

Review

Halofuginone and muscular dystrophy

Mark Pines¹ and Orna Halevy²

¹Institute of Animal Sciences, Volcani Center, Bet Dagan and

²Department of Animal Sciences, the Hebrew University of Jerusalem, Rehovot, Israel

Summary. Muscular dystrophies (MDs) include different inherited diseases that all result in progressive muscle degeneration, impaired locomotion and often premature death. The major focus of MD research has been on alleviating the primary genetic deficit - using gene therapy and myoblast-transfer approaches to promote expression of the deficient or mutated genes in the muscle fibers. Although promising, these approaches have not yet entered into clinical practice and unfortunately for MD patients, there is currently no cure. Thus, the development of complementary and supportive therapies that slow disease progression and improve patients' quality of life is critically important. The main features of MDs are sarcolemmal instability and increased myofiber vulnerability to mechanical stress, resulting in myofiber degeneration. Fibrosis, with progressive replacement of muscle tissue, is a prominent feature in some MDs, preventing complete regeneration and hampering muscle functions. TGF β is the leading candidate for activating fibroblasts and eliciting overproduction of extracellular matrix (ECM) proteins. Halofuginone, an inhibitor of Smad3 phosphorylation downstream of TGF β signaling, inhibits the activation of fibroblasts and their ability to synthesize ECM, regardless of their origin or location. In animal models of MDs with prominent muscle fibrosis, halofuginone treatment has resulted in both prevention of collagen production in young animals and resolution of established fibrosis in older ones: the reduction in muscle collagen content was associated with improved muscle histopathology and major improvements in muscle function. Recently, these halofuginone-dependent improvements were also observed in MD with minor fibrosis involvement, probably due to a direct effect of halofuginone on muscle cells, resulting in myotube fusion that is dependent on Akt and MAPK

pathway activation. In summary, halofuginone improves muscle histopathology and muscle functions in various MDs, via inhibition of muscle fibrosis on the one hand, and increased myotube fusion on the other.

Key words: Collagen, Fibrosis, TGF β , Smad3

Introduction

Muscular dystrophies (MDs) include more than 30 different inherited diseases caused by mutations that affect distinct genes, which all result in progressive muscle degeneration, impaired locomotion and, in many cases, premature death (Dalkilic and Kunkel, 2003; Davies and Nowak, 2006). The mutations in MDs involve loss of structural proteins, defective enzymes, disruption of sarcolemma-repair mechanisms and loss of signaling molecules. The membranes of the fibers are fragile and suffer extensive damage that leads to massive infiltration of immune cells, chronic inflammation, necrosis, and severe muscle degeneration. The microenvironment of dystrophic muscles consists of an elevated number of inflammatory cells that act as a complex interface for cytokine signaling (Gorospe et al., 1994). Key features of dystrophic muscle include central nuclei, variation in muscle fiber size with small regenerating fibers, and accumulation of connective and fatty tissues (McNally and Pytel, 2007). Although spectacular progress has been made in understanding the molecular and genetic bases of MDs, cell-transplantation, gene-replacement, and gene-repair approaches have met with great difficulties and are not yet available in the clinic. Thus, a more realistic goal is to treat secondary defects in MDs, such as the muscle fibrosis which is common to various forms of MDs and a major cause of morbidity and mortality, and for which there is a huge unmet therapeutic need.

In this review, we focus on halofuginone, a novel therapy that inhibits fibroblast activation, resulting in

prevention of tissue fibrosis and resolution of pre-existing fibrosis in pre-clinical models and in humans. Recently, additional non-fibrosis-related effects of halofuginone have been discovered. We describe the effects of halofuginone in animal models of MDs: Duchenne (DMD) and congenital (CMD) muscular dystrophies which lack dystrophin and laminin $\alpha 2$ chain, respectively, and in which fibrosis is the hallmark of the disease, and in a model of dysferlinopathy with a loss-of-function mutation in the dysferlin gene and no apparent involvement of fibrosis. In addition, the direct effects of halofuginone on a myogenic cell line and primary dystrophic mouse myoblasts are described.

MDs and fibrosis

In MDs such as DMD and CMD, a progressive loss of muscle and its ability to function are associated with significant fibrosis that correlates with poor motor outcome (Desguerre et al., 2009). In DMD, the leading causes of death—respiratory and heart failure—result from weakness in the diaphragm and myocardium, which are the tissues most affected by fibrosis (Finsterer and Stollberger, 2003). Cardiac involvement in DMD is characterized by degeneration and fibrosis of the myocardium, probably because of myofibroblast activity, centering around the posterolateral wall of the left ventricle. Functionally, abnormalities in electrocardiograms, valve motion, wall thickness and wall motion are observed in DMD patients with cardiomyopathy (Finsterer and Stollberger, 2003). The significance of fibrosis in cardiac muscle function was demonstrated by enhanced cardiomyocyte sarcolemmal and treadmill performance after prevention of cardiac fibrosis by a dystrophin minigene (Bostick et al., 2009). The levels of TGF β were higher in the muscle (Murakami et al., 1999) and plasma of CMD and DMD patients (Bernasconi et al., 1999; Ishitobi et al., 2000), and in muscle biopsies of DMD and Becker MD (Bernasconi et al., 1995). DMD fibroblasts had a profibrotic phenotype which was enhanced by TGF β treatment, and in culture they secreted higher levels of collagen than normal muscle fibroblasts (Ionasescu et al., 1977; Zanotti et al., 2010). On the other hand, reduced TGF β signaling resulted in decreased fibrosis and improved muscle pathology (Heydemann et al., 2009). In the X-linked golden retriever (Passerini et al., 2002) and the *mdx* mouse (Quinlan et al., 2004), both models of DMD, myocardium and diaphragm are the tissues most affected by fibrosis, similar to human patients, and their failure makes a major contribution to morbidity and mortality. The *mdx* diaphragm exhibits a pattern of degeneration, fibrosis and severe functional deficit and although diaphragm dysfunction precedes gross collagen deposition, prevention of fibrosis enhances muscle regeneration and facilitates more efficient muscle repair (Marshall et al., 1989; Stedman et al., 1991; Goldspink et al., 1994; Chan et al., 2003; Coirault et al., 2003; Krupnick et al., 2003). A

comparison between molecular signatures of affected and spared muscles indicated that the absence of dystrophin is necessary, but not sufficient, to cause patterned fibrosis, inflammation and failed muscle regeneration (Porter et al., 2003).

Muscle fibrosis also occurs in other MDs such as CMD but unlike in DMD, increased connective tissue is the hallmark of CMD muscle histopathology from birth. In both DMD and CMD, increases in type I and III collagens were observed in the skeletal muscle (Dunace et al., 1980; Hantai et al., 1985) leading to fibrosis, which correlated with muscle destruction (Zhao et al., 2003). The progression of muscular weakness in CMD patients is directly correlated with the progressive loss of myofibers, which is accompanied by replacement of the connective and adipose tissue. This results in a thick collagen layer around the muscle fibers, as well as the capillary walls, reducing the blood supply to individual muscle fibers and delaying muscle-fiber regeneration following necrosis (Ishitobi et al., 2000). This association between the structural integrity of myofibril basal lamina and fibrosis was further demonstrated in the CMD mouse models after somatic gene delivery (Qiao et al., 2005). In the *dy^{2J}/dy^{2J}* mouse model of CMD with laminin $\alpha 2$ -chain deficiency, the fibrosis process is first segmental but becomes generalized by the age of 3 months (MacPike and Meier, 1976; Meier and MacPike, 1977). Scattered necrotic fibers were noted at 10 days of age followed by poorly compensated regeneration, leading to muscle-fiber loss, marked variation in fiber size, and proliferation of fibrous and adipose tissue (Kuang et al., 1998; Besse et al., 2003; Guo et al., 2003). Gene-expression profiling of muscle in Fukuyama-type MD (FCMD) and CMD type 1A (MDC1A) patients (Taniguchi et al., 2006), and of *dy/dy* mouse muscle (van Lunteren et al., 2006) revealed a number of genes related to the increases in connective tissue, fibrosis and collagen content. The myogenic regulatory factors MyoD and myogenin are expressed at reasonably high levels in parallel with the active regenerating process in *mdx* mice, whereas in *dy/dy* mice, MyoD and myogenin levels decrease as fibrosis progresses (Jin et al., 2000).

