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Glutathione peroxidase 3, a new retinoid target gene, is crucial for human skeletal muscle precursor cell survival

Marina El Haddad^{1,*}, Elise Jean^{1,*}, Ahmed Turki¹, Gérald Hugon¹, Barbara Vernus², Anne Bonniou², Emilie Passerieux¹, Aline Hamade³, Jacques Mercier^{1,4}, Dalila Laoudj-Chenivesse¹ and Gilles Carnac^{1,‡}

¹Inserm U1046, Université Montpellier 1, Université Montpellier 2, 34295 Montpellier, France

²INRA, UMR866, Université Montpellier 1, Université Montpellier 2, 34060 Montpellier, France

³Department of Biology, Faculty of Sciences II, Lebanese University, Jdeidet el Matr, Lebanon

⁴CHRU de Montpellier, Département de Physiologie Clinique, 34295 Montpellier cedex 5, France

*These authors contributed equally to this work

‡Author for correspondence (gilles.carnac@inserm.fr)

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Summary

Protection of satellite cells from cytotoxic damages is crucial to ensure efficient adult skeletal muscle regeneration and to improve therapeutic efficacy of cell transplantation in degenerative skeletal muscle diseases. It is therefore important to identify and characterize molecules and their target genes that control the viability of muscle stem cells. Recently, we demonstrated that high aldehyde dehydrogenase activity is associated with increased viability of human myoblasts. In addition to its detoxifying activity, aldehyde dehydrogenase can also catalyze the irreversible oxidation of vitamin A to retinoic acid; therefore, we examined whether retinoic acid is important for myoblast viability. We showed that when exposed to oxidative stress induced by hydrogen peroxide, adherent human myoblasts entered apoptosis and lost their capacity for adhesion. Pre-treatment with retinoic acid reduced the cytotoxic damage *ex vivo* and enhanced myoblast survival in transplantation assays. The effects of retinoic acid were maintained in dystrophic myoblasts derived from facioscapulohumeral patients. RT-qPCR analysis of antioxidant gene expression revealed glutathione peroxidase 3 (*Gpx3*), a gene encoding an antioxidant enzyme, as a potential retinoic acid target gene in human myoblasts. Knockdown of *Gpx3* using short interfering RNA induced elevation in reactive oxygen species and cell death. The anti-cytotoxic effects of retinoic acid were impaired in GPx3-inactivated myoblasts, which indicates that GPx3 regulates the antioxidative effects of retinoic acid. Therefore, retinoid status and GPx3 levels may have important implications for the viability of human muscle stem cells.

Key words: GPx3, Vitamin A, Retinoic acid, Myoblasts, Facioscapulohumeral dystrophy (FSHD), Transplantation

Introduction

Because transplanted myoblasts (skeletal muscle precursor cells) can fuse with endogenous muscle fibers to form hybrid myotubes (Partridge et al., 1989), myoblast transplantation represents a viable approach for the treatment of inherited myopathies and diseases that are characterized by fiber necrosis and muscle weakness (Gussoni et al., 1997). Although limitations such as immune rejection or limited spread into host tissue are important, the failure of myoblast transfer in initial clinical trials was also partially attributed to poor survival rates of transplanted myoblasts (Gussoni et al., 1997; Mendell et al., 1995; Partridge et al., 1989; Tremblay et al., 1993). Recent data have suggested that oxidative stress, which is presumably derived from damage resulting from intramuscular implantation, might cause rapid cell death in transplantation experiments. The pre-treatment of muscle precursor cells with antioxidant molecules improves graft survival (Drowley et al., 2010; Rodriguez-Porcel et al., 2010; Suzuki et al., 2004). Conversely, the inhibition of antioxidant capacity by decreasing glutathione activity impairs the regenerative capacity of muscle-derived stem cells (Drowley et al., 2010). Although such pro-survival strategies were found to

