

Inhibition of muscle fibrosis and improvement of muscle histopathology in dysferlin knock-out mice treated with halofuginone

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Summary. Absence of, or loss-of-function mutations in the dysferlin gene (*dysf*) result in dysferlinopathy, characterized by increased muscle inflammation, collagen deposition and deterioration in muscle function. We evaluated halofuginone efficacy in improving muscle histopathology in mice with deleted *dysf* transmembrane domain. Quadriceps sublumbar and longissimus muscles of 9-month-old *dysf*^{-/-} mice treated with halofuginone for 4 months exhibited a reduction in centrally-nucleated myofibers, inflammatory infiltrates and collagen content. Late onset of dysferlinopathy makes it ideal for evaluating the efficacy of early treatments on late outcome. The *dysf*^{-/-} mice were treated with halofuginone for 3 to 4 months starting at 1, 5 or 9 months of age, and quadricep muscle histopathology was evaluated at 12 months. Collagen content and number of centrally nucleated myofibers decreased after early halofuginone treatment, administered when myofibers with central nuclei and inflammatory infiltrates are evident, but there was almost no fibrosis. When administered at the beginning of fibrosis it resulted in a further decrease in the number of centrally-nucleated myofibers with no additional decrease in collagen levels. Cardiac fibrosis was almost completely abolished following early halofuginone treatment. Halofuginone inhibited Smad3 phosphorylation and its translocation to the nucleus and increased the activity of matrix metalloproteinases 9 and 2 responsible for resolution of pre-existing collagen. Macrophage and myofibroblast invasion into the dystrophic muscle at the site of myofibers with central nuclei was inhibited by halofuginone. These results suggest that early halofuginone treatment can prevent

the late outcome of dysferlinopathy and can cause resolution of the established fibrosis when administered at later stages.

Key words: Muscular dystrophies, Myofibroblast, Macrophage, Fibrosis, Fusion, Dysferlinopathies, Halofuginone

Introduction

In muscle, contraction-induced sarcolemmal injury is a common event, and myofibers have an efficient sarcolemmal repair system that mediates responses to local damage, such that small tears within membranes are normally sealed within seconds (Bansal et al., 2003). Dysferlin is a 230-kDa protein that is highly expressed in skeletal and cardiac muscle (Bashir et al., 1998; Barthélémy et al., 2011) and is an important constituent of the muscle-membrane repair system and a major mediator of membrane resealing in muscle (Lennon et al., 2003). In addition, dysferlin is required for proper muscle differentiation, regeneration and fusion events (De Luna et al., 2004; Roche et al., 2008; Chiu et al., 2009; Millay et al., 2009; Demonbreun et al., 2011). Dysferlinopathies encompass a large variety of muscle dystrophies (MDs) characterized by the absence of, or loss-of-function mutations in the dysferlin gene (*dysf*) in skeletal muscle (Liu et al., 1998; Bushby, 2000; Klinge et al., 2010; Cacciottolo et al., 2011). Although rare, dysferlinopathies can account for up to 30% of progressive recessive MDs in certain geographical regions, such as the Middle East and the Indian subcontinent (Urtizberea et al., 2008). Clinically, dysferlinopathies are relatively mild diseases characterized by late onset in the second or third decade with normal musculature prior to this time. To date,

three main phenotypes have been reported: limb girdle muscular dystrophy type 2B (LGMD 2B), Miyoshi myopathy (MM), and distal myopathy with anterior tibial onset. The first phenotype exhibits predominantly proximal slowly progressive involvement of the pelvic and shoulder girdle, while the latter two forms present a predominantly distal pattern (Kawabe et al., 2004). Dysferlin is also expressed in cardiomyocytes (Guglieri et al., 2005) and in its absence cardiomyocyte membrane damage occurs; however, unlike in skeletal muscle, the cell injury is sub-lethal and causes only mild cardiomyopathy, even at an advanced age (Chase et al., 2009). In patients with dysferlinopathy, inflammatory and degenerative changes and fibrosis are observed at later ages (Brunn et al., 2006).

One of the characteristics of dystrophic muscle is infiltration of myofibroblasts expressing P₄H β , a major collagen cross-linking enzyme (Nevo et al., 2010). These cells are the main source of fibrillar collagens in the tissue. In addition to the myofibroblasts, muscle inflammation is a prominent feature in several MDs. In patients with dysferlinopathy (Gallardo et al., 2001; Confalonieri et al., 2003) and in animal models of dysferlinopathies (Bittner et al., 1999; Ho et al., 2004), the inflammation consists mainly of macrophages. The fibrosis in dysferlinopathies is mild compared to that in other MDs such as Duchenne (DMD) and congenital MD (CMD). It has been hypothesized that autocrine release of TGF β from necrotic myofibers contributes to the progressive loss of muscle regeneration and the increase in fibrosis (Bernasconi et al., 1995; Melone et al., 1999). Inhibition of TGF β /Smad3 signaling ameliorates the pathological phenotype in dystrophic mice, a finding consistent with the importance of TGF β signaling in regulating both regeneration and fibrosis (Cohn et al., 2007). In murine models of dysferlinopathies and in patients with the disease, a reduction in Smad2/Smad3 protein levels (Wenzel et al., 2005) and an increase in connective tissue are observed, together with late onset of fibrosis (Weller et al., 1997; Vinit et al., 2010).

Halofuginone is an inhibitor of Smad3 phosphorylation downstream of the TGF β -signaling pathway (Pines et al., 2000; Pines, 2008). Halofuginone inhibits Smad3 phosphorylation in a variety of cell types and animal models (McGaha et al., 2002; Yee et al., 2006; Roffe et al., 2010), TGF β -dependent fibrosis in scores of animal models (Levi-Schaffer et al., 1996; Bruck et al., 2001; Pines et al., 2001; Zion et al., 2009), and extracellular matrix (ECM) production in tumor xenografts associated with a major reduction in tumor growth and development (Gavish et al., 2002; Pinthus et al., 2005; Spector et al., 2010). In murine models of DMD and CMD, halofuginone was shown to inhibit Smad3 phosphorylation in cardiac and skeletal muscles and this was associated with decreased muscle fibrosis and enhanced motor coordination and balance (Turgeman et al., 2008; Nevo et al., 2010). Moreover, halofuginone treatment decreased muscle fibrosis and

improved lung and cardiac muscle functions in older *mdx* mice that exhibited established fibrosis (Huebner et al., 2008). The ability to resolve pre-existing fibrosis derives from the regulation of MMP activity, as these enzymes play a crucial role in the fine regulation of ECM turnover, which is altered in most pathological states associated with fibrosis (Bruck et al., 2001). In addition to the profound effect on muscle fibrosis, halofuginone also elicits a direct effect on muscle cells and promotes myotube fusion in primary myoblasts derived from normal and dystrophic muscles (Roffe et al., 2010).

In this study, we employed a murine model of dysferlinopathy with late onset of the disease to evaluate the efficacy of halofuginone at improving histopathology at both early stages of the dystrophy before the onset of fibrosis, and at later stages, when muscle fibrosis is established.

Materials and methods

Materials

Halofuginone bromhydrate was obtained from Halo Therapeutics, LLC (Boston, MA, USA). Monoclonal prolyl 4-hydroxylase β (P₄H β) and polyclonal anti-macrophage antibodies were purchased from Acris (Hiddenhausen, Germany). Monoclonal anti-dysferlin antibody was obtained from Novocastra (Newcastle Upon Tyne, UK). A rabbit polyclonal anti-collagen type I antibody was purchased from Abcam (Cambridge, UK), polyclonal pSmad3 antibodies were from Cell Signaling (Beverly, MA), polyclonal Smad3 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sirius red F3B was obtained from BDH Laboratory Supplies (Poole, UK).

Animals and experimental design

Homozygous *dysf*^{-/-} male mice of a mixed 129SvJ and C57BL/g background (Stock 006830, Jackson Laboratories, Bar Harbor ME, USA), in which a 12-kb region of the *dysf* gene containing the last three exons (exons 53-55, aa 1983-2080) is deleted, removing the transmembrane domain, were used; C57/Bl/6J (termed, C57/Bl) mice served as wild-type controls. The mice were housed in cages under constant photoperiod (12:12 h L:D) with free access to food and water. All animal experiments were carried out according to the guidelines of the Volcani Center Institutional Committee for Care and Use of Laboratory Animals (IL-234/10). The mice were divided into three groups (n=10 per group) according to age (one, five and nine months, respectively) and were injected intraperitoneally (ip) with 7.5 μ g halofuginone per mouse three times a week for a period of three to four months. This protocol has been shown to be effective in other MD models (Turgeman et al., 2008; Nevo et al., 2010). A fourth group of *dysf*^{-/-} mice was not injected and formed the

control group. All mice were sacrificed at 12 months of age.

Motor coordination

Motor coordination and balance were evaluated with an accelerating single-station RotaRod treadmill (Med Associates, Inc., St. Albans, VT) as previously described (Turgeman et al., 2008; Nevo et al., 2010). The mice were placed one at a time on the rod, rotating at an initial speed of 3.0 rpm; the speed was gradually increased to 30 rpm over a period of 5 min, and the amount of time that the mice stayed on the rod was recorded. RotaRod performance is highly dependent on training, and the mice were therefore subjected to three successive trials in each session, and the tests were repeated on two consecutive days. The performance of each mouse was measured as the mean of its best individual performances over the three trials on the second day.

Preparation of sections, collagen staining and immunohistochemistry

At the end of the experiments, biopsies from quadriceps, longissimus, sublumbar, gastrocnemius and cardiac muscles were collected and fixed, and sections were prepared and stained as previously described (Turgeman et al., 2008; Nevo et al., 2010). The level of fibrosis was analyzed with ImagePro software (Media Cybernetics, Inc., Silver Spring, MD, USA). Photographs (n=15, i.e., five sections from three different mice from each group of 10 mice were analyzed) were taken at each time point for analysis at x20 magnification. The results were calculated as red area divided by the total area (red + green) and presented as the proportion of fibrotic muscle area (mean $\pm$ SE). Special care was taken to exclude blank areas, which were assumed to be artefactual. Tukey-Kramer HSD test was applied to the results, at $p=0.05$, using JMP software, version 6 (SAS Institute Inc., Cary, NC, USA). Immunohistochemistry was performed with antibodies against P₄H₈ (1:25), collagen type I (1:100), phospho-Smad3 (1:50) and macrophages (1:250). Peroxidase activity was determined with 3,3'-diaminobenzidine (DAB) as the chromogen. Nuclei were detected with 4',6-diamidino-2-phenylindole (DAPI).

Confocal microscopy

Microscopic observation and image acquisition were performed with an Olympus IX 81 inverted confocal laser-scanning microscope (Fluoview 500; Olympus, Tokyo, Japan) equipped with a 405-nm diode laser, a 488-nm argon-ion laser, a 543-nm helium-neon laser, and a 60x1.0 NA PlanApo water-immersion objective. DAPI was excited at 405 nm and the emission was collected through a BA 430-460 filter; "GREEN" was excited at 488 nm and the emission was collected through a BA 505-525 filter; "RED" was excited at 543

nm and the emission was collected through a BA 560 IF filter. The transmitted-light images were obtained by Nomarski differential interference contrast. Sections were stained with DAPI (1:1000) for 2 min and washed with PBS for 5 min. Goat anti-mouse IgG antibodies with Alexa Fluor dye (Molecular Probes, Carlsbad, CA, USA) were used.