Various attempts to inhibit muscle fibrosis have achieved modest degrees of success, accompanied by various adverse effects. For example, reduction in TGF β levels by neutralizing antibodies resulted in increased inflammation, probably due to its pleiotropic role in various processes (Andreetta et al., 2006). The use of corticosteroids such as prednisone, although causing a reduction in TGF β levels in the *mdx* mouse, also caused an increase in collagen cross-linking (Hartel et al., 2001) and subsequent collagen stiffness, which may have a profound impact on chest wall compliance and subsequently on breathing. TNF α blockage reduced levels of TGF β and collagen (Gosselin and Martinez, 2004) but long-term treatment, especially in pediatric patients, is questionable. Pentoxifylline, which inhibits collagen synthesis in fibroblasts *in vitro*, failed to reduce collagen synthesis in *mdx* mice (Gosselin and Williams,

2006). Pirfenidone, which inhibits lung fibrosis, had a very modest effect in *mdx* mice (Gosselin et al., 2007). Thus, a specific inhibitor of fibrosis with low side effects that can improve quality of life is greatly needed for MD patients.

Halofuginone and fibrosis

The roots and leaves of *Dichroa febrifuga* have been used in China for centuries against malarial fever. An active alkaloid, febrifugine ($C_{16}H_{21}O_3N_3$), was isolated but the high anti-malarial activity was accompanied by gastrointestinal toxicity. Thus, febrifugine was used as a lead compound in the synthesis of active molecules with lower toxicity (Takaya et al., 1999). Halofuginone hydrobromide, one of these febrifugine analogues, is a FDA-approved feed additive used for the prevention of coccidiosis in poultry (for reviews see Pines et al., 2000; Pines, 2008).

Serendipitously, halofuginone was also found to inhibit fibroblast activation, resulting in inhibition of ECM synthesis (Pines and Nagler, 1996, 1998; Pines et al., 2000). At first, halofuginone was identified as an inhibitor of collagen synthesis. The observation that halofuginone inhibits collagen $\alpha 1(I)$ gene expression in various cells, including fibroblasts derived from scleroderma and chronic graft-versus-host disease (cGvHD) patients (Halevy et al., 1996; Pines et al., 1997; Nagler et al., 1998), paved the way for the use of halofuginone as an anti-fibrotic drug (Pines et al., 2003). In animal models of fibrosis for which excess collagen is the hallmark of the disease, administration of halofuginone—regardless of the route of administration—prevented the increase in the number of activated fibroblasts expressing the collagen $\alpha 1(I)$ gene and synthesizing large amounts of collagen. These models included post-operational adhesions (Nagler et al., 1998, 2000; Nagler and Pines, 1999), radiation-induced fibrosis (Xavier et al., 2004; Ishii et al., 2009) and chemically induced liver, pancreas and pulmonary fibrosis (Nyska et al., 1996; Pines et al., 1997; Bruck et al., 2001; Zion et al., 2009). In cGvHD-afflicted mice, halofuginone inhibited collagen synthesis and the proliferation of dermis fibroblasts without affecting other resident cell populations (Levi-Schaffer et al., 1996; Pines et al., 2001, 2003). Halofuginone-dependent inhibition of fibroblast activation and fibrosis affects other physiological systems as well: for example, it improves liver regeneration in cirrhotic rats (Spira et al., 2002), reduces the levels of autoantibodies specific for human target antigens (anti-topoisomerase I and antifibrillin antibodies) in the tight skin (Tsk+) mouse (McGaha et al., 2002a), and reduces tumor development (Yee et al., 2006; Sheffer et al., 2007; Genin et al., 2008). In addition to preventing fibrosis, halofuginone elicits the resolution of established fibrosis, setting it well apart from all other anti-fibrotic agents. In the Tsk+ mouse model of scleroderma with skin fibrosis and in rats with established liver fibrosis, halofuginone

treatment caused a decrease in the pre-existing fibrotic condition by inhibiting the fibroblast-to-myofibroblast transition (Bruck et al., 2001; Pines et al., 2001). Moreover, halofuginone successfully decreased fibrosis in a patient who had developed severe cGvHD after a successful bone marrow transplant, demonstrating human clinical efficacy (Nagler and Pines, 1999).

In most animal models of fibrosis, regardless of the tissue, halofuginone has a minimal effect on ECM content in the control, non-fibrotic animals, whereas it has a profound inhibitory effect in the fibrotic organs. These results suggest different regulation of the normally low housekeeping expression levels of ECM genes on the one hand, and their TGF β -driven over-expressed levels induced by the fibrogenic stimulus, which is usually an aggressive and rapid process, on the other. Halofuginone was found to overcome the collagen synthesis by human skin fibroblasts activated by TGF β (Halevy et al., 1996) and to inhibit TGF β -dependent phosphorylation of Smad3 and collagen synthesis by Tsk+ mouse fibroblasts (McGaha et al., 2002b). In chemically induced liver fibrosis, halofuginone affected TGF β -regulated genes as a result of inhibition of Smad3 phosphorylation of activated hepatic stellate cells (Gnainsky et al., 2007). No effect of halofuginone was observed on the gene expression of the TGF β receptors, supporting the hypothesis that the halofuginone target(s) is downstream in the TGF β pathway.

Halofuginone reduces fibrosis and improves muscle histopathology

Recent studies in *mdx* and *dy^{2J}/dy^{2J}* mice with massive fibrosis involvement representing DMD and CMD, respectively, have demonstrated the inhibitory effect of halofuginone on muscle fibrosis. Halofuginone treatment resulted in timely and dose-dependent inhibition of collagen $\alpha 1(I)$ gene synthesis, as shown by *in-situ* hybridization in the *mdx* mouse diaphragm (Turgeman et al., 2008) and *dy^{2J}/dy^{2J}* hind limbs (Nevo et al., 2010)—the most affected muscles in these mice. These findings are supported in Fig. 1, which uses immunofluorescence staining with a specific antibody to show protein inhibition of collagen type I in halofuginone-treated *mdx* mice, and in Fig. 2, which shows serial z-stack images of Sirius Red-stained gastrocnemius from *dy^{2J}/dy^{2J}* mice. Halofuginone had no effect on diaphragm or hind limb collagen levels or on any other histological properties when administered to wild-type mice, implying different regulation of collagen synthesis in normal tissues versus those with chronic trauma. In agreement with other studies, fibrosis in the *mdx* mice was also evident in muscles other than the diaphragm, albeit to a lesser extent (Gosselin et al., 2007). In muscles with lower collagen content, such as the gastrocnemius and tibialis cranialis, higher doses of halofuginone were needed to affect collagen synthesis. Cardiac involvement due to progressive accumulation of myocardial fibrosis has become increasingly important

