* (1998). A gene related to Caenorhabditis elegans spermatogenesis factor fer-1 is mutated in limb-girdle muscular dystrophy type 2B. *Nat Genet* **20**: 37–42.
3. Illa, I, Serrano-Munuera, C, Gallardo, E, Lasa, A, Rojas-García, R, Palmer, J *et al.* (2001). Distal anterior compartment myopathy: a dysferlin mutation causing a new muscular dystrophy phenotype. *Ann Neurol* **49**: 130–134.
4. Liu, J, Aoki, M, Illa, I, Wu, C, Fardeau, M, Angelini, C *et al.* (1998). Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. *Nat Genet* **20**: 31–36.
5. Illa, I, De Luna, N, Domínguez-Perles, R, Rojas-García, R, Paradas, C, Palmer, J *et al.* (2007). Symptomatic dysferlin gene mutation carriers: characterization of two cases. *Neurology* **68**: 1284–1289.
6. Klinge, L, Dean, AF, Kress, W, Dixon, P, Charlton, R, Müller, JS *et al.* (2008). Late onset in dysferlinopathy widens the clinical spectrum. *Neuromuscul Disord* **18**: 288–290.
7. Paradas, C, González-Quereda, L, De Luna, N, Gallardo, E, García-Consuegra, I, Gómez, H *et al.* (2009). A new phenotype of dysferlinopathy with congenital onset. *Neuromuscul Disord* **19**: 21–25.
8. Lerario, A, Cogiamanian, F, Marchesi, C, Belicchi, M, Bresolin, N, Porretti, L *et al.* (2010). Effects of rituximab in two patients with dysferlin-deficient muscular dystrophy. *BMC Musculoskelet Disord* **11**: 157.
9. Nemoto, H, Konno, S, Sugimoto, H, Nakazora, H, Nomoto, N, Murata, M *et al.* (2011). Anti-TNF therapy using etanercept suppresses degenerative and inflammatory changes in skeletal muscle of older SJL/J mice. *Exp Mol Pathol* **90**: 264–270.
10. Leriche-Guérin, K, Anderson, LV, Wrogemann, K, Roy, B, Goulet, M and Tremblay, JP (2002). Dysferlin expression after normal myoblast transplantation in SCID and in SJL mice. *Neuromuscul Disord* **12**: 167–173.
11. Kong, KY, Ren, J, Kraus, M, Finklestein, SP and Brown, RH Jr (2004). Human umbilical cord blood cells differentiate into muscle in *SJL* muscular dystrophy mice. *Stem Cells* **22**: 981–993.
12. Vieira, NM, Bueno, CR Jr, Brandalise, V, Moraes, LV, Zucconi, E, Secco, M *et al.* (2008). SJL dystrophic mice express a significant amount of human muscle proteins following systemic delivery of human adipose-derived stromal cells without immunosuppression. *Stem Cells* **26**: 2391–2398.
13. Díaz-Manera, J, Touvier, T, Dellavalle, A, Tonlorenzi, R, Tedesco, FS, Messina, G *et al.* (2010). Partial dysferlin reconstitution by adult murine mesoangioblasts is sufficient for full functional recovery in a murine model of dysferlinopathy. *Cell Death Dis* **1**: e61.

14. Lostal, W, Bartoli, M, Bourg, N, Roudaut, C, Bentaïb, A, Miyake, K *et al.* (2010). Efficient recovery of dysferlin deficiency by dual adeno-associated vector-mediated gene transfer. *Hum Mol Genet* **19**: 1897–1907.
15. Krahn, M, Wein, N, Bartoli, M, Lostal, W, Courrier, S, Bourg-Alibert, N *et al.* (2010). A naturally occurring human minidysferlin protein repairs sarcolemmal lesions in a mouse model of dysferlinopathy. *Sci Transl Med* **2**: 50ra69.
16. Anderson, LV, Davison, K, Moss, JA, Young, C, Cullen, MJ, Walsh, J *et al.* (1999). Dysferlin is a plasma membrane protein and is expressed early in human development. *Hum Mol Genet* **8**: 855–861.
17. Klinge, L, Laval, S, Keers, S, Haldane, F, Straub, V, Barresi, R *et al.* (2007). From T-tubule to sarcolemma: damage-induced dysferlin translocation in early myogenesis. *FASEB J* **21**: 1768–1776.
18. Ho, M, Gallardo, E, McKenna-Yasek, D, De Luna, N, Illa, I and Brown Jr, RH (2002). A novel, blood-based diagnostic assay for limb girdle muscular dystrophy 2B and Miyoshi myopathy. *Ann Neurol* **51**: 129–133.
19. De Luna, N, Freixas, A, Gallano, P, Caselles, L, Rojas-García, R, Paradas, C *et al.* (2007). Dysferlin expression in monocytes: a source of mRNA for mutation analysis. *Neuromuscul Disord* **17**: 69–76.
20. Miyaura, C, Abe, E, Kuribayashi, T, Tanaka, H, Konno, K, Nishii, Y *et al.* (1981). 1 alpha,25-Dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells. *Biochem Biophys Res Commun* **102**: 937–943.
21. Tanaka, H, Abe, E, Miyaura, C, Shiina, Y and Suda, T (1983). 1 alpha,25-dihydroxyvitamin D3 induces differentiation of human promyelocytic leukemia cells (HL-60) into monocyte-macrophages, but not into granulocytes. *Biochem Biophys Res Commun* **117**: 86–92.
22. Boland, R, Buitrago, C and De Boland, AR (2005). Modulation of tyrosine phosphorylation signalling pathways by 1alpha,25(OH)2-vitamin D3. *Trends Endocrinol Metab* **16**: 280–287.