Determination of number of myofibers with central nuclei

Muscle sections were stained with hematoxylin and eosin (H&E) and at least 20 evenly distributed photographs from each treatment group were analyzed. The number of myofibers containing central nuclei was expressed as a proportion of the total number of myofibers observed (with central or peripheral nuclei) and is presented as mean $\pm$ SE (Turgeman et al., 2008). Data were subjected to one-way analysis of variance (ANOVA) and to all-pairs Tukey-Kramer HSD test using JMP software.

Zymography and western blot

Matrix metalloproteinase (MMP) activity was determined in quadriceps and gastrocnemius muscle homogenates by gelatin-impregnated (0.01% w/v) 10% SDS-PAGE. Proteins were separated on the gel under non-reducing conditions, followed by 2 h of incubation in 2.5% (w/v) Triton X-100 and 24 h of incubation in 50 mM Tris pH 7.6, 0.2 M NaCl, and 5 mM CaCl₂ at 37°C. After the incubation, the gels were stained with 0.5% (w/v) Coomassie blue G250 in methanol/acetic acid/H₂O (30:10:60, v/v). Each lane contained a quadriceps muscle homogenate sample from a single mouse and the assay was performed 3 times with homogenates from 3 different mice. Western blot for muscle homogenates was performed as described (Roffe et al., 2010).

Fusion assay

Primary myoblasts from the hind-limb muscles of 8-week-old *dysf*^{-/-} mice were prepared as described previously (Ben Dov et al., 1999). Cells were plated at 5x10⁴ cell/cm² and grown in DMEM-supplemented with 20% (v/v) fetal calf serum (growing medium). The growing myoblasts were induced to differentiate with 2% (v/v) horse serum-containing DMEM for one day, then the medium was switched back to growing medium for an additional 2 h before halofuginone addition (10 nM) for one additional day. Myotube formation was verified by immunofluorescence assay for myosin heavy chain (MHC). Myotube fusion was analyzed by nuclear number assay (Horsley et al., 2001; Roffe et al., 2010). The number of nuclei in individual myotubes was counted for 600 to 700 myotubes and these were grouped into categories of cells exhibiting 2-5, 6-15, or >15 nuclei. The percentage of myotubes in each category was calculated. The data were subjected to ANOVA and to all-pairs Tukey-Kramer HSD test using

JMP software.

Results

Development of fibrosis in *dysf*^{-/-} mice

Mouse genotype was verified by immunofluorescence analysis, which revealed localization of *dysf* in the sarcolemma of wild-type (Wt) quadriceps muscle but no staining in the *dysf*^{-/-} mice, along with a complete loss of protein expression, as confirmed by western blot analysis (Fig. 1A). The mice exhibited a normal life span without any significant decrease in locomotion activity but their RotaRod performance deteriorated with age (1800±200 seconds at 4 weeks; 900±100 at 10 weeks; 200±20 at 20 weeks and 40±10 at 52 weeks). In patients with *dysf* deficiency, centrally-nucleated myofibers and inflammatory infiltrates are

usually observed near necrotic fibers and perivascular regions (Prelle et al., 2003). In the *dysf*^{-/-} mice, centrally nucleated myofibers were observed as early as 10 weeks of age and inflammatory infiltrates were observed at 20 weeks of age (Fig. 1B). Muscle fibrosis, as detected by Sirius red staining, was apparent at 36 weeks of age, and at 52 weeks, major collagen deposition was observed (Fig. 1B).

Effect of halofuginone on muscle histopathology

Quadriceps, sublumbar and longissimus muscles of 9-month-old *dysf*^{-/-} mice treated with halofuginone from 5 months of age exhibited a reduction in the percentage of centrally-nucleated myofibers out of total myofibers compared to controls (quadriceps - untreated 58±3% vs. treated 39±3%; sublumbar - untreated 49±4% vs. treated 29±2%; longissimus - untreated 53±4% vs. treated

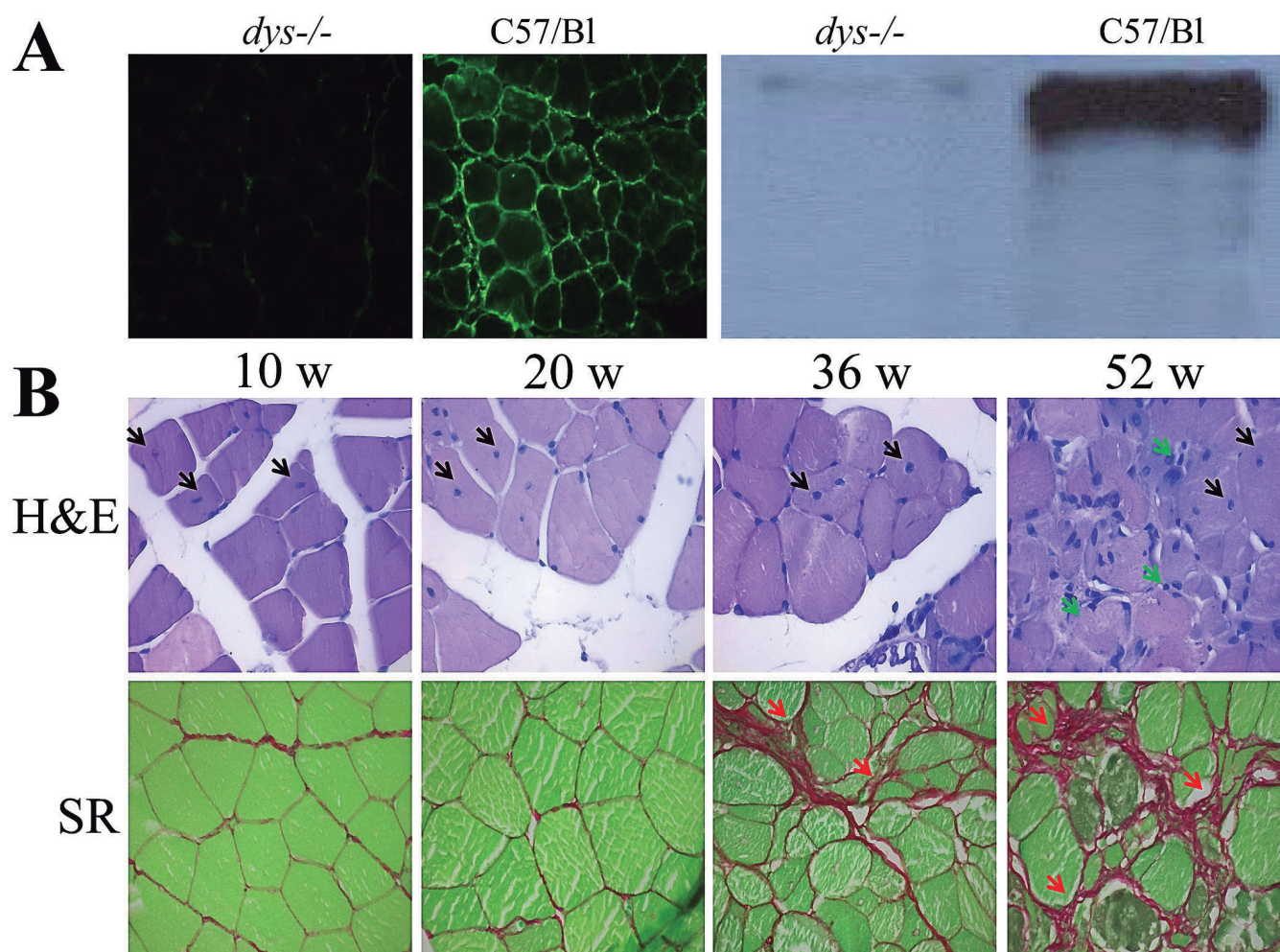


Fig. 1. **A.** Immunofluorescence staining (IHC) and western blot (WB) analysis of dysferlin in quadriceps muscle. Dysferlin was detected in the sarcolemma of C57/Bl muscle, with no equivalent staining in *dysf*^{-/-} muscle. A complete loss of dysferlin expression is observed in the western blot analysis in *dysf*^{-/-} muscle. **B.** Biopsies of quadriceps muscle sampled at various ages were stained with H&E and Sirius red (SR; collagen stains red). Black arrows point to central nuclei, green arrows point to inflammatory infiltrate and red arrows point to collagen bundles. x 40

Fibrosis in dysferlinopathy

31±2%). This was accompanied with reduced inflammatory infiltrates as well as a major reduction in collagen deposits (Fig. 2). Quantification of collagen content, expressed as red area divided by the total muscle area and presented as the proportion of fibrotic muscle area, revealed a significant reduction of 65, 62 and 64% in quadriceps, sublumbal and longissimus muscles, respectively (quadriceps - untreated 16.6±3.7 vs. treated 5.9±2.9; sublumbal - 21±4 vs. treated 8±2; longissimus - untreated 22±4 vs. treated 7.5±4) at 9 months. Similar results were observed in the longissimus muscle while almost no fibrosis was observed in the gastrocnemius (data not shown). A small but significant improvement in the RotaRod performance was observed (from 60±9 seconds in the untreated mice to 120±10 in the halofuginone-treated mice).

We took into consideration the late onset of the disease in *dysf*^{-/-} mice to evaluate the efficacy of early

and late halofuginone treatment. The *dysf*^{-/-} mice were treated with halofuginone for 3 to 4 months starting at 1 (1-5m), 5 (5-9m) or 9 (9-12m) months of age, and untreated mice were used as controls. At 12 months of age, quadriceps muscle biopsies were taken from all mice for histopathology. In the untreated *dysf*^{-/-} mice, high levels of inflammatory infiltrates and collagen content (monitored by Sirius red staining) were observed (Fig. 3A,B), as well as a high number of myofibers with central nuclei (Fig. 3A,C). Collagen content and number of myofibers with central nuclei were lower in 12-month-old mice that had undergone early halofuginone treatment (i.e., 1-5m, at a time when centrally-nucleated myofibers and inflammatory infiltrates are already evident but almost no fibrosis is observed) compared to the untreated mice. Halofuginone treatment at the beginning of fibrosis (5-9m) did not cause any further reduction in collagen levels in the 12-month-old mice.