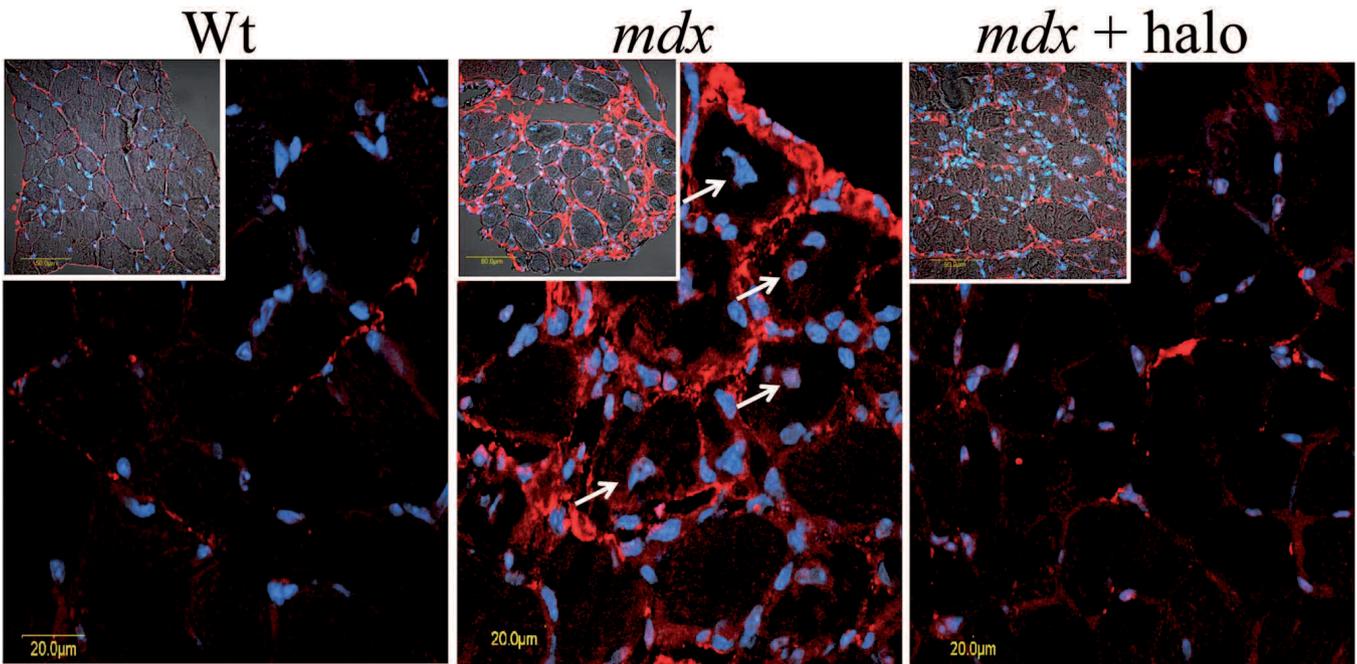


Fig. 1. Halofuginone reduces collagen type I levels in *mdx* diaphragm. Confocal microscopy of immunofluorescence staining with collagen type I antibodies (collagen stains red) in diaphragm of C57/BL (Wt), *mdx* and *mdx* mice treated with halofuginone. Halofuginone was administered IP at 7.5 $\mu\text{g}/\text{ml}$, three times a week for 4 weeks starting at 4 weeks of age. Cell nuclei were stained with DAPI. Fibers with central nuclei are indicated by arrows. Note the low levels of collagen and absence of fibers with central nuclei in the Wt mice and in the *mdx* mice after halofuginone treatment.

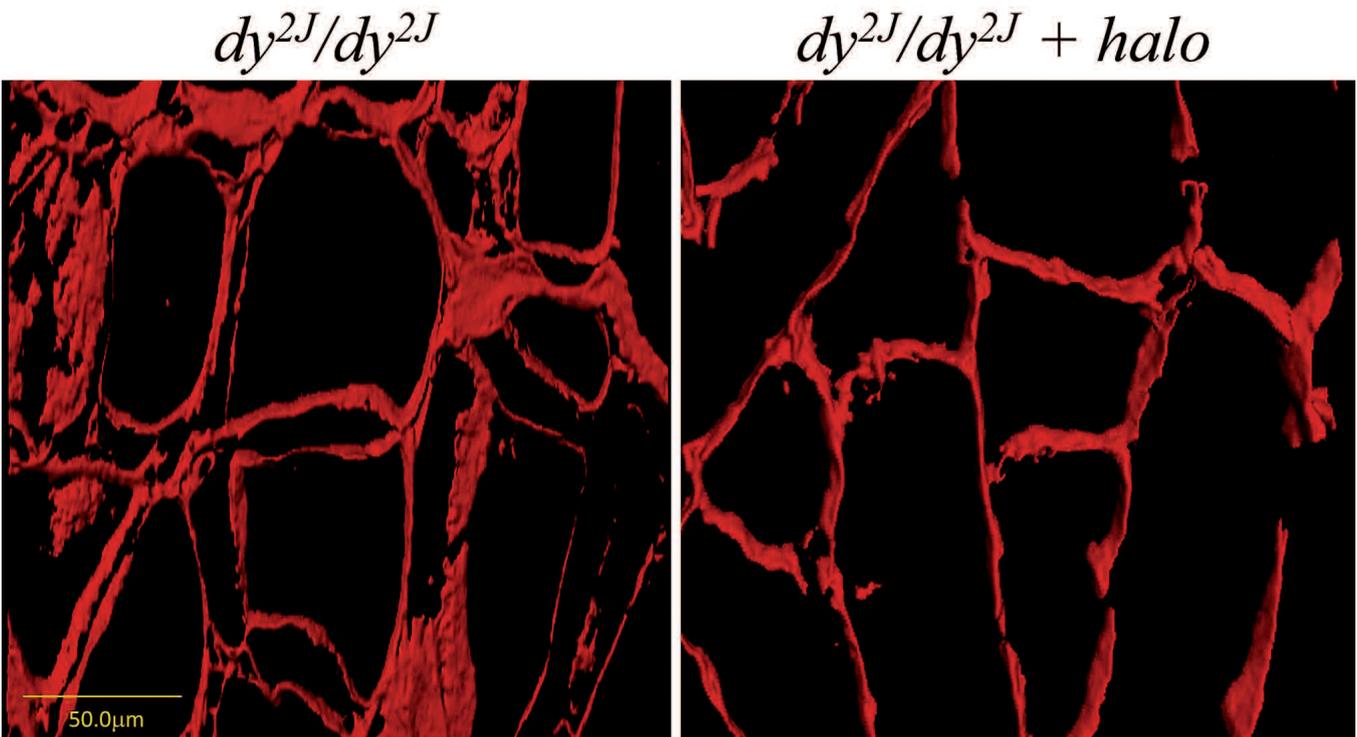


Fig. 2. Halofuginone inhibits collagen synthesis in *dy^{2J}/dy^{2J}* mice. Gastrocnemius from *dy^{2J}/dy^{2J}* mice and *dy^{2J}/dy^{2J}* mice treated with halofuginone (5 $\mu\text{g}/\text{mouse}$, IP, three times a week for 15 weeks, starting at 3 weeks of age) were stained with Sirius red. Serial z-stack images were taken by confocal microscopy (excitation 543 nm, emission 560 nm). Sections are 1.2- μm each and extend 7 μm into the surface of the tissue. The images with the highest collagen staining are presented. Note the reduction in collagen levels after halofuginone treatment.

Halofuginone and MD

in DMD (Marques et al., 2009). The cardiac tissue of *mdx* mice exhibited high expression of the collagen $\alpha 1(I)$ gene and high collagen levels compared to the wild type, which were abrogated by halofuginone treatment (Huebner et al., 2008; Turgeman et al., 2008). Halofuginone caused a reduction in infiltrating fibroblasts that were located close to centrally nucleated myofibers, suggesting cross-talk between the two cell types (Nevo et al., 2010). These cells, which are distinct from the satellite cells, express, in addition to collagen type I, prolyl 4 hydroxylase β (P_4HB), a major collagen cross-linking enzyme, pSmad3 and MyoD. The origin of these cells is unknown; they could be resident fibroblasts that have undergone differentiation or they could be myofibroblasts recruited by factors secreted by the regenerated areas or central nucleated myofibers. TGF β may be one such candidate, since it promotes the fibroblast-to-myofibroblast transition and is released locally from necrotic myofibers into the ECM and thus promotes progressive impairment of muscle regeneration (Melone et al., 1999). Inhibition of TGF β signaling ameliorated the pathological phenotype in dystrophic mice, a finding consistent with the importance of TGF β signaling in the regulation of both regeneration and fibrosis (Cohn et al., 2007). It has been hypothesized that autocrine release of TGF β from necrotic myofibers contributes to the increasing loss of muscle regeneration and the increase in fibrosis. In both *mdx* and *dy^{2J}/dy^{2J}* mice, halofuginone inhibited Smad3 phosphorylation downstream of TGF β in cardiac and skeletal muscles (Turgeman et al., 2008; Nevo et al., 2010; Roffe et al., 2010).

Areas of necrosis, small myofibers and the existence of myofibers with central nuclei are the main characteristics of MDs; in mice they appear in various muscle tissues as markers of previously damaged muscle and regeneration (Coulton et al., 1988). Accumulation of connective tissue has been suggested to be a major factor in the progressive failure of muscle regeneration and, ultimately, of muscle dysfunction; i.e., the fibrotic process in MD may involve positive feedback whereby collagen type I directly inhibits muscle regeneration (Alexakis et al., 2007). In both *mdx* and *dy^{2J}/dy^{2J}* mice, halofuginone treatment resulted in reductions in degenerated area, cell proliferation and number of myofibers with central nuclei, and in an increase in myofiber diameter (Turgeman et al., 2008; Nevo et al., 2010). These results suggest that halofuginone, by inhibiting fibrosis, prevents its deleterious effect and provides some protection against muscle damage, and therefore lessens the need for excessive muscle regeneration and exhaustion of the satellite cell pool.

Whether halofuginone should be given continuously or can be discontinued after fibrosis inhibition is a question of major clinical relevance. Previous reports have demonstrated a transient effect of halofuginone on collagen synthesis in human skin fibroblasts in culture and in a cGvHD patient (Halevy et al., 1996; Nagler and

Pines, 1999). The collagen content of the *mdx* diaphragm following discontinuation of halofuginone treatment was significantly higher than in the halofuginone-treated *mdx* mice (Turgeman et al., 2008). This was manifested in the number of myofibers with central nuclei in the *mdx* diaphragm that were restored after halofuginone withdrawal, reaching the same level as that in the untreated *mdx* mice. These transient effects are due to the chronic pathological process that presents a continuous stimulus for fibrogenesis. Thus, in order to sustain low levels of muscle fibrosis, halofuginone needs to be given continuously.