23. Norman, AW, Nemere, I, Zhou, LX, Bishop, JE, Lowe, KE, Maiyar, AC *et al.* (1992). 1,25(OH)2-vitamin D3, a steroid hormone that produces biologic effects via both genomic and nongenomic pathways. *J Steroid Biochem Mol Biol* **41**: 231–240.
24. Tsai, MJ and O'Malley, BW (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* **63**: 451–486.
25. Morelli, S, Buitrago, C, Boland, R and de Boland, AR (2001). The stimulation of MAP kinase by 1,25(OH)(2)-vitamin D(3) in skeletal muscle cells is mediated by protein kinase C and calcium. *Mol Cell Endocrinol* **173**: 41–52.
26. Kliewer, SA, Umesono, K, Mangelsdorf, DJ and Evans, RM (1992). Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature* **355**: 446–449.
27. Toell, A, Polly, P and Carlberg, C (2000). All natural DR3-type vitamin D response elements show a similar functionality *in vitro*. *Biochem J* **352 Pt 2**: 301–309.
28. Wang, TT, Tavera-Mendoza, LE, Laperriere, D, Libby, E, MacLeod, NB, Nagai, Y *et al.* (2005). Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D3 target genes. *Mol Endocrinol* **19**: 2685–2695.
29. Favata, MF, Horiuchi, KY, Manos, EJ, Daulerio, AJ, Stradley, DA, Feeser, WS *et al.* (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* **273**: 18623–18632.
30. Gallardo, E, de Luna, N, Diaz-Manera, J, Rojas-García, R, Gonzalez-Quereda, L, Flix, B *et al.* (2011). Comparison of dysferlin expression in human skeletal muscle with that in monocytes for the diagnosis of dysferlin myopathy. *PLoS ONE* **6**: e29061.
31. Ronda, AC, Buitrago, C, Colicheo, A, de Boland, AR, Roldán, E and Boland, R (2007). Activation of MAPKs by 1alpha,25(OH)2-vitamin D3 and 17beta-estradiol in skeletal muscle cells leads to phosphorylation of Elk-1 and CREB transcription factors. *J Steroid Biochem Mol Biol* **103**: 462–466.
32. de Luna, N, Gallardo, E, Soriano, M, Dominguez-Perles, R, de la Torre, C, Rojas-García, R *et al.* (2006). Absence of dysferlin alters myogenin expression and delays human muscle differentiation "*in vitro*". *J Biol Chem* **281**: 17092–17098.
33. Stephan, L, Bouchentouf, M, Mills, P, Lafreniere, JF and Tremblay, JP (2007). 1,25-dihydroxyvitamin D3 increases the transplantation success of human muscle precursor cells in SCID mice. *Cell Transplant* **16**: 391–402.
34. Glover, L and Brown, RH Jr (2007). Dysferlin in membrane trafficking and patch repair. *Traffic* **8**: 785–794.
35. Wallace, GQ and McNally, EM (2009). Mechanisms of muscle degeneration, regeneration, and repair in the muscular dystrophies. *Annu Rev Physiol* **71**: 37–57.
36. Pike, JW and Meyer, MB (2010). The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). *Endocrinol Metab Clin North Am* **39**: 255–69, table of contents.
37. Nagaraju, K, Rawat, R, Veszelovszky, E, Thapliyal, R, Kesari, A, Sparks, S *et al.* (2008). Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B. *Am J Pathol* **172**: 774–785.
38. Gallardo, E, Rojas-García, R, de Luna, N, Pou, A, Brown, RH Jr and Illa, I (2001). Inflammation in dysferlin myopathy: immunohistochemical characterization of 13 patients. *Neurology* **57**: 2136–2138.
39. McNally, EM, Ly, CT, Rosenmann, H, Mitrani Rosenbaum, S, Jiang, W, Anderson, LV *et al.* (2000). Splicing mutation in dysferlin produces limb-girdle muscular dystrophy with inflammation. *Am J Med Genet* **91**: 305–312.
40. De Luna, N, Gallardo, E, Rojas-Garcia, R, Dominguez-Perles, R, Diaz-Manera, J, De la Torre, C*et al.* (2008). Quantification of dysferlin in monocytes: a useful tool for the detection of patients and carriers of dysferlinopathy. *Neuromuscul Disord* **18**: 790–791.
41. Wang, B, Yang, Z, Brisson, BK, Feng, H, Zhang, Z, Welch, EM *et al.* (2010). Membrane blebbing as an assessment of functional rescue of dysferlin-deficient human myotubes via nonsense suppression. *J Appl Physiol* **109**: 901–905.
42. Ward, KA, Das, G, Roberts, SA, Berry, JL, Adams, JE, Rawer, R *et al.* (2010). A randomized, controlled trial of vitamin D supplementation upon musculoskeletal health in postmenarchal females. *J Clin Endocrinol Metab* **95**: 4643–4651.
43. Jacobsen, RB, Hronek, BW, Schmidt, GA and Schilling, ML (2011). Hypervitaminosis D associated with a vitamin D dispensing error. *Ann Pharmacother* **45**: e52.
44. Hathcock, JN, Shao, A, Vieth, R and Heaney, R (2007). Risk assessment for vitamin D. *Am J Clin Nutr* **85**: 6–18.
45. Azakir, BA, Di Fulvio, S, Kinter, J and Sinnreich, M (2012). Proteasomal inhibition restores biological function of mis-sense mutated dysferlin in patient-derived muscle cells. *J Biol Chem* **287**: 10344–10354.