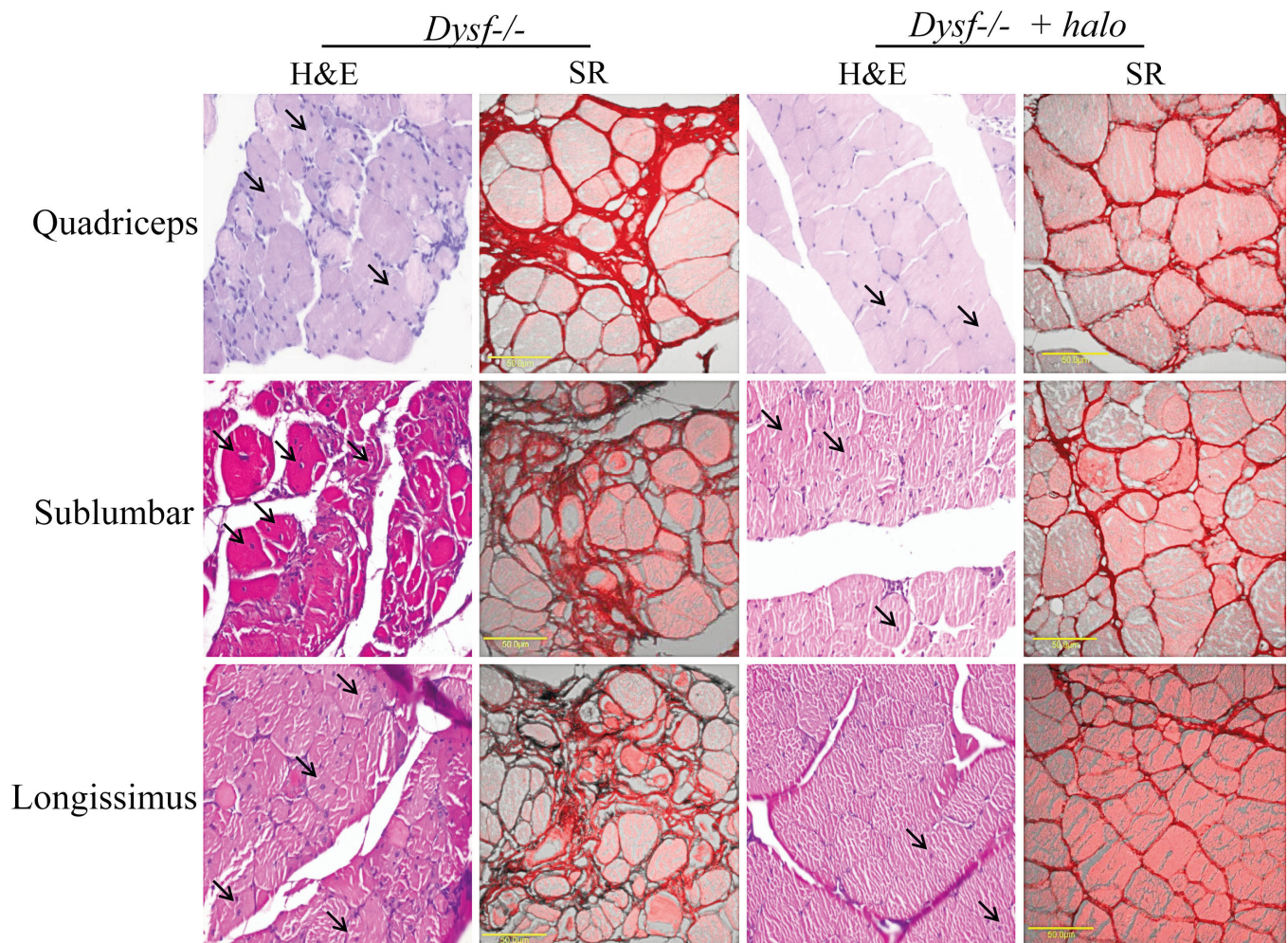


Fig. 2. Effect of halofuginone administered at early stage of dysferlinopathy development on muscle histopathology. Halofuginone (7.5 µg/mouse) was injected ip three times a week from 5 to 9 months. Quadriceps, sublumbal and longissimus muscles were stained with H&E for visualization of nuclei and inflammatory infiltrates or with Sirius red for collagen and visualized by confocal microscopy. Arrows point to myofibers with central nuclei. Note the reduction in the number of myofibers with central nuclei, in inflammatory infiltrates and the major reduction in collagen content in all muscles.

However, an additional decrease in the number of centrally nucleated myofibers, which differed significantly from their numbers in the untreated mice, was observed. The lowest number of centrally-nucleated myofibers was observed when the *dysf* knock-out mice were treated with halofuginone at later stages of the dystrophy (9-12m), without any further change in muscle collagen content.

Effect of halofuginone on cardiac fibrosis

At 12 months of age, cardiac fibrosis, as detected by Sirius red staining, was observed in the *dysf*^{-/-} mice, most markedly in the myocardium. A reduction in fibrosis and collagen type I content at 12 months of age was observed after halofuginone treatment, irrespective

of time of administration (Fig. 4). The fibrotic area covered 3.3% of the total cardiac area in the untreated mice and was reduced to 0.25% when treated with halofuginone from 1-5 months and to 0.04 and 0.03% when treated with halofuginone from 5-9 and 9-12 months, respectively. Almost identical results were obtained when quantified the collagen area in the cardiac tissue using collagen type I antibodies suggesting that the fibrosis consisted mainly of collagen type I.

Effect of halofuginone on infiltrating myofibroblasts and macrophages

The quadriceps muscle of 12-month-old untreated *dysf*^{-/-} mice was characterized by infiltration of myofibroblasts expressing P₄HB adjacent to myofibers

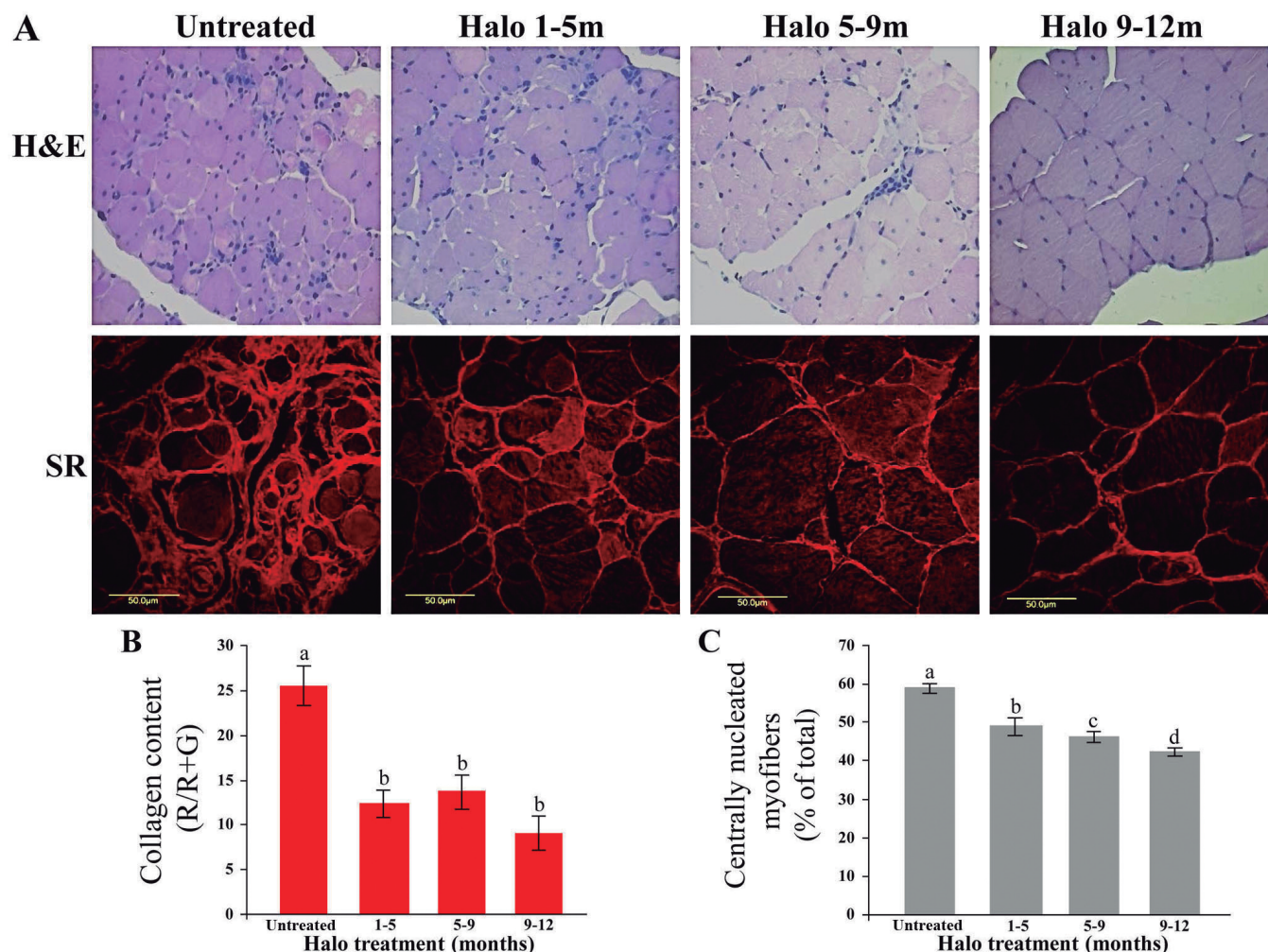


Fig. 3. Effect of early and late treatment with halofuginone on muscle histopathology of the *dysf*^{-/-} mice. Halofuginone (Halo; 7.5 μ g/mouse) was injected ip three times a week for the indicated time period. All mice were sacrificed at 12 months of age. **A.** Biopsies of quadriceps muscle were stained with H&E and Sirius red (SR) (collagen stains red), the latter was visualized by confocal microscopy. **B.** Image analysis of the Sirius red-stained collagen content was calculated from x20 magnification photos (n=15; five sections from three mice from each group); R/R+G, red area divided by the total area = proportion of fibrotic muscle area. **C.** Percentage of myofibers with central nuclei. Results are means $\pm$ SE and columns with different letters within each experiment differ significantly (P<0.05).

with central nuclei (Fig. 5, upper panels). Treatment with halofuginone at the very early stages of the disease progression (1-5m) almost completely eliminated myofibroblasts expressing P₄H β . The disappearance of these myofibroblasts was also seen in mice treated with halofuginone at later stages of the disease (5-9 m and 9-12 m). Many macrophages were observed in the quadriceps muscle of the untreated 12-month-old *dysf*^{-/-} mice, particularly adjacent to the degenerative area (Fig. 5, lower panels). A marked reduction in macrophage numbers was observed after halofuginone treatment, regardless of time of administration, starting from the very early stages of disease progression. As in the case of myofibroblasts, the few macrophages left after halofuginone treatment were usually localized adjacent to centrally-nucleated myofibers. To reveal the point of entry, double-immunostaining for P₄H β -expressing cells and macrophages in the quadriceps muscle of 12-month-old *dysf*^{-/-} mice was performed (Fig. 6). The macrophages and myofibroblasts were seen to infiltrate the muscle at the same location in areas adjacent to centrally-nucleated myofibers (Fig. 6) and from there to spread out to the entire muscle.

Effect of halofuginone on MMP activity

In the quadriceps muscle biopsies, activities of two major matrix MMPs were observed, namely MMP 9 and

2. There was almost no MMP 9 or 2 activity in the quadriceps muscle biopsies from C57/Bl or untreated *dysf*^{-/-} mice (Fig. 7). A major increase in the activity of both MMPs was observed in 12-month-old biopsies after halofuginone treatment, independent of the time of treatment. Similar results were observed in gastrocnemius muscle biopsies (data not shown).