Halofuginone improves muscle function

Cardiac abnormalities cause early morbidity and mortality in DMD patients (McNally, 2007; McNally and Pytel, 2007). Halofuginone has been shown to prevent posterior cardiac wall thickness and wall motion abnormalities, as well as the deterioration in motor coordination and balance observed in young *mdx* mice, as evidenced by echocardiography and Rota-Rod, respectively (Turgeman et al., 2008). In *dy^{2J}/dy^{2J}* mice, halofuginone improved motor function and balance without affecting muscle strength or muscle force (Nevo et al., 2010). The disparity between the effect on grip strength and grasping reflex on the one hand, and motor function and balance on the other, implies the existence of additional, as yet unknown effects of halofuginone on peripheral-nerve abnormalities in these mice.

In older *mdx* mice with established fibrosis, halofuginone decreased collagen content in the diaphragm and quadriceps (Huebner et al., 2008). This was correlated with reduced muscle fiber damage and increased voluntary run distances, suggesting a reduction in exercise-induced damage. The functional improvement in exercise endurance in *mdx* mice after halofuginone treatment is of great importance, since exercise often exacerbates the progression of symptoms (DeLuca et al., 2003). There was significant improvement in *mdx* cardiac and respiratory functions following halofuginone treatment, and the ventricular contraction was synchronous in the *mdx*-treated mice and not dyskinetic as in the untreated mice. The treated mice displayed a lower response to methacholine challenge, as demonstrated by barometric plethysmography. These results were in agreement with other studies demonstrating resolution of established fibrosis of the liver and skin in animal models and in a cGvHD patient (Nagler and Pines, 1999; Bruck et al., 2001; Pines et al., 2001). The ability to elicit resolution of pre-existing fibrosis is probably due to halofuginone-mediated regulation of matrix metalloproteinase (MMP) activity (Popov et al., 2006) and effects on the synthesis of tissue inhibitors of metalloproteinases (TIMPs) (Bruck et al., 2001; Zion et al., 2009). The MMPs and their inhibitors play a crucial role in the fine regulation of ECM turnover, which is altered in most pathological

states associated with fibrosis, wound healing and cancer.

Halofuginone and gene profiling in the *mdx* mouse

Proteomic (Lewis et al., 2009) and global gene-expression profiling of dystrophic muscle has been previously studied in both patients with DMD (Bakay et al., 2002; Haslett et al., 2002, 2003) and *mdx* mice (Porter et al., 2001; Turk et al., 2005; Marotta et al., 2009). The impact of halofuginone treatment on the transcriptome of the diaphragm of *mdx* mice was determined using Affymetrix GeneChip oligonucleotide microarray-based transcriptional profiling. Genes that were differentially expressed during disease progression

and which affected by halofuginone were described (Fig. 3). Preliminary analysis suggested that halofuginone affects different sets of genes (Fig. 3A) involved in different biological processes (Fig. 3B) during disease progression. For example, at an early phase of the disease, halofuginone affects genes involved in chemotaxis, cell immunity and collagen biosynthesis, while at a later stage it affects genes involved in cell differentiation and apoptosis, and at the final stages it affects genes involved in wound healing, lipid and carbohydrate metabolism. Genes involved in inflammation are affected during the entire period. There is a close relationship between the regeneration-associated processes of inflammation and fibrosis. Ultimately, the most desirable strategy is to enhance the

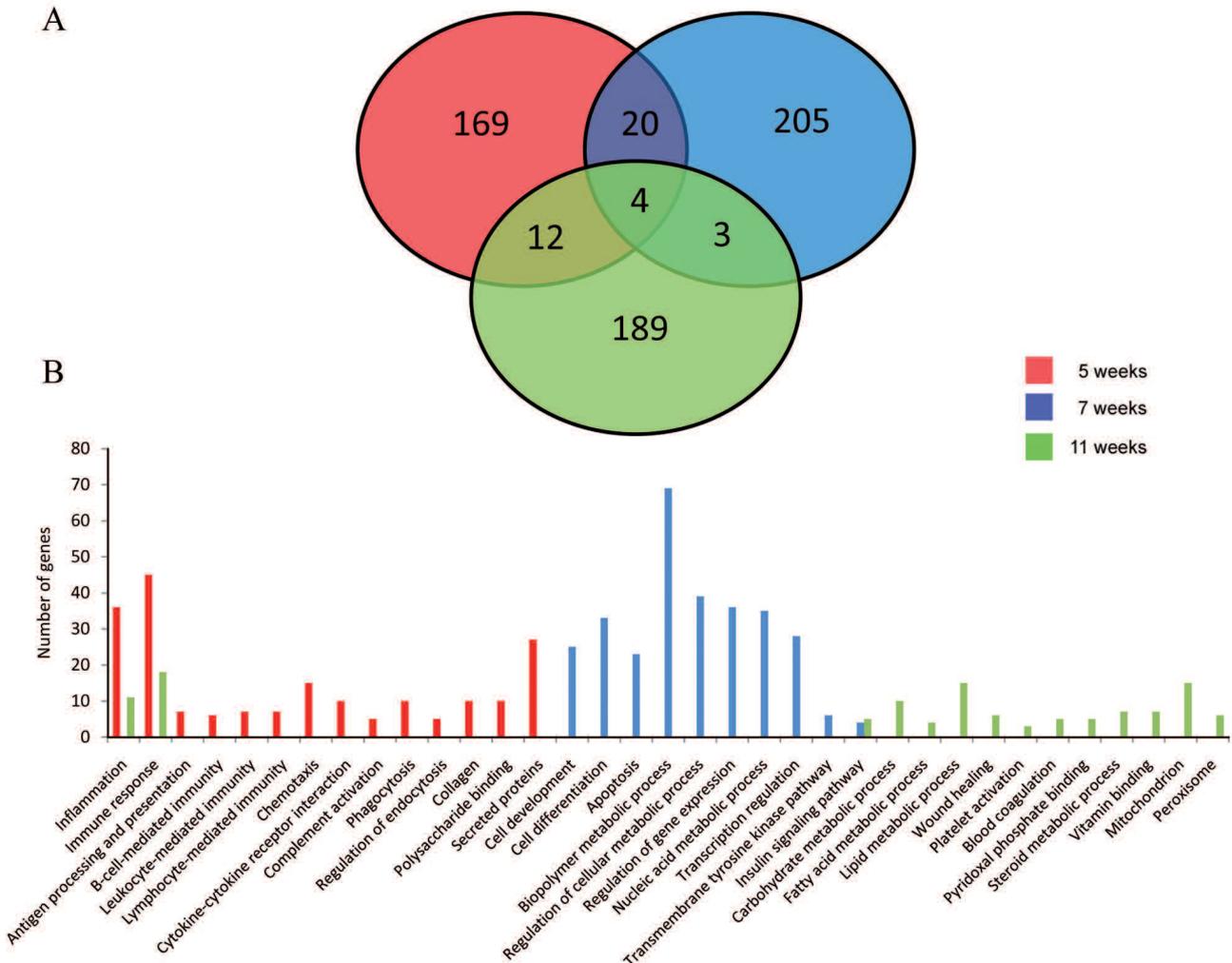


Fig. 3. Effect of halofuginone on gene profiling in the *mdx* mouse. Halofuginone (7.5 µg/mouse, three times a week, from 4 weeks of age) was injected IP to *mdx* mice for various intervals. Diaphragms were then collected and subjected to Affymetrix GeneChip analysis. **A.** Number of genes at each time period that were differentially expressed in the *mdx* mice compared to the wild type and were affected by halofuginone. **B.** Gene ontology of the halofuginone-affected genes. The DAVID (Database for Annotation, Visualization and Integrated Discovery) 2.0 software tool was used to produce gene ontology classification (at least two genes per category).

Halofuginone and MD

inflammatory signals that promote regeneration, while inhibiting the inflammation-derived pathways that cause muscle necrosis/fibrosis—the most deleterious outcome of MDs. The link between tissue inflammation and fibrosis is supported by observations that fibrinogen-Mac-1 receptor binding promotes the synthesis of TGF β in *mdx* macrophages, which in turn inducing fibrosis in *mdx* fibroblasts (Vidal et al., 2008). Comparing these results to those observed during the progression of

chemically induced liver fibrosis (Gnainsky et al., 2007) will enable a determination of general and tissue-specific genes and pathways affected by halofuginone.

Halofuginone and late onset of MDs with minor fibrosis involvement

Many of the genes affected by halofuginone in the *mdx* mouse were not related to the TGF β -ECM pathway,

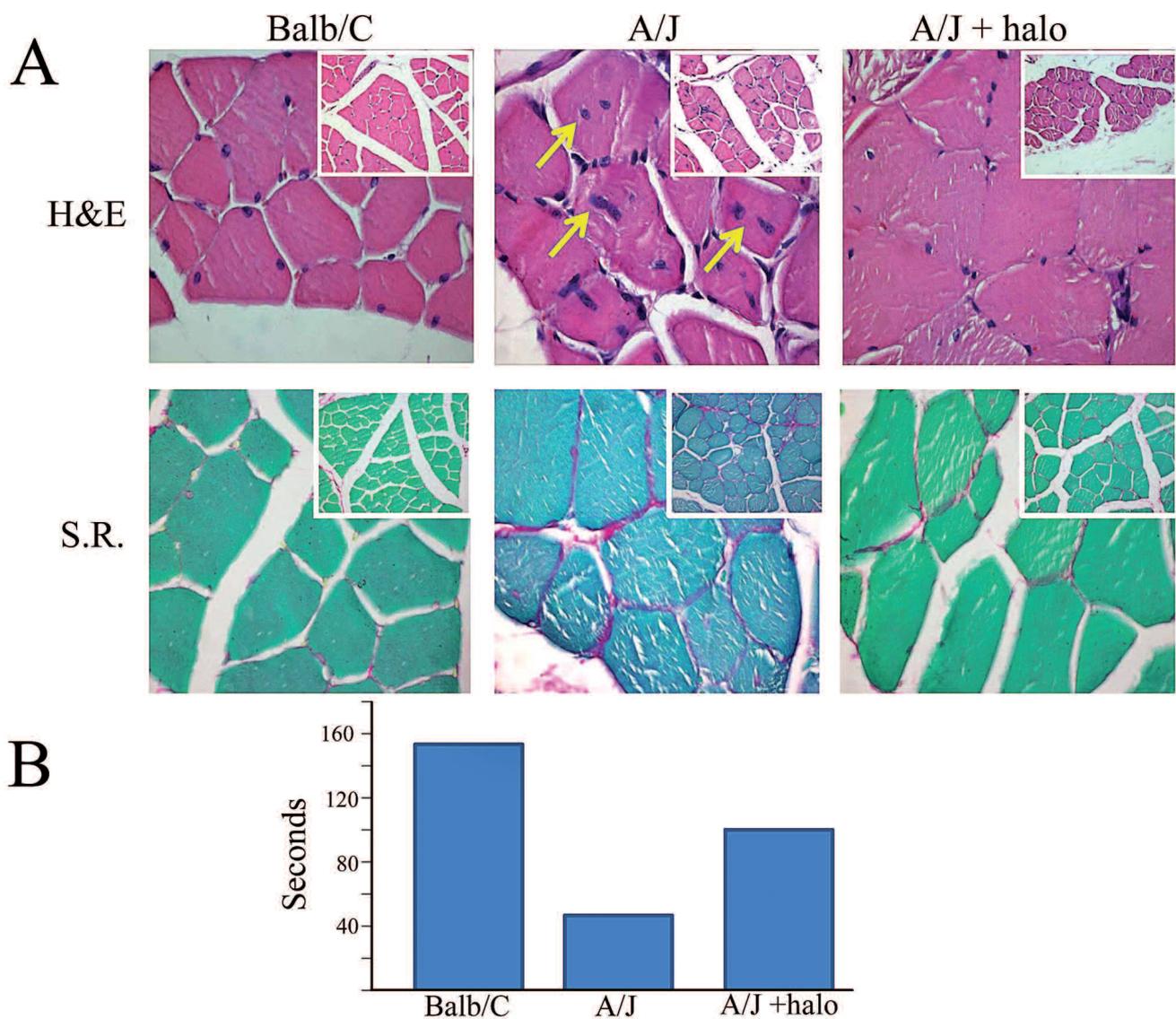


Fig. 4. Histopathology of A/J muscle. A/J mice at 9 months of age were injected with 10 μ g/mouse halofuginone (IP) three times a week, for 3 months. **A.** Longissimus muscles were then collected and fixed, and sections were stained with H&E or Sirius red (S.R.). Yellow arrows point to myofibers with central nuclei. Note that the A/J mouse muscle exhibits a large proportion of myofibers with central nuclei relative to the controls (Balb/C), which disappeared after halofuginone treatment. x 400; insets, x 100. **B.** Performance on the Rota-Rod was assessed in terms of time spent on the rod as a function of drum speed. The Wt mice performed much better on the Rota-Rod than the A/J mice. After halofuginone treatment, there was significant improvement in the latter's performance.

suggesting that apart from fibrosis, halofuginone affects other physiological and metabolic processes. Thus, the efficacy of halofuginone was evaluated in dysferlinopathy, a MD with late onset and only minor fibrosis involvement. Dysferlinopathies encompass a wide variety of MDs, characterized by the absence of, or loss-of-function mutations in the dysferlin (*dysf*) gene in skeletal muscle (Bushby, 2000, 2009; Klinge et al., 2010). In contrast to DMD and CMD, dysferlinopathies begin in the second or third decade, with previously normal musculature. Centrally nucleated myofibers, variations in muscle diameter, and inflammatory infiltrates near the necrotic fibers and perivascular regions, probably directed against degenerating myofibers, are usually observed (Prelle et al., 2003). As a model we used A/J mice with an ETn retrotransposon insertion in *dysf* intron 4 (Kobayashi et al., 2010). Many of the muscle fibers of the A/J muscle which contained central nuclei disappeared after halofuginone treatment. This occurred despite the relative absence of fibrosis involvement (Fig. 4A). The halofuginone-dependent improvement in muscle histopathology was accompanied by better performance on a Rota-Rod (Fig. 4B). These results suggest that at least some of halofuginone's effects are not mediated by fibrosis inhibition but rather by a direct effect on muscle.

Direct effects of halofuginone on muscle

Halofuginone's actions could potentially be targeted to muscle cells, myofibers or satellite cells, the source for progenitors during muscle regeneration. Preliminary studies conducted in our laboratory in which halofuginone presence was monitored by reverse-phase chromatography followed by its detection on quad mass

selective detector (LC/MS/MS analysis), revealed its ability to penetrate in a matter of minutes the muscle-cell membrane. In addition, halofuginone affected the kinetics of biotin-labeled membrane-cell-surface protein expression. (S. Roffe, M. Pines and O. Halevy, unpublished data). In proliferating myoblasts derived from C57/BL (wild-type) and *mdx* mouse diaphragms, halofuginone promoted the phosphorylation of Akt and mitogen-activated protein kinase (MAPK) family members and enhanced the association of Akt and MAPK/extracellular signal-regulated protein kinase (MAPK/ERK) with the non-phosphorylated form of Smad3, resulting in decreased Smad3 phosphorylation (Roffe et al., 2010). These results suggest that at least part of the halofuginone-dependent inhibition of Smad3 phosphorylation is due to activation of Akt and ERK and demonstrate cross-talk between TGF β and the Akt and MAPK/ERK pathways in muscle cells. The Akt and Smad3-mediated effects of halofuginone were also observed in myotubes, resulting in their increased fusion. This finding correlates with the increase in diameter of centrally nucleated myofibers observed in halofuginone-treated *mdx* and *dy^{2J/dy^{2J}}* mice (Turgeman et al., 2008; Nevo et al., 2010), and is consistent with the observation that induction of the Smad3 pathway inhibits myotube fusion and repair of old muscles (Zhu et al., 2004; Carlson et al., 2008). Increased Akt activity has been reported to prevent the excessive reduction in maximal tetanic force that follows eccentric contractions observed in the dystrophic muscle, and to increase skeletal muscle mass (Blaauw et al., 2008, 2009). 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 dysferlin will cause insufficiency in membrane fusion (Hino et al.,