Inhibition of Smad3 phosphorylation

TGF β , the major cytokine driving tissue fibrosis, signals via Smad3 (Flanders, 2004). The TGF β -dependent phosphorylated Smad3 binds with Smad4 and this complex translocates to the nucleus where it regulates transcription of target genes. pSmad-positive cells were hardly detected in sections of quadriceps muscle of 12-month-old C57/Bl mice, confirming earlier reports (Turgeman et al., 2008), while in *dysf*^{-/-} mice these numbers were greatly increased (Fig. 8A). Halofuginone treatment (9-12m) greatly reduced the number of pSmad-positive cells. A western blot analysis of protein lysate from this muscle revealed a 3.9-fold decrease in pSmad3/Smad3 ratio in halofuginone-treated relative to non-treated *dysf*^{-/-} mice. In C57/Bl mice muscle, no centrally-nucleated myofibers were noted and hardly any nuclei exhibiting pSmad3 were observed (Fig. 8B,C). However, in the *dysf*^{-/-} mice, the level of pSmad3 protein was markedly higher, localizing in both

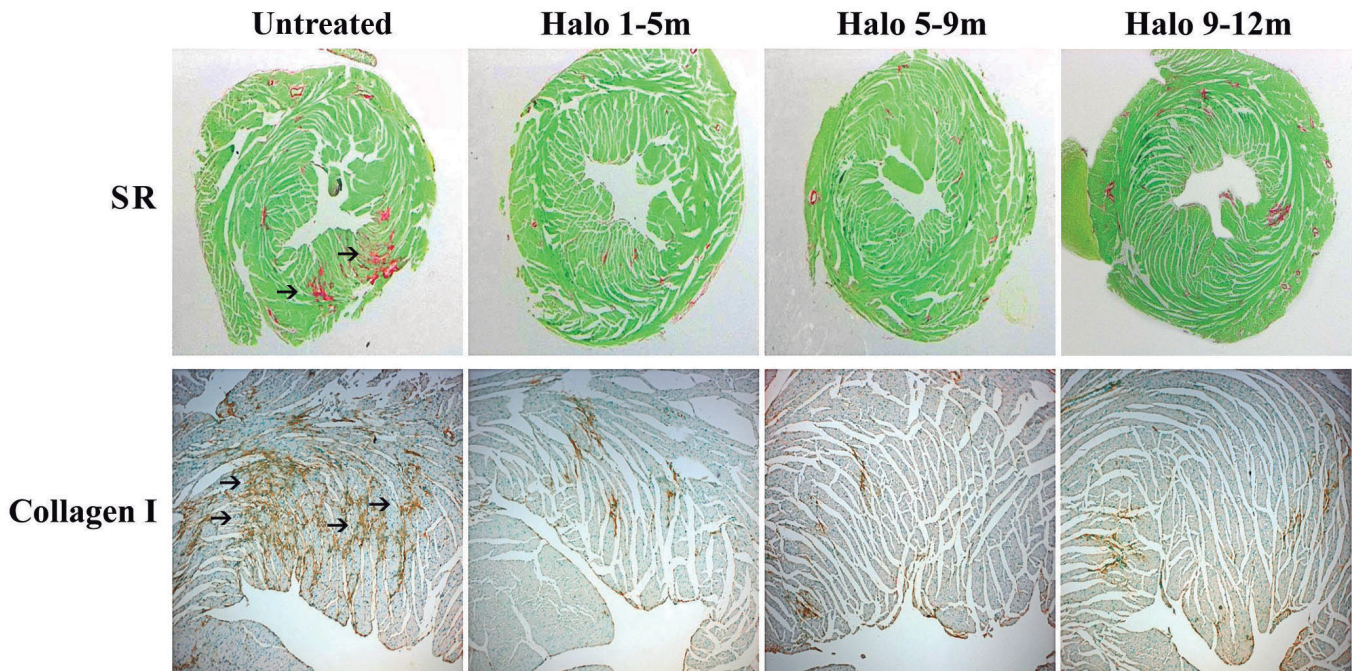


Fig. 4. Effect of halofuginone on collagen synthesis in the cardiac tissue of *dysf*^{-/-} mice. Halofuginone (Halo; 7.5 μ g/mouse) was injected ip three times a week for the indicated time period. At 12 months of age, the myocardium exhibits high levels of collagen as demonstrated by Sirius red (SR) staining (upper panels, x 4 magnification). The collagen is mainly collagen type I as demonstrated by immunohistochemistry with anti-collagen type I antibody (lower panels, x 40 magnification). Note the reduction in myocardium collagen content after treatment with halofuginone.

central and interstitial nuclei. In the halofuginone-treated muscle, almost no myofibers with central nuclei were observed and the treatment almost completely eliminated the number of pSmad3-positive nuclei.

Effect of halofuginone enhancing myotube fusion

Fusion of cultured *dysf*^{-/-} myotubes was ranked according to number of nuclei. The percentage of larger myotubes (for myofibers with 6 to 15 myonuclei) tended to be higher, and it was significantly higher (for myofibers with more than 15 nuclei) after incubation with halofuginone (10 nM) compared to the controls, indicating a promotive effect of halofuginone on myotube fusion (Fig. 9).

Discussion

Inflammatory changes (Gallardo et al., 2001; Confalonieri et al., 2003) and increased endomysial and perimysial connective tissue (Selcen et al., 2001) are key features of muscle histopathology in dysferlinopathies. In *dysf*^{-/-} mice, the presence of myofibers with central

nuclei, indicating at least one cycle of degeneration-regeneration process (Fig. 1B), precedes the increase in collagen content. A similar phenomenon has been reported for *mdx* mice, in which diaphragm muscle dysfunction preceded gross collagen deposition (Coirault et al., 2003). Together, these findings suggest that part of the muscle dysfunction is an intrinsic characteristic which cannot be accounted for only by fibrosis. Notably, endomysial fibrosis is the only myopathological parameter that significantly correlates with poor motor outcome (Desguerre et al., 2009), and inhibition of muscle fibrosis facilitates more efficient muscle recovery and increased muscle performance (Chan et al., 2003; Huebner et al., 2008).

Treatment of *dysf*^{-/-} mice with halofuginone for 4 months starting at 5 months of age resulted in decreased inflammatory infiltrates and collagen deposition, along with a decrease in the number of myofibers with central nuclei (Fig. 2). These results demonstrate the efficacy of halofuginone in preventing muscle pathology in a mouse model of dysferlinopathy at early stages of disease progression, as observed previously using mouse models of DMD and CMD (Turgeman et al., 2008; Nevo et al.,

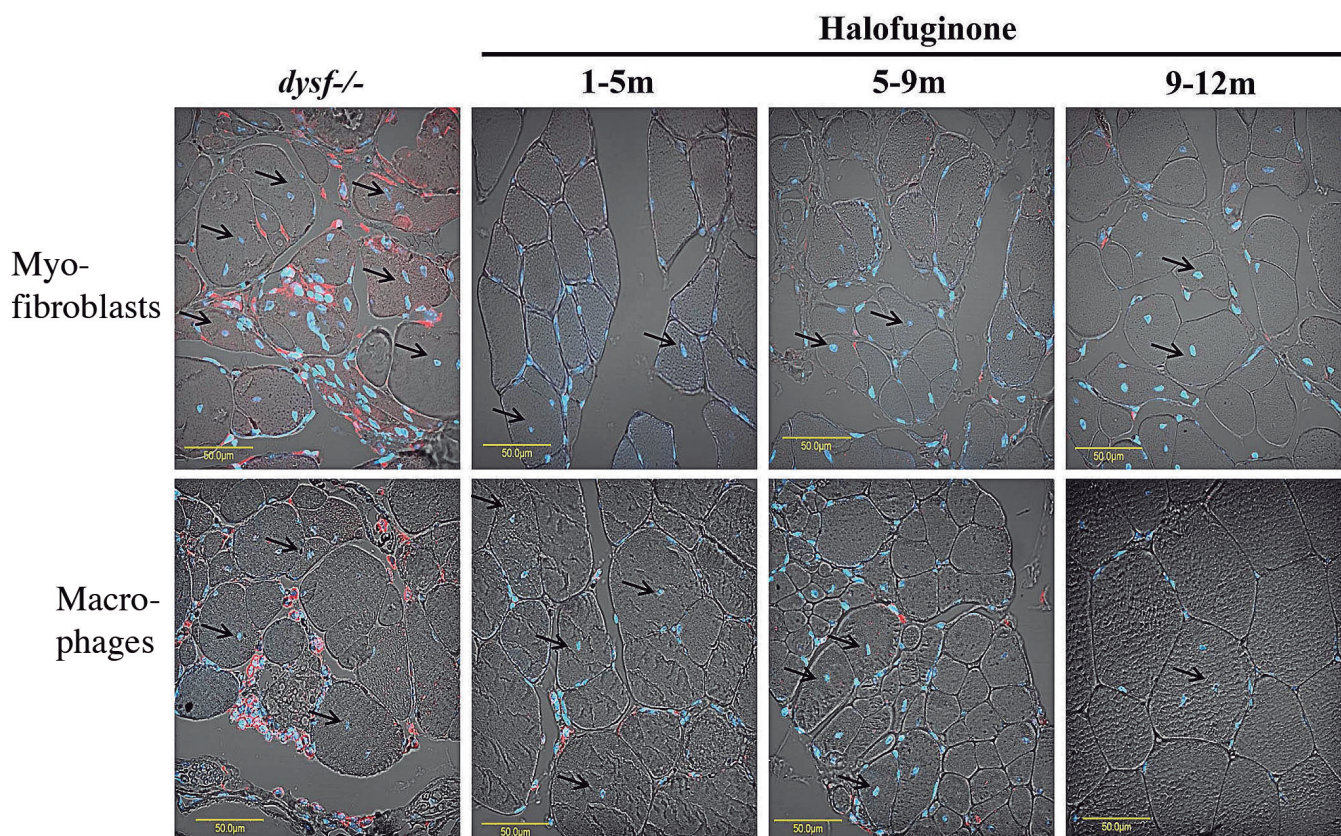


Fig. 5. Infiltration of myofibroblasts expressing P₄H β and macrophages into quadriceps muscle of *dysf*^{-/-} mice. Mice were treated with halofuginone for the indicated time period and all mice were sacrificed at 12 months of age. Muscle sections were immunostained with anti-P₄H β monoclonal and anti-macrophage polyclonal antibodies and observed by confocal microscopy. Upper panels, myofibroblasts (red); lower panels, macrophages (red). Nuclei were detected with DAPI. Arrows indicate myofibers with central nuclei. Note the reduction in the number of P₄H β -expressing cells and macrophages after treatment with halofuginone at the very early stages of the disease.

2010). Moreover, halofuginone administration to *dysf*^{-/-} mice, regardless of the timing of muscle degeneration, resulted in an approximately 50% reduction in muscle collagen content at 12 months of age that was accompanied with improved muscle performance. We suggest that different mechanisms are involved in halofuginone-dependent inhibition of collagen content at early and late stages of disease progression. At the early stages, halofuginone prevents the increase in collagen synthesis by inhibiting muscle infiltration of macrophages, the source of the cytokines needed for activation/recruitment of myofibroblasts. At a later stage of the disease, in addition to inhibition of collagen synthesis, halofuginone causes resolution of the established fibrosis, as observed in rat liver fibrosis, tight-skin mice and a patient with graft-versus-host disease (Nagler and Pines, 1999; Bruck et al., 2001; Pines et al., 2001), probably by activating MMPs (Popov et al., 2006). Indeed, in the *dysf*^{-/-} mice, MMP 2 and 9 activities were increased by halofuginone and remained high for months after the end of the treatment (Fig. 7). One possible mechanism underlying halofuginone's effect on MMP activity might be inhibition of the synthesis of tissue inhibitors of MMPs (TIMPs), as has been observed in other fibrotic conditions (Bruck et al.,

2001; Zion et al., 2009).