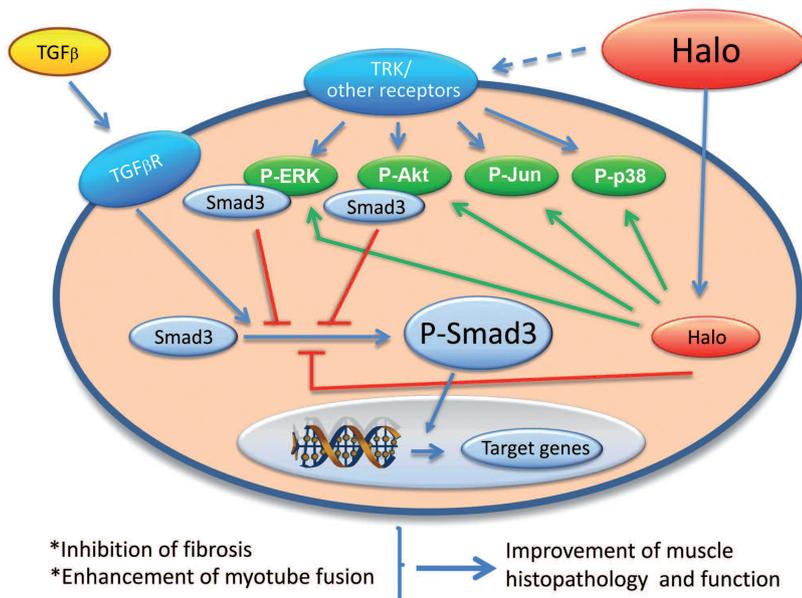


Fig. 5. Inhibition of Smad3 pathway by halofuginone in muscle cells. TGF β is the major activator of the Smad3 pathway. The phosphorylated Smad3 translocates to the nucleus and regulates target gene transcription. Halo that binds to tyrosine kinase receptors (TRK) or other cell membrane receptors and/or directly penetrates the cell activates the PI3K and MAPK family signaling pathways. The phosphorylated Akt and ERK, but not the phosphorylated JUN and p38 associate with the unphosphorylated form of Smad3, thereby inhibiting its phosphorylation. Halo may also inhibit Smad3 phosphorylation either directly or via other molecules (e.g., Smad7). By activating the PI3K and MAPK family pathways and by inhibiting the Smad3 pathway, halo inhibits fibrosis and promotes myotube fusion, thereby improving the histopathology and function of the dystrophic muscle.

2009) and hamper muscle regeneration. The fact that there were hardly any centrally nucleated myofibers in the halofuginone-treated A/J mice (Fig. 4A) supports the notion that halofuginone plays a direct role in controlling early stages of muscle regeneration, and perhaps also during myofiber maturation, thereby improving muscle repair. This is of importance because in MDs small immature centrally nucleated fibers are observed that reflect muscle regeneration from myoblasts in the early phase of the disease resulting in reduced muscle strength (Deconinck and Dan, 2007).

Concluding remarks

Research into therapeutic approaches for MD has progressed rapidly over the past decade and holds great promise for the future. The development of practical gene- and cell-based therapies is still in its early stages, but at present the cornerstones of current treatment are symptomatic and supportive healthcare. Interventions that implement muscle regeneration appear particularly appropriate to patients in early stages of MDs, when muscles are in the regenerative stage. In patients at later stages of disease progression, however, prevention of secondary events, such as collagen deposition and fibrosis, which preclude efficient regeneration and limit the success of gene transfer and cell transplantation, is required. The observation that non-muscle tissues in MDs exhibit increased levels of collagen suggests a systemic stimulus for fibrosis which may be triggered in the muscle but spreads to other tissues as well. One explanation lies in the involvement of the T-cell “repertoire”, since collagen deposition in the diaphragm and heart of the immunosuppressed *mdx*-nu/nu mouse was delayed relative to its deposition in the *mdx* mouse (Morrison et al., 2000). Halofuginone restores the muscle's regeneration ability on the one hand and inhibits muscle fibrosis on the other. Halofuginone probably reaches its versatile therapeutic targets by inhibiting both differentiation of T helper 17 cells (Sundrud et al., 2009) and Smad3 phosphorylation downstream of TGF β signaling (Roffe et al., 2010). Figure 5 summarize halofuginone's mode of action.

Halofuginone can be administered orally, making it a clinically attractive therapy. Therapeutically effective plasma levels of halofuginone can be achieved at well-tolerated dosages. Dose-limiting toxicities, maximum tolerated dose (MTD), pharmacokinetics and the recommended dose for chronic administration were evaluated in a dose-escalating phase I study with patients with malignant solid tumors refractory to standard forms of therapy (de Jonge et al., 2006). Thanks to its low molecular weight, halofuginone can be easily synthesized in large quantities under clinical pharmaceutical guidelines. Thus, halofuginone, which demonstrates a significant impact on molecular, tissue and functional phenotypes in dystrophic mice, meets the criteria for a potential novel therapy for patients exhibiting MDs of various etiologies.

Acknowledgements. This study was supported in part by the Muscle Dystrophy Association (MDA 68007), the Israel Science Foundation (ISF 207/05), the Association Francaise contre les myopathies (AFM 14105) and from the EEU 6th Framework Program Network of Excellence MYORES (contract 511978). We thank Suzy Roffe and Adi Lavi for their assistance and helpful discussions. 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-C669.
- Andretta 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.
- Bakay M., Zhao P., Chen J. and Hoffman E.P. (2002). A web-accessible complete transcriptome of normal human and DMD muscle. *Neuromuscular Disord.* 12, S125-S141.
- 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.
- Bernasconi P., Di Blasi C., Mora M., Morandi L., Galbiati S., Confalonieri P., Cornelio F. and Mantegazza R. (1999). Transforming growth factor- β 1 and fibrosis in congenital muscular dystrophies. *Neuromuscular Disord.* 9, 28-33.
- Besse S., Allamand V., Vilquin J.T., Li Z., Poirier C., Vignier N., Hori H., Guénet J.L. and Guicheney P. (2003). Spontaneous muscular dystrophy caused by a retrotransposal insertion in the mouse laminin alpha2 chain gene. *Neuromuscular Disord.* 13, 216-222.
- 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.
- Blaauw B., Canato M., Agatea L., Toniolo L., Mammucari C., Masiero E., Abraham R., Sandri M., Schiaffino S. and Reggiani C. (2009). Inducible activation of Akt increases skeletal muscle mass and force without satellite cell activation. *FASEB J.* 23, 3896-3905.
- Bostick B., Yue Y., Long C., Marschalk N., Fine D.M., Chen J. and Duan D. (2009). Cardiac expression of a mini-dystrophin that normalizes skeletal muscle force only partially restores heart function in aged *Mdx* mice. *Mol. Ther.* 17, 253-261.
- Bruck R., Genin O., Aeed H., Alexiev R., Nagler A. and Pines M. (2001). Halofuginone to prevent and treat thioacetamide-induced liver fibrosis in rats. *Hepatology* 33, 379-386.
- Bushby K.M. (2000). Dysferlin and muscular dystrophy. *Acta Neurol. Belg.* 100, 142-145.
- Bushby K.M. (2009). Diagnosis and management of the limb girdle muscular dystrophies. *Pract. Neurol.* 9, 314-323.
- Carlson M.E., Hsu M. and Conboy I.A. (2008). Imbalance between

- pSmad3 and Notch induces CDK inhibitors in old muscle stem cells, *Nature* 45, 528-532.
- 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.
- 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-beta-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.
- Coulton G.R., Morgan J.E., Partridge T.A. and Sloper J.C. (1988). The *mdx* mouse skeletal muscle myopathy: I. A histological, morphometric and biochemical investigation. *Neuropathol. Appl. Neurobiol.* 14, 53-70.
- Dalkilic I. and Kunkel L.M. (2003). Muscular dystrophies: genes to pathogenesis. *Curr. Opin. Genet. Dev.* 13, 231-238.
- Davies K.E. and Nowak K.J. (2006). Molecular mechanisms of muscular dystrophies: old and new players. *Nat. Rev. Mol. Cell Biol.* 10, 762-773.
- Deconinck N. and Dan B. (2007). Pathophysiology of duchenne muscular dystrophy: current hypotheses. *Pediatr. Neurol.* 36,1-7.
- de Jonge M.J., Dumez H., Verweij J., Yarkoni S., Snyder D., Lacombe D., Marréaud S., Yamaguchi T., Punt C.J., van Oosterom A. and EORTC New Drug Development Group (NDDG) (2006). Phase I and pharmacokinetic study of halofuginone, an oral quinazolinone derivativ in patients with advanced solid tumours. *Eur. J. Cancer* 42, 1768-1774.
- DeLuca A., Pierno S., Liantonio A., Cetrone M., Camerino C., Fraysse B., Mirabella M., Servidei S., Ruegg U.T. and Camerino D.C. (2003). Enhanced dystrophic progression in *mdx* mice by exercise and beneficial effects of taurine and insulin-like growth factor-1. *J. Pharmacol. Exp. Ther.* 304, 453-472.
- 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.
- Dunace V.C., Stephens H.R., Dunn M., Bailey A.J. and Dubowitz V. (1980). A role for collagen in the pathogenesis of muscular dystrophy? *Nature* 284, 470-472.
- Finsterer J. and Stollberger C. (2003). The heart in human dystrophinopathies. *Cardiology* 99, 1-19.
- 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.
- Gnainsky Y., Kushnirsky Z., Bilu G., Hagai Y., Genina O., Volpin H., Bruck R., Spira G., Nagler A., Kawada N., Yoshizato K., Reinhardt D.P., Libermann T.A. and Pines M. (2007). Gene expression during chemically induced liver fibrosis: effect of halofuginone on TGF-beta signaling. *Cell Tissue Res.* 328, 153-166.
- Goldspink G., Fernandes K., Williams P.E. and Wells D.J. (1994). Age-related changes in collagen gene expression in the muscles of *mdx* dystrophic and normal mice. *Neuromuscular Disord.* 4, 183-191.
- Gorospe J.R., Tharp M., Demitsu T. and Hoffman E.P. (1994). Dystrophin-deficient myofibers are vulnerable to mast cell granule-induced necrosis. *Neuromuscular Disord.* 4, 325-333.
- Gosselin L.E. and Martinez D.A. (2004). Impact of TNF-alpha blockade on TGF-beta1 and type I collagen mRNA expression in dystrophic muscle. *Muscle Nerve* 30, 244-246.
- Gosselin L.E. and Williams J.E. (2006). Pentoxifylline fails to attenuate fibrosis in dystrophic (*mdx*) diaphragm muscle. *Muscle Nerve* 33, 820-823.
- Gosselin L.E., Williams J.E., Personius K. and Farkas G.A. (2007). A comparison of factors associated with collagen metabolism in different skeletal muscles from dystrophic (*mdx*) mice: impact of pirfenidone. *Muscle Nerve* 35, 208-216.
- Guo L.T., Zhang X.U., Kuang W., Xu H., Liu L.A., Vilquin J.T., Miyagoe-Suzuki Y., Takeda S., Ruegg M.A., Wewer U.M. and Engvall E. (2003). Laminin alpha2 deficiency and muscular dystrophy; genotype-phenotype correlation in mutant mice. *Neuromuscular Disord.* 13, 207-215.
- Halevy O., Nagler A., Levi-Schaffer F., Genin O. and Pines M. (1996). Inhibition of collagen type I synthesis by skin fibroblasts of graft versus host disease and scleroderma patients: effect of halofuginone. *Biochem. Pharmacol.* 52, 1057-1063.
- Hantai D., Labat-Robert J., Grimaud J.A. and Fardeau M. (1985). Fibronectin, laminin, type I, III and IV collagens in Duchenne's muscular dystrophy, congenital muscular dystrophies and congenital myopathies: an immunocytochemical study. *Connect. Tissue Res.* 13, 273-281.
- Hartel J.V., Granchelli J.A., Hudecki M.S., Pollina C.M. and Gosselin L.E. (2001). Impact of prednisone on TGF-beta1 and collagen in diaphragm muscle from *mdx* mice. *Muscle Nerve* 24, 428-432.
- Haslett J.N., Sanoudou D., Kho A.T., Bennett R.R., Greenberg S.A., Kohane I.S., Beggs A.H. and Kunkel L.M. (2002). Gene expression comparison of biopsies from Duchenne muscular dystrophy (DMD) and normal skeletal muscle. *Proc. Natl. Acad. Sci. USA* 99, 15000-15005.
- Haslett J.N., Sanoudou D., Kho A.T., Han M., Bennett R.R., Kohane I.S., Beggs A.H. and Kunkel L.M. (2003). Gene expression profiling of Duchenne muscular dystrophy skeletal muscle. *Neurogenetics* 4, 163-171.
- Heydemann A., Ceco E., Lim J.E., Hadhazy M., Ryder P., Moran J.L., Beier D.R., Palmer A.A. and McNally E.M. (2009). Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. *J. Clin. Invest.* 119, 3703-3712.
- 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.
- 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-H1561.
- Ionasescu V., Lara-Braud C., Zellweger H., Ionasescu R. and Burmeister L. (1977). Fibroblast cultures in Duchenne muscular dystrophy. Alterations in synthesis and secretion of collagen and noncollagen proteins. *Acta Neurol. Scand.* 55, 407-417.
- Ishii H., Choudhuri R., Mathias A., Sowers A.L., Flanders K.C., Cook J.A. and Mitchell J.B. (2009). Halofuginone mediated protection against radiation-induced leg contracture. *Int. J. Oncol.* 35, 315-319.
- Ishitobi M., Haginoya K., Zhao Y., Ohnuma A., Minato J., Yanagisawa T., Tanabu M., Kikuchi M. and linuma K. (2000). Elevated plasma

Halofuginone and MD

- levels of transforming growth factor beta1 in patients with muscular dystrophy. *Neuroreport* 11, 4033-4035.
- Jin Y., Murakami N., Saito Y., Goto Y., Koishi K. and Nonaka I. (2000). Expression of MyoD and myogenin in dystrophic mice, *mdx* and *dy*, during regeneration. *Acta Neuropathol.* 99, 619-627.
- 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.
- Kobayashi K., Izawa T., Kuwamura M. and Yamate J. (2010). The distribution and characterization of skeletal muscle lesions in dysferlin-deficient SJL and A/J mice. *Exp. Toxicol. Pathol.* 62, 509-517.
- Krupnick A.S., Zhu J., Nguyen T., Kreisel D., Balsara K.R., Lankford E.B., Clark C.C., Levine S., Stedman H.H. and Shrager J.B. (2003). Inspiratory loading does not accelerate dystrophy in *mdx* mouse diaphragm: implications for regenerative therapy. *J. Appl. Physiol.* 94, 411-419.
- Kuang W., Xu H., Vachon P.H., Liu L., Loechel F., Wewer U.M. and Engvall E. (1998). Merosin-deficient congenital muscular dystrophy. Partial genetic correction in two mouse models. *J. Clin. Invest.* 102, 844-852.
- 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, 84-88.
- Lewis C., Carberry S. and Ohlendieck K. (2009). Proteomic profiling of x-linked muscular dystrophy. *J. Muscle Res. Cell. Motil.* 30, 267-269.
- MacPike A.D. and Meier H. (1976). Comparison of *dy* and *dy2J*, two alleles expressing forms of muscular dystrophy in the mouse. *Proc. Soc. Exp. Biol. Med.* 151, 670-672.
- Marotta M., Ruiz-Roig C., Sarria Y., Peiro J.L., Nuñez F., Ceron J., Munell F. and Roig-Quilis M. (2009). Muscle genome-wide expression profiling during disease evolution in *mdx* mice. *Physiol. Genomics* 37, 119-132.
- Marques M.J., Oggiam D.S., Barbin I.C., Ferretti R. and Santo Neto H. (2009). Long-term therapy with deflazacort decreases myocardial fibrosis in *mdx* mice. *Muscle Nerve* 40, 466-468.
- Marshall P.A., Williams P.E. and Goldspink G. (1989). Accumulation of collagen and altered fiber-type ratios as indicators of abnormal muscle gene expression in the *mdx* dystrophic mouse. *Muscle Nerve* 12, 528-537.
- McGaha T.L., Kodera T., Phelps R., Spiera H., Pines M. and Bona C. (2002a). Effect of halofuginone on the development of tight skin (TSK) syndrome. *Autoimmunity* 35, 277-282.
- McGaha T.L., Phelps R.G., Spiera H. and Bona C. (2002b). 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.
- McNally E.M. (2007). New approaches in the therapy of cardiomyopathy in muscular dystrophy. *Ann. Rev. Med.* 58, 75-88.
- McNally E.M. and Pytel P. (2007). Muscle diseases: the muscular dystrophies. *Annu. Rev. Pathol. Mech. Dis.* 2, 87-109.
- Meier H. and MacPike A.D. (1977). Myopathies caused by three mutations of the mouse. *J. Hered.* 68, 383-385.
- 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.
- Morrison J., Lu Q.L., Pastoret C., Partridge T. and Bou-Gharios G. (2000). T-cell-dependent fibrosis in the *mdx* dystrophic mouse. *Lab. Invest.* 80, 881-891.
- Murakami N., McLennan I.S., Nonaka I., Koishi K., Baker C. and Hammond-Tooke G. (1999). Transforming growth factor-beta2 is elevated in skeletal muscle disorders. *Muscle Nerve* 22, 889-898.
- 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.
- Nagler A., Rivkind A.I., Raphael J., Levi-Schaffer F., Genina O., Lavelin I. and Pines M. (1998). Halofuginone—an inhibitor of collagen type I synthesis—prevents post-operation abdominal adhesions formation. *Ann. Surg.* 227, 575-582.
- Nagler A., Genina O., Lavelin I., Ohana M. and Pines M. (1999). Halofuginone—an inhibitor of collagen type I synthesis—prevents formation of post-operative adhesions formation in the rat uterine horn model. *Am. J. Obstet. Gyn.* 180, 558-563.
- Nagler A., Gofrit O., Ohana M., Pode D., Genina O. and Pines M. (2000). The effect of halofuginone, an inhibitor of collagen type I synthesis, on urethral stricture formation in vivo and in vitro study in a rat model. *J. Urol.* 164, 1776-1780.
- Nevo Y., Halevy O., Genin O., Turgeman T., Harel M., Biton E., Reif S. and Pines M. (2010). Inhibition of fibrosis in laminin- α 2-deficient congenital muscular dystrophy mice: effect of halofuginone. *Muscle Nerve* 42, 218-229.
- Nyska M., Nyska A., Rivlin E., Porat S., Pines M., Shoshan S. and Nagler, A. (1996). Topically applied halofuginone, an inhibitor of collagen type I transcription, reduced peritendinous fibrous adhesions following surgery. *Conn. Tissue Res.* 34, 97-103.
- Passerini L., Bernasconi P., Baggi F., Confalonieri P., Cozzi F., Cornelio F. and Mantegazza R. (2002). Fibrogenic cytokines and extent of fibrosis in muscle of dogs with X-linked golden retriever muscular dystrophy. *Neuromuscular Disord.* 12, 828-835.
- Pines M. (2008). Targeting TGF β signaling to inhibit fibroblasts activation as a therapy for fibrosis and cancer. *Exp. Opin. Drug Dis.* 3(1), 11-20.
- Pines M. and Nagler A. (1996). Halofuginone—a novel anti-fibrotic therapy. *Drug of the Future* 21, 569-599.
- Pines M. and Nagler A. (1998). Halofuginone: a novel antifibrotic therapy. *Gen. Pharmacol.* 30, 445-450.
- Pines M., Knopov V., Genin O., Lavelin I. and Nagler A. (1997). Halofuginone, a specific inhibitor of collagen type I synthesis, prevents dimethylnitrosamine-induced liver cirrhosis. *J. Hepatol.* 27, 391-398.
- 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., Aleviev 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.
- Pines M., Snyder D., Yarkoni S. and Nagler A. (2003). Halofuginone to treat fibrosis in chronic graft versus host disease and scleroderma. *Biol. Blood Marrow Transplant.* 9, 417-425.
- 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 NF κ B. *J. Biol. Chem.* 281, 15090-15098.
- Porter J.D., Khanna S., Kaminski H.J., Rao J.S., Merriam A.P.,