The infiltrating myofibroblasts are derived from either resident fibroblasts or recruited from circulating bone marrow-derived cells, although an additional source has been attributed to myofiber-associated satellite cells (Alexakis et al., 2007). Myofibroblasts are the main source of muscle collagen, as has been observed in *dy*^{2j}*dy*^{2j} and *mdx* mice (Turgeman et al., 2008; Nevo et al., 2010; Zannotti et al., 2010). The thick collagen layer, which is deposited around the muscle fibers and capillary walls by these cells, reduces the blood supply to individual myofibers and delays muscle-fiber regeneration following necrosis (Ishitobi et al., 2000). TGF β is the major activator of quiescent fibroblasts into differentiated myofibroblasts with migration and ECM-production properties (Serrano et al., 2011). Halofuginone, which inhibits TGF β signaling, almost completely abolished the P₄H β -expressing myofibroblast infiltration into the dystrophic muscle of *dysf*^{-/-} mice from all treatment groups (Fig. 5). These results are consistent with the observation of halofuginone-dependent inhibition of myofibroblast activation in the stroma and infiltration into tumors (Sheffer et al., 2007; Genin et al., 2008), suggesting a general effect of halofuginone on the fibroblast-to-

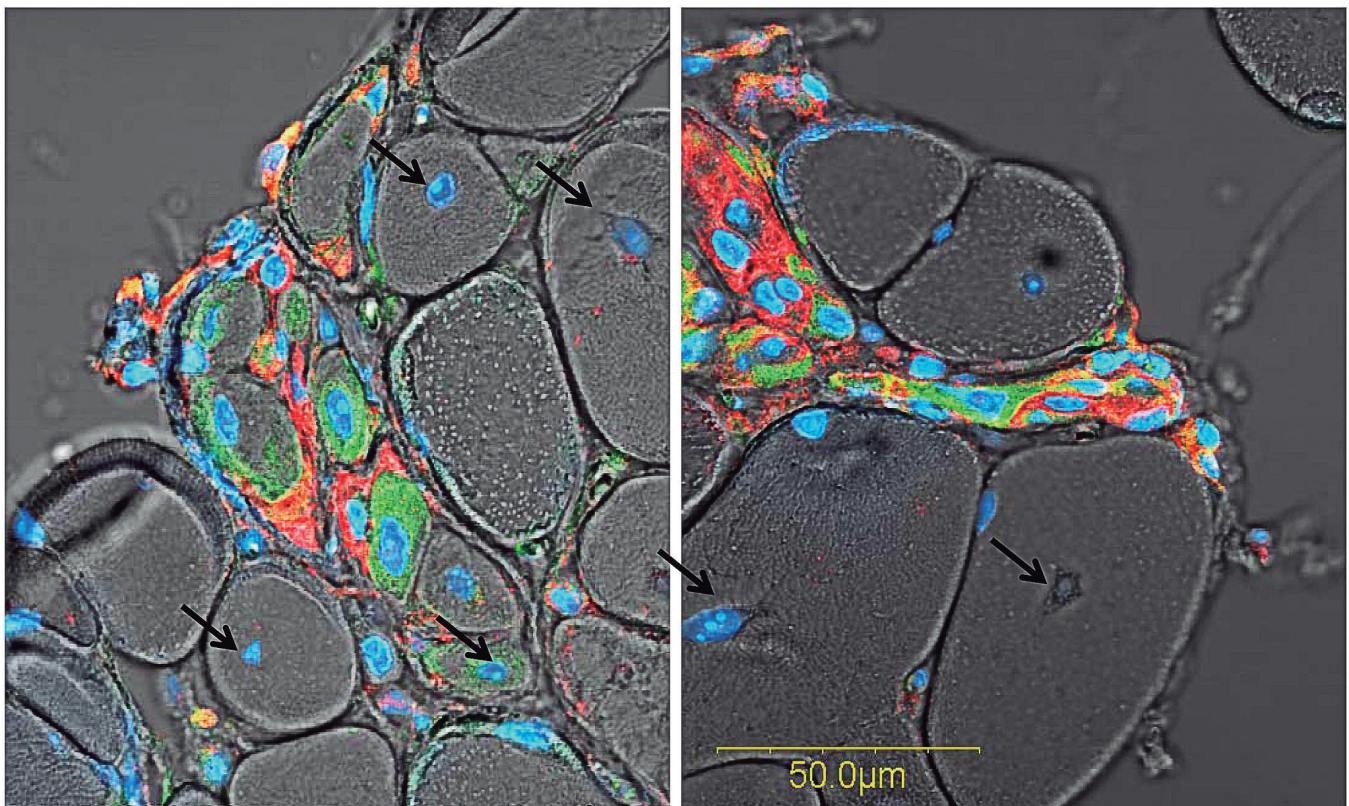


Fig. 6. Double-immunostaining for P₄H β -expressing cells (green) and macrophages (red) in quadriceps muscle of *dysf*^{-/-} mice. Arrows indicate myofibers with central nuclei. Note that the myofibroblasts and macrophages are next to each other and adjacent to myofibers with central nuclei.

myofibroblast transition and migration, regardless of disease status.

Myofiber damage in *dysf*-deficient muscle stimulates an inflammatory cascade that may initiate, exacerbate, and possibly perpetuate the underlying myofiber-specific dystrophic process (Nagaraju et al., 2008). Macrophages are the most common inflammatory cells in patients and mouse models of dysferlinopathies (Gallardo et al., 2001; Confalonieri et al., 2003; Nemoto et al., 2007). Activated tissue macrophages are a rich source of inflammatory cytokines that are capable of causing extensive tissue damage (Rawat et al., 2010) and/or recruit myofibroblasts (Segawa et al., 2008). The present study showed that the point of entry of the macrophages and myofibroblasts resides near the centrally-nucleated myofibers (Fig. 6), from which they spread to the entire muscle (Fig. 5). A recent study has reported that *dysf*-deficient myotubes release more soluble factors involved in monocyte chemotaxis than control myotubes, suggesting that endogenous chemotactic factors are crucial to the sustained inflammatory process observed in dysferlinopathies (De Luna et al., 2010). In agreement with these findings, the close proximity of myofibroblasts and macrophages to myofibers with central nuclei, as found in this study, suggests cross-talk between inflammatory cells, ECM-producing cells and regenerating myofibers which replace the necrotic ones. Therefore, a vicious cycle is created: myofiber necrosis results in migration of monocytes into the muscle, where they become tissue macrophages. This is probably a general mechanism since in *dy^{2j}/dy^{2j}* mice myofibroblasts also reside near myofibers with central nuclei (Nevo et al., 2010). The fact that the number of macrophages and myofibroblasts

in the muscle was significantly reduced by halofuginone may explain the lesser need for vicious cycles of muscle degeneration-regeneration, hence the lesser numbers of centrally-nucleated myofibers (Fig. 3).

Other drugs have been shown to reduce muscle macrophage numbers, although without affecting muscle histopathology in mouse models of dysferlinopathies (Rayavarapu et al., 2010). This is in contrast to halofuginone's effect demonstrating a major improvement in both parameters. Whether halofuginone directly inhibits the transition of fibroblasts to myofibroblasts with migration capabilities and macrophage invasion into the dystrophic muscle, or inhibits the secretion of chemotactic factors from damaged myofibers remains to be determined.

Dysferlin is involved in cardiomyocyte membrane repair and *dysf* deficiency leads to cardiomyopathy (Han et al., 2007). MM patients demonstrate fibrosis-related myocardial dysfunction (Choi et al., 2010) and cardiac abnormalities, together with interstitial fibrosis, have been observed in LGMD 2B patients (Wenzel et al., 2007). Mechanical stress has been shown to increase cardiac fibrosis in various mouse models of dysferlinopathy. Dysferlin-deficient and control mice had different gene expression patterns regarding the mechanism of cardiomyocyte Z-disc and signal transduction proteins (Wenzel et al., 2007). In the present study, halofuginone treatment beginning at different stages of the dystrophic process almost totally inhibited the development of myocardium fibrosis consisting mainly of collagen type I. As occurs in the quadriceps muscle, when the mice were treated for 4 months at a very early stage of disease progression and then remained untreated, the inhibition of myocardium

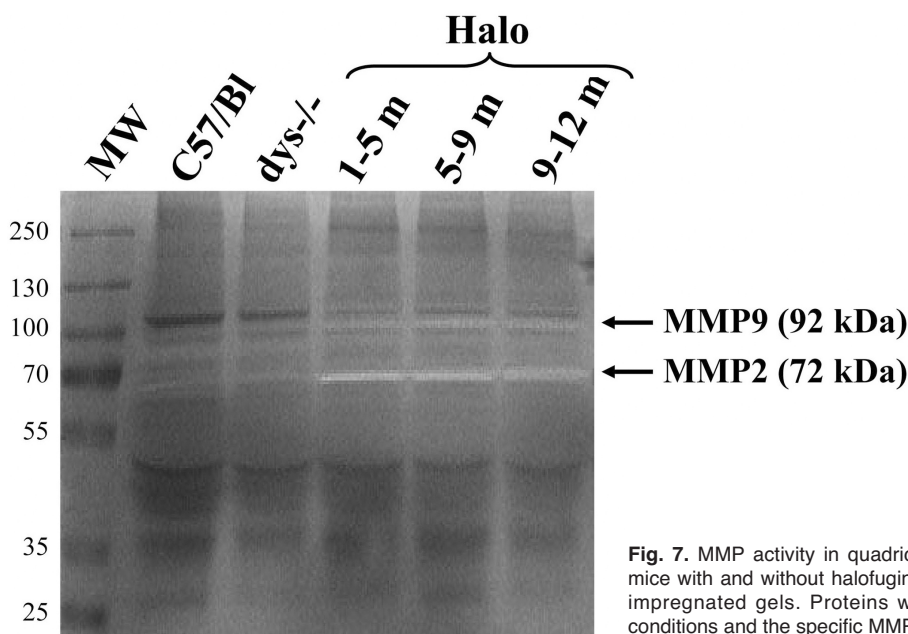


Fig. 7. MMP activity in quadriceps muscle homogenates of C57/Bl and *dysf*^{-/-} mice with and without halofuginone (Halo) treatment was determined on gelatin-impregnated gels. Proteins were separated on the gel under non-reducing conditions and the specific MMPs were determined based on their MW.

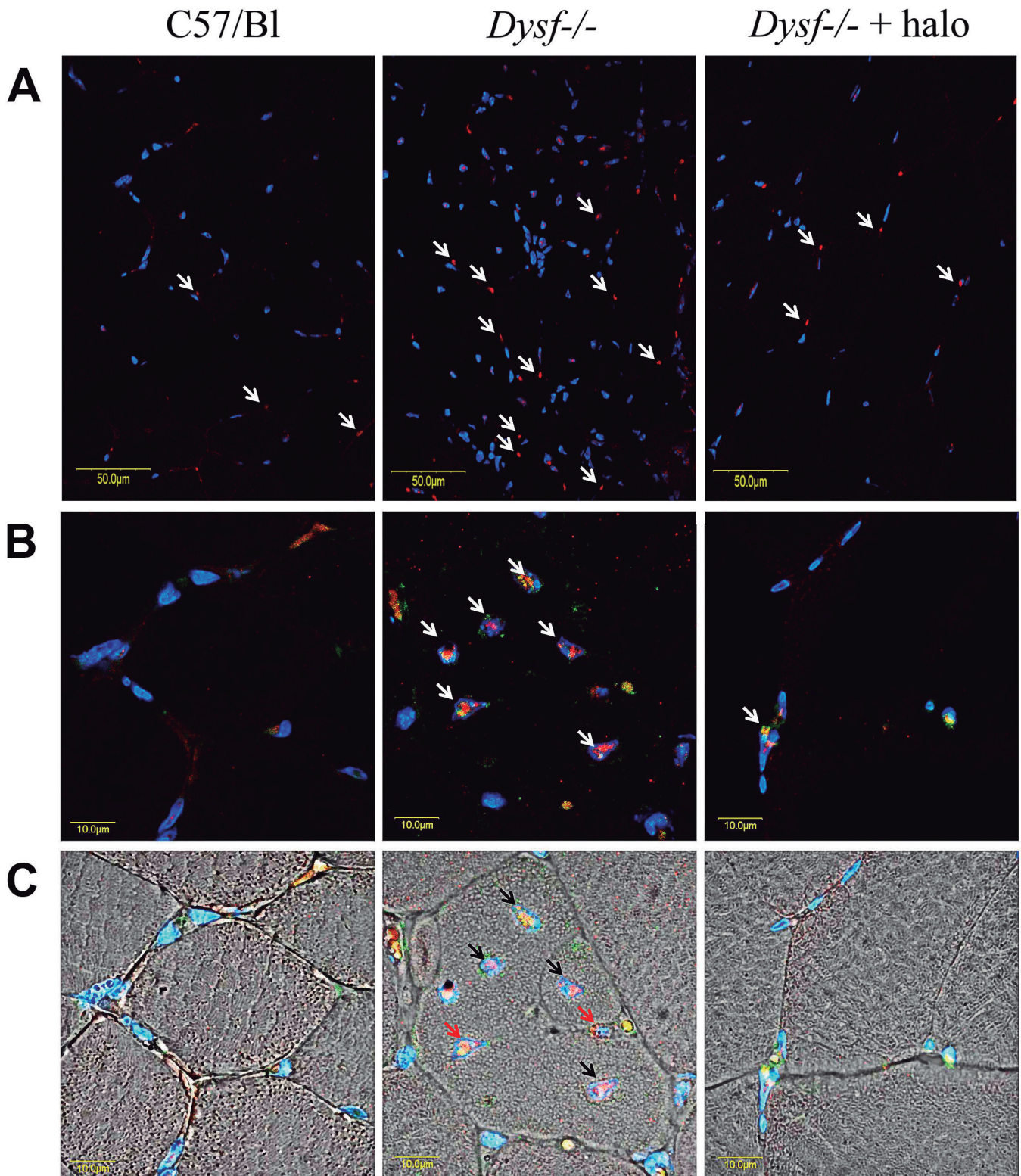
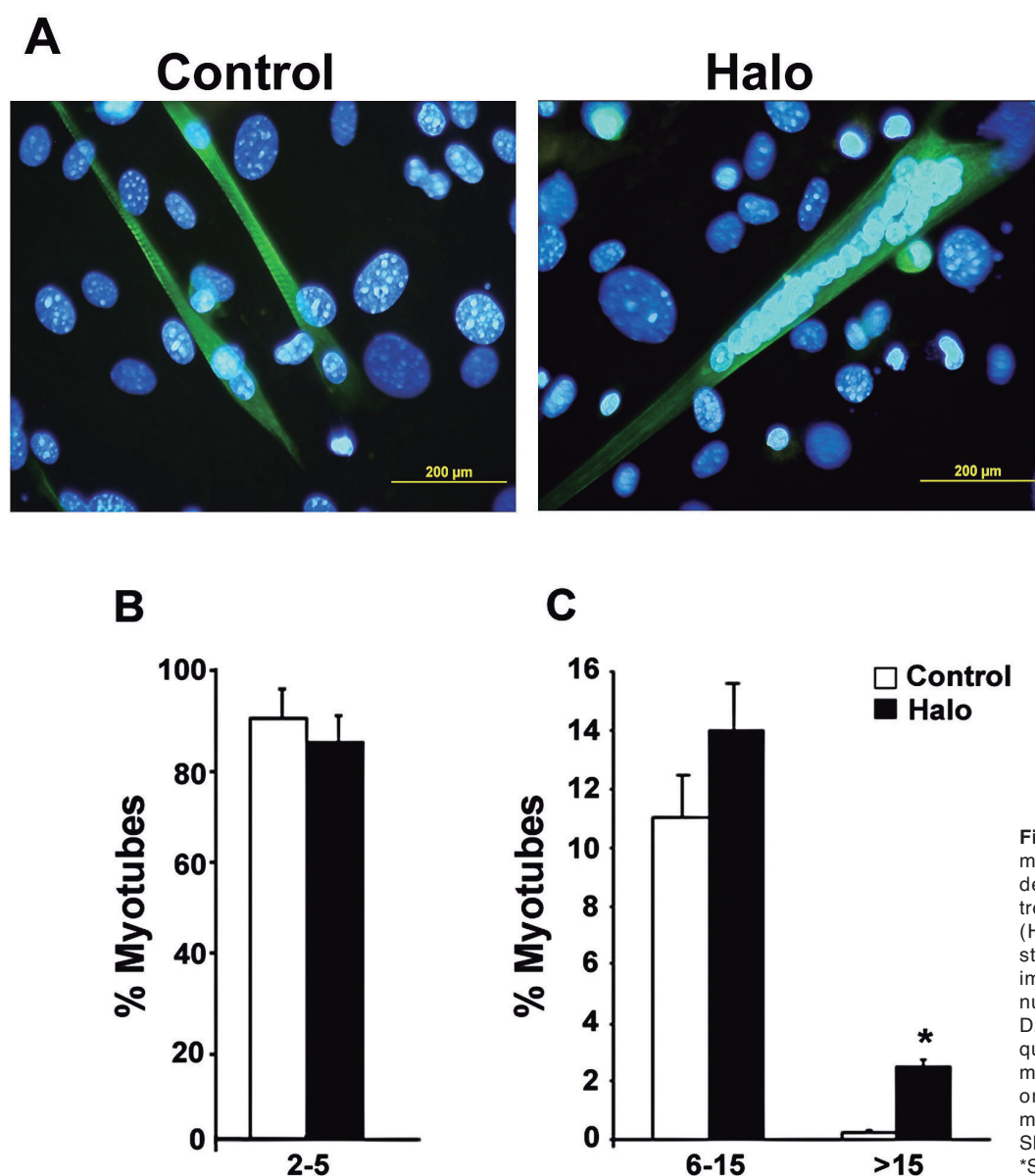


Fig. 8. Inhibition of Smad3 phosphorylation by halofuginone. Quadriceps muscle sections from C57/Bl and *dysf*^{-/-} mice before and after treatment with halofuginone (Halo) (7.5 μg/mouse, 3 times a week from 9 to 12 months) were immunostained with anti-pSmad3 antibody (stained red) and the nuclei were counterstained with DAPI. **A.** Low magnification. Arrows point to pSmad3 signal. **B.** High magnification. White arrows (dark field,) indicate pSmad3-positive nuclei. **C.** Some of the myofiber nuclei are central nuclei as indicating by the black arrows (bright field,) and some are interstitial nuclei (red arrows). Note halofuginone inhibition of the increase in pSmad3-positive nuclei in the *dysf*^{-/-} muscle.

fibrosis continued to be observed until at least 12 months of age (Fig. 4). Halofuginone has also been shown to inhibit cardiac fibrosis and to improve cardiac function in young *mdx* mice and in old mice with established fibrosis (Turgeman et al., 2008; Huebner et al., 2008). Together, the findings of fibrosis reduction in skeletal muscles in other MDs (Turgeman et al., 2008; Nevo et al., 2010) and in other tissues, such as lung, liver and pancreas (Pines, 2008), highlight a canonical mechanism for halofuginone's antifibrotic effect, regardless of the tissue.

In *dysf*^{-/-} mice, early halofuginone treatment resulted in reduced fibrosis and improved muscle histopathology, which lasted for months after

termination of the treatment (Fig. 3). In contrast, in *mdx* mice, halofuginone-dependent reduction in fibrosis requires continuous treatment, as termination of halofuginone treatment resulted in reversal of muscle fibrosis (Turgeman et al., 2008). This disparity between the two mouse models is probably due to differences in the disease progression and in the involvement of the innate immune system which promotes inflammatory changes. The *mdx* mice exhibit an early onset/severe phenotype with disruption of the dystrophin-associated glycoprotein complex (DGC) and increased susceptibility to contraction-induced injury, whereas the *dysf*^{-/-} mice, with a late onset/mild phenotype, possess a structurally intact and stable DGC and skeletal muscles



that are essentially normal with respect to resistance to contraction-induced injury (Chiu et al., 2009). In addition, complement system-mediated muscle injury is central to the pathogenesis of dysferlinopathy; a recent report has shown that genetic disruption of the central component (C3) of the complement system ameliorates muscle pathology in *dysf*-deficient mice but has no significant beneficial effect in *mdx* mice (Han et al., 2010).

An increase in TGF β activity leads to an increase in nuclear accumulation of pSmad3 and in muscle fibrosis, resulting in failed muscle regeneration and impaired muscle repair in various MDs (Cohn et al., 2007). A major increase in pSmad3-expressing cells was observed in the *dysf*^{-/-} mice compared to the wild type (Fig. 8), which co-localized with both myofibers' central nuclei and the nuclei of cells surrounding the myofibers. Halofuginone treatment resulted in a major reduction in pSmad3 content. The central role of Smad3 as a downstream mediator of TGF β signal in MDs highlights its potential as a target for pharmacological intervention. Because of the extremely pleiotropic nature of TGF β , therapies that target its expression or its receptors are likely to be associated with an array of adverse effects, including abnormal cell proliferation, inflammatory changes and autoimmunity (Andreotta et al., 2006). Therefore, drugs that selectively target downstream events are likely to be more successful by eliciting fewer side effects. Halofuginone has been shown to be effective and well tolerated in many animal models of fibrosis (Pines et al., 2000; Pines, 2008) as well as in humans (Nagler and Pines, 1999; De Jonge et al., 2006). Moreover, inhibition of Smad3 phosphorylation would probably target pathological mechanisms common to other forms of MDs as was found in the mouse models for DMD and CMD (Turgeman et al., 2008; Nevo et al., 2010).

The existence of an additional direct effect of halofuginone on the muscle tissue cannot be ruled out, as supported by different findings: the prevention of muscle histopathology of the *dysf*^{-/-} mouse; the increase in average of myofiber diameter by halofuginone treatment at early stages of disease progression before the onset of collagen deposition (Fig. 3 and Barzilai-Tutsch, unpublished data.); the inhibition of Smad3 phosphorylation in muscle in general and in myofibers with central nuclei in particular (Fig. 8); the inhibition of Smad3 phosphorylation in myoblasts derived from both *mdx* and *dy*^{2J/dy}^{2J} mice *in vitro* (Roffe et al., 2010), and the increase of myotube fusion *in vitro* (Fig. 9). It is important to note that dysferlin functions as a membrane-fusion protein in the wound-healing system of the plasma membrane, so that any defect in *dysf* gene will cause insufficiency in membrane fusion (Hino et al., 2009). Mouse (Demonbreun et al., 2011) and human (De Luna et al., 2006) *dysf*-null myoblasts are defective in myoblast-myotube fusion, resulting in smaller myotubes in culture. One of the explanations for halofuginone's beneficial effect on myotube fusion might be its

promotion of mitogen-activated protein kinase/extracellular signal regulated protein kinase (MAPK/ERK) and phosphoinositide 3-kinase/Akt -signaling pathways. In *mdx* mice, myotube fusion is at least partially dependent on halofuginone's ability to phosphorylate Akt and MAPK/ERK (Roffe et al., 2010). This is probably a general mechanism since in preliminary experiments using isolated *dysf*^{-/-} myofibers and myoblasts, induction of Akt and MAPK/ERK phosphorylation was also observed in response to halofuginone (Barzilai-Tutsch, Mimon and Halevy, unpublished results). Akt signaling attenuates muscular degeneration, promotes myofiber regeneration and improves muscle function in *mdx* mice (Kim et al., 2011). In some models of myopathies, a reduction in dysferlin was associated with perturbed MAPK/ERK activation during contraction (Shaw et al., 2006), and activation of Akt was associated with an increase in dysferlin levels (Blaauw et al., 2008). In preliminary experiments no effect of halofuginone was observed on Evans blue uptake in *dysf*^{-/-} isolated myofibers (data not shown).