- Richmonds C.R., Leahy P., Li J. and Andrade FH. (2001). Extraocular muscle is defined by a fundamentally distinct gene expression profile. *Proc. Natl. Acad. Sci. USA* 98, 12062-12067.
- Porter J.D., Merriam A.P., Leahy P., Gong B. and Khanna S. (2003). Dissection of temporal gene expression signatures of affected and spared muscle groups in dystrophin-deficient (*mdx*) mice. *Hum. Mol. Genet.* 12, 1813-1821.
- Prelle A., Sciacco M., Tancredi L., Fagiolari 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.
- Qiao C., Li J., Zhu T., Draviam R., Watkins S., Ye X., Chen C., Li J. and Xiao X. (2005). Amelioration of laminin-alpha2-deficient congenital muscular dystrophy by somatic gene transfer of miniagrin. *Proc. Natl. Acad. Sci. USA* 102, 11999-12004.
- Quinlan J.G., Hahn H.S., Wong B.L., Lorenz J.N., Wenisch A.S. and Levin L.S. (2004). Evolution of the *mdx* mouse cardiomyopathy: physiological and morphological findings. *Neuromuscular Disord.* 14, 491-496.
- 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.
- 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 contributes to the chemotherapy-mediated anti-tumoral effect. *Mol. Cancer Ther.* 6, 570-577.
- Spira G., Mawasi N., Paizi M., Anbinder N., Genina O., Alexiev R. and Pines M. (2002). Halofuginone, a collagen type I inhibitor, improves liver regeneration in cirrhotic rats. *J. Hepatol.* 37, 331-339.
- Stedman H.H., Sweeney H.L., Shrager J.B., Maguire H.C., Panettieri R.A., Petrof B., Narusawa M., Leferovich J.M., Sladky J.T. and Kelly A.M. (1991). The *mdx* mouse diaphragm reproduces the degenerative changes of Duchenne muscular dystrophy. *Nature* 352, 536-539.
- Sundrud M.S., Koralov S.B., Feuerer M., Calado D.P., Kozhaya A.E., Rhule-Smith A., Lefebvre R.E., Unutmaz D., Mazitschek R., Waldner H., Whitman M., Keller T. and Rao A. (2009). Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. *Science* 324, 1334-1338.
- Takaya Y., Tasaka H., Chiba T., Uwai K., Tanitsu M. and Kim H.S. (1999). New type of febrifugine analogues, bearing a quinolizidine moiety, show potent antimalarial activity against plasmodium malaria parasite. *J. Med. Chem.* 42, 3163-3166.
- Taniguchi M., Kurahashi H., Noguchi S., Sese J., Okinaga T., Tsukahara T., Guicheney P., Ozono K., Nishino I., Morishita S. and Toda T. (2006). Expression profiling of muscles from Fukuyama-type congenital muscular dystrophy and laminin-alpha 2 deficient congenital muscular dystrophy; is congenital muscular dystrophy a primary fibrotic disease? *Biochem. Biophys. Res. Commun.* 342, 489-502.
- Turgeman T., Hagai Y., Huebner K., Jassal D.S., Anderson J., Genin O., Nagler A., Halevy O. and Pines M. (2008). Prevention of muscle fibrosis and improvement in muscle performance in the *mdx* mouse by halofuginone. *Neuromuscular Disord.* 18, 857-868.
- Turk R., Sterrenburg E., de Meijer E.J., van Ommen G.J., den Dunnen J.T. and 't Hoen P.A. (2005). Muscle regeneration in dystrophin-deficient *mdx* mice studied by gene expression profiling. *BMC Genomics* 6, 98.
- van Lunteren E., Moyer M. and Leahy P. (2006). Gene expression profiling of diaphragm muscle in alpha2-laminin (merosin)-deficient *dy/dy* dystrophic mice. *Physiol. Genomics* 25, 85-95.
- Vidal B., Serrano A.L., Tjwa M., Suelves M., Ardite E., De Mori R., Baeza-Raja B., Martínez de Lagrán M., Lafuste P., Ruiz-Bonilla V., Jardí M., Gherardi R., Christov C., Dierssen M., Carmeliet P., Degen J.L., Dewerchin M. and Muñoz-Cánoves P. (2008). Fibrinogen drives dystrophic muscle fibrosis via a TGFbeta/alternative macrophage activation pathway. *Genes Dev.* 22, 1747-1752.
- Xavier S., Piek E., Fujii M., Javelaud D., Mauviel A., Flanders K.C., Samuni A.M., Felici A., Reiss M., Yarkoni S., Sowers A., Mitchell J.B., Roberts A.B. and Russo A. (2004). Amelioration of radiation-induced fibrosis: inhibition of transforming growth factor-beta signaling by halofuginone. *J. Biol. Chem.* 279, 15167-15176.
- 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 Tiss. Res.* 339, 397-410.
- Zhao Y., Haginoya K., Sun G., Dai H., Onuma A. and Inuma K. (2003). Platelet-derived growth factor and its receptors are related to the progression of human muscular dystrophy: an immunohistochemical study. *J. Pathol.* 201, 149-159.
- Zhu S., Goldschmidt-Clermont P.J. and Dong C. (2004). Transforming growth factor-beta-induced inhibition of myogenesis is mediated through Smad pathway and is modulated by microtubule dynamic stability. *Circ. Res.* 94, 617-625.
- 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* 3, 427-435.