In summary, in patients with dysferlinopathy at late stages of disease progression, the prevention of secondary events, such as fibrosis and inflammation, which preclude efficient regeneration and limit the success of gene transfer and cell transplantation, is of great importance. We demonstrate that early halofuginone treatment, even before any typical changes in histopathology are observed, can improve muscle histopathology in *dysf*^{-/-} mice. These effects are associated with a reduction of infiltrating myofibroblasts and macrophages, muscle and cardiac fibrosis, inflammation, and number of centrally nucleated myofibers. These improvements are probably achieved by the combined effects of halofuginone on myofibroblast and macrophage infiltration to the dystrophic muscle on one hand and on myofibers on the other. Thus, halofuginone, which has significant impacts on molecular, tissue and functional phenotypes in dystrophic mice, meets the criteria for a potential novel antifibrotic drug for patients exhibiting MDs of various etiologies.

Acknowledgments. This study was supported in part by the Muscle Dystrophy Association (MDA 68007), the Israel Science Foundation (ISF 1315/10), the Association Française contre les myopathies (AFM 14105) and the Legacy Heritage Bio-Medical Program of the Israel Science Foundation (grant No. 1315/10). We thank Edouard Belauso for his help with the confocal microscopy. This paper is a contribution from the Agricultural Research Organization, the Volcani Center, Bet Dagan, Israel.

References

- Alexakis C., Partridge T. and Bou-Gharios G. (2007). Implication of the satellite cell in dystrophic muscle fibrosis: a self-perpetuating mechanism of collagen overproduction. *Am. J. Physiol. Cell. Physiol.*

- 293, C661-669.
- Andreetta F., Bernasconi P., Baggi F., Ferro P., Oliva L., Arnoldi E., Cornelio F., Mantegazza R. and Confalonieri P. (2006). Immunomodulation of TGF- β 1 in mdx mouse inhibits connective tissue proliferation in diaphragm but increases inflammatory response: implications for antifibrotic therapy. *J. Neuroimmunol.* 175, 77-86.
- Bansal D., Miyake K., Vogel S.S., Groh S., Chen C.C., Williamson R., McNeil P.L. and Campbell K.P. (2003). Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature* 423, 168-172.
- Barthélémy F., Wein N., Krahn M., Lévy N. and Bartoli M. (2011). Translational research and therapeutic perspectives in dysferlinopathies. *Mol. Med.* 17, 875-882.
- Bashir R., Britton S., Strachan T., Keers S., Vafiadaki E., Lako M., Richard I., Marchand S., Bourg N., Argov Z., Sadeh M., Mahjneh I., Marconi G., Passos-Bueno M.R., Moreira Ede S., Zatz M., Beckmann J.S. and Bushby K. (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.
- Ben-Dov N., Shefer G., Irintchev A., Wernig A., Oron U. and Halevy O. (1999). Low-energy laser irradiation affects satellite cell proliferation and differentiation in vitro. *Biochim. Biophys. Acta* 1448, 372-380.
- Bernasconi P., Torchiana E., Confalonieri P., Brugnoli R., Barresi R., Mora M., Cornelio F., Morandi L. and Mantegazza R. (1995). Expression of transforming growth factor- β 1 in dystrophic patient muscles correlates with fibrosis. Pathogenetic role of a fibrogenic cytokine. *J. Clin. Invest.* 96, 1137-1144.
- Bittner R.E., Anderson L.V., Burkhardt E., Bashir R., Vafiadaki E., Ivanova S., Raffelsberger T., Maerk I., Höger H., Jung M., Karbasiyan M., Storch M., Lassmann H., Moss J.A., Davison K., Harrison R., Bushby K.M. and Reis A. (1999). Dysferlin deletion in SJL mice (SJL-Dysf) defines a natural model for limb girdle muscular dystrophy 2B. *Nat. Genet.* 23, 141-142.
- Blaauw B., Mammucari C., Toniolo L., Agatea L., Abraham R., Sandri M., Reggiani C. and Schiaffino S. (2008). Akt activation prevents the force drop induced by eccentric contractions in dystrophin-deficient skeletal muscle. *Hum. Mol. Genet.* 17, 3686-3696.
- Bruck R., Genina O., Aeed H., Alexiev R., Nagler A., Avni Y. and Pines M. (2001). Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. *Hepatology* 33, 379-386.
- Brunn A., Schröder R. and Deckert M. (2006). The inflammatory reaction pattern distinguishes primary dysferlinopathies from idiopathic inflammatory myopathies: an important role for the membrane attack complex. *Acta Neuropathol.* 112, 325-332.
- Bushby K.M. (2000). Dysferlin and muscular dystrophy. *Acta Neurol. Belg.* 100, 142-145.
- Cacciottolo M., Numitone G., Aurino S., Caserta I.R. and Fanin M. (2011). Muscular dystrophy with marked Dysferlin deficiency is consistently caused by primary dysferlin gene mutations. *Eur. J. Hum. Genet.* 19, 974-980.
- Chan Y.S., Li Y., Foster W., Horaguchi T., Somogyi G., Fu F.H. and Huard J. (2003). Antifibrotic effects of suramin in injured skeletal muscle after laceration. *J. Appl. Physiol.* 95, 771-780.
- Chase T.H., Cox G.A., Burzenski L., Foreman O. and Shultz L.D. (2009). Dysferlin deficiency and the development of cardiomyopathy in a mouse model of limb-girdle muscular dystrophy 2B. *Am. J. Pathol.* 175, 2299-2308.
- Chiu Y.H., Hornsey M.A., Klinge L., Jørgensen L.H., Laval S.H., Charlton R., Barresi R., Straub V., Lochmüller H. and Bushby K. (2009). Attenuated muscle regeneration is a key factor in dysferlin-deficient muscular dystrophy. *Hum. Mol. Genet.* 18, 1976-1989.
- Choi E.R., Park S.J., Choe Y.H., Ryu D.R., Chang S.A., Choi J.O., Lee S.C., Park S.W., Kim B.J., Kim D.K. and Oh J.K. (2010). Early detection of cardiac involvement in Miyoshi myopathy: 2D strain echocardiography and late gadolinium enhancement cardiovascular magnetic resonance. *J. Cardiovasc. Magn. Reson.* 12, 31.
- Cohn R.D., van Erp C., Habashi J.P., Soleimani A.A., Klein E.C., Lisi M.T., Gamradt M., ap Rhys C.M., Holm T.M., Loeys B.L., Ramirez F., Judge D.P., Ward C.W. and Dietz H.C. (2007). Angiotensin II type 1 receptor blockade attenuates TGF- β -induced failure of muscle regeneration in multiple myopathic states. *Nat. Med.* 13, 204-210.
- Coirault C., Pignol B., Cooper R.N., Butler-Browne G., Chabrier P.E. and Lecarpentier Y. (2003). Severe muscle dysfunction precedes collagen tissue proliferation in mdx mouse diaphragm. *J. Appl. Physiol.* 94, 1744-1750.
- Confalonieri P., Oliva L., Andreetta F., Lorenzoni R., Dassi P., Mariani E., Morandi L., Mora M., Cornelio F. and Mantegazza R. (2003). Muscle inflammation and MHC class I up-regulation in muscular dystrophy with lack of dysferlin: an immunopathological study. *J. Neuroimmunol.* 142, 130-136.
- De Jonge M.J., Dumez H., Verweij J., Yarkoni S., Snyder D., Lacombe D., Marréaud S., Yamaguchi T., Punt C.J. and van Oosterom A.; EORTC New Drug Development Group (NDDG) (2006). Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone derivative in patients with advanced solid tumours. *Eur. J. Cancer* 42, 1768-1774.
- De Luna N., Gallardo E. and Illa I. (2004). In vivo and in vitro dysferlin expression in human muscle satellite cells. *J. Neuropathol. Exp. Neurol.* 63, 1104-1113.
- De Luna N., Gallardo E., Soriano M., Dominguez-Perles R., de la Torre C., Rojas-García R., García-Verdugo J.M. and Illa I. (2006). Absence of dysferlin alters myogenin expression and delays human muscle differentiation 'in vitro'. *J. Biol. Chem.* 281, 17092-17098.
- De Luna N., Gallardo E., Sonnet C., Chazaud B., Dominguez-Perles R., Suarez-Calvet X., Gherardi R.K. and Illa I. (2010). Role of thrombospondin 1 in macrophage inflammation in dysferlin myopathy. *J. Neuropathol. Exp. Neurol.* 69, 643-653.
- Demonbreun A.R., Fahrenbach J.P., Deveaux K., Earley J.U., Pytel P. and McNally E.M. (2011). Impaired muscle growth and response to insulin-like growth factor 1 in dysferlin-mediated muscular dystrophy. *Hum. Mol. Genet.* 20, 779-789.
- Desguerre I., Mayer M., Leturcq F., Barbet J.P., Gherardi R.K. and Christov C. (2009). Endomysial fibrosis in Duchenne muscular dystrophy: a marker of poor outcome associated with macrophage alternative activation. *J. Neuropathol. Exp. Neurol.* 68, 762-773.
- Flanders K.C. (2004). Smad3 as a mediator of the fibrotic response. *Int. J. Exp. Pathol.* 85, 47-64.
- Gallardo E., Rojas-García R., de Luna N., Pou A., Brown R.H. Jr. and Illa I. (2001). Inflammation in dysferlin myopathy: immunohistochemical characterization of 13 patients. *Neurology* 57, 2136-2138.
- Gavish Z., Pinthus J.H., Barak V., Ramon J., Nagler A., Eshhar Z. and Pines M. (2002). Growth inhibition of prostate cancer xenografts by halofuginone. *Prostate* 51, 73-83.
- Genin O., Rechavi G., Nagler A., Ben-Itzhak O., Nazemi K. and Pines M. (2008). Myofibroblasts in pulmonary and brain metastases of alveolar soft-part sarcoma: a novel target for treatment? *Neoplasia* 10, 940-948.

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- Guglieri M., Magri F. and Comi G.P. (2005). Molecular etiopathogenesis of limb girdle muscular and congenital muscular dystrophies: boundaries and contiguities. *Clin. Chim. Acta* 361, 54-79.
- Han R., Bansal D., Miyake K., Muniz V.P., Weiss R.M., McNeil P.L. and Campbell K.P. (2007). Dysferlin-mediated membrane repair protects the heart from stress-induced left ventricular injury. *J. Clin. Invest.* 117, 1805-1813.
- Han R., Frett E.M., Levy J.R., Rader E.P., Lueck J.D., Bansal D., Moore S.A., Ng R., Beltrán-Valero de Bernabé D., Faulkner J.A. and Campbell K.P. (2010). Genetic ablation of complement C3 attenuates muscle pathology in dysferlin-deficient mice. *J. Clin. Invest.* 120, 4366-4374.
- Hino M., Hamada N., Tajika Y., Funayama T., Morimura Y., Sakashita T., Yokota Y., Fukamoto K., Kobayashi Y. and Yorifuji H. (2009). Insufficient membrane fusion in dysferlin-deficient muscle fibers after heavy-ion irradiation. *Cell Struct. Funct.* 34, 11-15.
- Ho M., Post C.M., Donahue L.R., Lidov H.G., Bronson R.T., Goolsby H., Watkins S.C., Cox G.A. and Brown R.H. Jr. (2004). Disruption of muscle membrane and phenotype divergence in two novel mouse models of dysferlin deficiency. *Hum. Mol. Genet.* 13, 1999-2010.
- Horsley V., Friday B.B., Matesson S., Kegley M., Gephart J. and Pavlath G.K. (2001). Regulation of the growth of multinucleated muscle cells by an NFATC2-dependent pathway. *J. Cell Biol.* 153, 329-338.
- Huebner K.D., Jassal D.S., Halevy O., Pines M. and Anderson J.E. (2008). Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone. *Am. J. Physiol. Heart Circ. Physiol.* 294, H1550-1561.
- Ishitobi M., Haginoya K., Zhao Y., Ohnuma A., Minato J., Yanagisawa T., Tanabu M., Kikuchi M. and Iinuma K. (2000). Elevated plasma levels of transforming growth factor beta1 in patients with muscular dystrophy. *NeuroReport* 11, 4033-4035.
- Kawabe K., Goto K., Nishino I., Angelini C. and Hayashi Y.K. (2004). Dysferlin mutation analysis in a group of Italian patients with limb-girdle muscular dystrophy and *Miyoshi myopathy*. *Eur. J. Neurol.* 11, 657-661.
- Kim M.H., Kay D.I., Rudra R.T., Chen B.M., Hsu N., Izumiya Y., Martinez L., Spencer M.J., Walsh K., Grinnell A.D. and Crosbie R.H. (2011). Myogenic Akt signaling attenuates muscular degeneration, promotes myofiber regeneration and improves muscle function in dystrophin-deficient mdx mice. *Hum. Mol. Genet.* 20, 1324-1338.
- Klinge L., Aboumoussa A., Eagle M., Hudson J., Sarkozy A., Vita G., Charlton R., Roberts M., Straub V., Barresi R., Lochmüller H. and Bushby K. (2010). New aspects on patients affected by dysferlin deficient muscular dystrophy. *J. Neurol. Neurosurg. Psychiatry* 81, 946-953.
- Lennon N.J., Kho A., Bacskai B.J., Perlmutter S.L., Hyman B.T. and Brown R.H. Jr (2003). Dysferlin interacts with annexins A1 and A2 and mediates sarcolemmal wound-healing. *J. Biol. Chem.* 278, 50466-50473.
- Levi-Schaffer F., Nagler A., Slavin S., Knopov V. and Pines M. (1996). Inhibition of collagen synthesis and changes in skin morphology in murine graft-versus-host disease and tight skin mice: effect of halofuginone. *J. Invest. Dermatol.* 106, 4-8.
- Liu J., Aoki M., Illa I., Wu C., Fardeau M., Angelini C., Serrano C., Urtizbarea J.A., Hentati F., Hamida M.B., Bohlega S., Culper E.J., Amato A.A., Bossie K., Oeltjen J., Bejaoui K., McKenna-Yasek D., Hosler B.A., Schurr E., Arahata K., de Jong P.J. and Brown R.H. Jr (1998). Dysferlin, a novel skeletal muscle gene, is mutated in Miyoshi myopathy and limb girdle muscular dystrophy. *Nat. Genet.* 20, 31-36.
- McGaha T.L., Phelps R.G., Spiera H. and Bona C. (2002). Halofuginone, an inhibitor of type-I collagen synthesis and skin sclerosis, blocks transforming-growth-factor-beta-mediated Smad3 activation in fibroblasts. *J. Invest. Dermatol.* 118, 461-470.
- Melone M.A., Peluso G., Petillo O., Galderisi U. and Cotrufo R. (1999). Defective growth in vitro of Duchenne muscular dystrophy myoblasts: the molecular and biochemical basis. *J. Cell. Biochem.* 76, 118-132.
- Millay D.P., Maillat M., Roche J.A., Sargent M.A., McNally E.M., Bloch R.J. and Molkentin J.D. (2009). Genetic manipulation of dysferlin expression in skeletal muscle: novel insights into muscular dystrophy. *Am. J. Pathol.* 175, 1817-1823.
- Nagaraju K., Rawat R., Veszelszky E., Thapliyal R., Kesari A., Sparks S., Raben N., Plotz P. and Hoffman E.P. (2008). Dysferlin deficiency enhances monocyte phagocytosis: a model for the inflammatory onset of limb-girdle muscular dystrophy 2B. *Am. J. Pathol.* 172, 774-785.
- Nagler A. and Pines M. (1999). Topical treatment of cutaneous chronic graft versus host disease (cGvHD) with halofuginone: a novel inhibitor of collagen type I synthesis. *Transplantation* 68, 1806-1809.
- Nemoto H., Konno S., Nakazora H., Miura H. and Kurihara T. (2007). Histological and immunohistological changes of the skeletal muscles in older SJL/J mice. *Eur. Neurol.* 57, 19-25.
- Nevo Y., Halevy O., Genin O., Moshe I., Turgeman T., Harel M., Biton E., Reif S. and Pines M. (2010). Fibrosis inhibition and muscle histopathology improvement in Laminin-2-deficient mice. *Muscle Nerve* 42, 218-229.
- Pines M. (2008). Targeting TGFβ signaling to inhibit fibroblasts activation as a therapy for fibrosis and cancer. *Exp. Opin. Drug Dis.* 3, 11-20.
- Pines M., Vlodavsky I. and Nagler A. (2000). Halofuginone: from veterinary use to human therapy. *Drug Dev. Res.* 50, 371-378.
- Pines M., Domb A., Ohana M., Inbar J., Genina O., Alexiev R. and Nagler A. (2001). Reduction in dermal fibrosis in the tight-skin (Tsk) mouse after local application of halofuginone. *Biochem. Pharmacol.* 62, 1221-1227.
- Pinthus J.H., Sheffer Y., Nagler A., Fridman E., Mor Y., Genina O. and Pines M. (2005). Inhibition of Wilms tumor xenografts progression by halofuginone is accompanied by activation of WT-1 gene expression. *J. Urol.* 174, 1527-1531.
- Popov Y., Patsenker E., Bauer M., Niedobitek E., Schulze-Krebs A. and Schuppan D. (2006). Halofuginone induces matrix metalloproteinases in rat hepatic stellate cells via activation of p38 and NFκappaB. *J. Biol. Chem.* 281, 15090-15098.
- Prele A., Sciacco M., Tancredi L., Fagioli G., Comi G.P., Ciscato P., Serafini M., Fortunato F., Zecca C., Gallanti A., Chiveri L., Bresolin N., Scarlato G. and Moggio M. (2003). Clinical, morphological and immunological evaluation of six patients with dysferlin deficiency. *Acta Neuropathol. (Berl)* 105, 537-542.
- Rawat R., Cohen T.V., Ampong B., Francia D., Henriques-Pons A., Hoffman E.P. and Nagaraju K. (2010). Inflammasome up-regulation and activation in dysferlin-deficient skeletal muscle. *Am. J. Pathol.* 176, 2891-2900.
- Rayavarapu S., Van der Meulen J.H., Gordish-Dressman H., Hoffman E.P., Nagaraju K. and Knoblach S.M. (2010). Characterization of dysferlin deficient SJL/J mice to assess preclinical drug efficacy: fasudil exacerbates muscle disease phenotype. *PLoS One* 5, e12981.

- Roche J.A., Lovering R.M. and Bloch R.J. (2008). Impaired recovery of dysferlin-null skeletal muscle after contraction-induced injury in vivo. *Neuroreport* 19, 1579-1584.
- Roffe S., Hagai Y., Pines M. and Halevy O. (2010). Halofuginone inhibits Smad3 phosphorylation via the PI3K/Akt and MAPK/ERK pathways in muscle cells: effect on myotube fusion. *Exp. Cell Res.* 316, 1061-1069.
- Segawa M., Fukada S., Yamamoto Y., Yahagi H., Kanematsu M., Sato M., Ito T., Uezumi A., Hayashi S., Miyagoe-Suzuki Y., Takeda S., Tsujikawa K. and Yamamoto H. (2008). Suppression of macrophage functions impairs skeletal muscle regeneration with severe fibrosis. *Exp. Cell Res.* 314, 3232-3244.
- Selcen D., Stilling G. and Engel A.G. (2001). The earliest pathologic alterations in dysferlinopathy. *Neurology* 56, 1472-1481.
- Serrano A.L., Mann C.J., Vidal B., Ardite E., Perdiguer E. and Muñoz-Cánoves P. (2011). Cellular and molecular mechanisms regulating fibrosis in skeletal muscle repair and disease. *Curr. Top. Dev. Biol.* 96, 167-201.
- Shaw C.A., Larochelle N., Dudley R.W., Lochmuller H., Danialou G., Petrof B.J., Karpati G., Holland P.C. and Nalbantoglu J. (2006). Simultaneous dystrophin and dysferlin deficiencies associated with high-level expression of the coxsackie and adenovirus receptor in transgenic mice. *Am. J. Pathol.* 169, 2148-2160.
- Sheffer Y., Leon O., Pinthus J.H., Nagler A., Mor Y., Genin O., Iluz M., Kawada N., Yoshizato K. and Pines M. (2007). Inhibition of fibroblast to myofibroblast transition by halofuginone contribute to the chemotherapy-mediated anti-tumoral effect. *Mol. Cancer Ther.* 6, 570-577.
- Spector I., Honig H., Kawada N., Nagler A., Genin O. and Pines M. (2010). Inhibition of pancreatic stellate cell activation by halofuginone prevents pancreatic xenograft tumor development. *Pancreas* 39, 1008-1015.
- Turgeman T., Hagai Y., Huebner K., Jassal D.S., Anderson J.E., Genin O., Nagler A., Halevy O. and Pines M. (2008). Inhibition of Smad3 phosphorylation by halofuginone prevents muscle fibrosis and improves muscle performance in the mdx mouse model of Duchenne muscular dystrophy. *Neromuscular Disorders* 18, 857-868.
- Urtizbarea J.A., Bassez G., Leturcq F., Nguyen K., Krahn M. and Levy N. (2008). Dysferlinopathies. *Neurol. India* 56, 289-297.
- Vinit J., Samson M. Jr, Gaultier J.B., Laquerriere A., Ollagnon E., Petiot P., Marie I., Levesque H. and Rousset H. (2010). Dysferlin deficiency treated like refractory polymyositis. *Clin. Rheumatol.* 29, 103-106.
- Weller A.H., Magliato S.A., Bell K.P. and Rosenberg N.L. (1997). Spontaneous myopathy in the SJL/J mouse: pathology and strength loss. *Muscle Nerve* 20, 72-82.
- Wenzel K., Zabojszcza J., Carl M., Taubert S., Lass A., Harris C.L., Ho M., Schulz H., Hummel O., Hubner N., Osterziel K.J. and Spuler S. (2005). Increased susceptibility to complement attack due to down-regulation of decay-accelerating factor/CD55 in dysferlin-deficient muscular dystrophy. *J. Immunol.* 175, 6219-6225.
- Wenzel K., Geier C., Qadri F., Hubner N., Schulz H., Erdmann B., Gross V., Bauer D., Dechend R., Dietz R., Osterziel K.J., Spuler S. and Ozcelik C. (2007). Dysfunction of dysferlin-deficient hearts. *J. Mol. Med. (Berl.)* 85, 1203-1214.
- Yee K.O., Connolly C.M., Pines M. and Lawler J. (2006). Halofuginone inhibits tumor growth in the polyoma middle T antigen mouse via a thrombospondin-1 independent mechanism. *Cancer Biol. Ther.* 5, 218-224.
- Zanotti S., Gibertini S. and Mora M. (2010) Altered production of extracellular matrix components by muscle-derived Duchenne muscular dystrophy fibroblasts before and after TGF-beta1 treatment. *Cell Tissue Res.* 339, 397-410.
- Zion O., Genin O., Kawada N., Yoshizato K., Roffe S., Nagler A., Iovanna J.L., Halevy O. and Pines M. (2009). Inhibition of TGFβ signaling by halofuginone as a modality for pancreas fibrosis prevention. *Pancreas* 38, 427-435.

Accepted July 27, 2012