be successful in these studies, the exact mechanisms of action of these molecules have not been determined. Furthermore, it is not clear whether pro-survival strategies can improve the poor adhesion of myoblasts to the cellular matrix under conditions of oxidative stress, which is a phenomenon previously observed upon transplantation of mesenchymal stem cells (MSCs) (Song et al., 2010). In addition to the difficulties described above, the specific physiopathology status of host dystrophic muscles may cause additional problems. Facioscapulohumeral dystrophy (FSHD) is an autosomal dominant neuromuscular disease characterized by a progressive weakness and atrophy of skeletal muscles (Statland and Tawil, 2011). In FSHD patients the coexistence of affected muscles and apparently healthy muscles has led to the proposal that myoblasts from unaffected muscles can be purified and implanted into the affected muscles to repair them (Vilquin et al., 2005). However, FSHD is associated with exacerbated oxidative stress (Barro et al., 2010; Turki et al., 2012; Winokur et al., 2003) that could further limit the efficacy of autologous myoblast transplantation. Therefore, the enhancement of cell survival should be a principal goal of cell transplantation techniques.

Aldehyde dehydrogenases (ALDHs) efficiently oxidize and detoxify aldehydic products of lipid peroxidation (LPO) initially generated by reactive oxygen species (Jackson et al., 2011) and contribute to stem cell self-protection, differentiation and/or self-renewal (Balber, 2011; Ma and Allan, 2011). Recently, we determined that high aldehyde dehydrogenase activity (ALDH^{high}) is associated with improved cell viability in human myoblasts (Jean et al., 2011). In addition, Vauchez et al. and Vella et al. have isolated ALDH^{high} muscle progenitors cells that exhibit increased stress resistance and regenerative capacity (Vauchez et al., 2009; Vella et al., 2011). Several ALDH enzymes, including Aldh1a1, Aldh1a2 and Aldh1a3 in humans, catalyze the irreversible oxidation of vitamin A (VA) to retinoic acid (RA), which binds and activates nuclear retinoic acid receptor (RAR)/retinoid X receptor (RXR) heterodimers to regulate the transcription of target genes that are important for development, morphogenesis and differentiation (Jackson et al., 2011; Samarut and Rochette-Egly, 2012). Many studies have been conducted to analyze the role of RA in muscle development and have shown that RA exerts a direct effect on skeletal muscle differentiation as well as on the metabolism of murine, zebrafish and chicken skeletal muscle cells (Albagli-Curiel et al., 1993; Amengual et al., 2008; Hamade et al., 2006; Maden et al., 2000; Reijntjes et al., 2009). However, the effect of RA on oxidative stress and survival of skeletal muscle cells has not been determined.

The cellular redox potential is maintained by a balanced regulation of pro-oxidative and antioxidative enzymes. Glutathione peroxidase (GPx) proteins along with superoxide dismutases and catalases are part of the enzymatic defense system that cell utilizes to fight free-radical-mediated attacks (Silva and Coutinho, 2010). GPx is a selenium-dependent enzyme containing a selenium atom incorporated within the selenocysteine residue. To date, several isoforms of GPx proteins have been identified. Of these, only GPx3 is secreted, and scavenges H₂O₂ and peroxidized organic molecules to reduce systemic oxidative stress (McCann and Ames, 2011; Reszka et al., 2012).

Because we demonstrated that the retinaldehyde Aldh1a1 contributes to most, if not all, ALDH activity in human myoblasts and promotes cell survival (Jean et al., 2011), our objectives were to determine whether RA, the final metabolite of Aldh1a1, could protect human myoblasts from oxidative stress *ex vivo* and *in vivo* transplantation assays. In this study, we demonstrated that

RA protected healthy and dystrophic (FSHD) human myoblasts from cytotoxic damage and improved cell survival in transplantation assays. In addition, we identified the gene for glutathione peroxidase 3 (GPx3) as a retinoid-responsive gene that mediates the antioxidant effects of RA in human myoblasts. We believe these studies, in addition to providing new information on the role of RA, may bring new insight into how myoblasts orchestrate their own protection.

Results

RA receptors are functional in human myoblasts

During skeletal muscle differentiation of human primary cultures, myoblasts, the progeny of satellite cells, exit the cell cycle and spontaneously differentiate, giving rise to myotubes, quiescent multinucleated cells expressing muscle-specific structural proteins. To determine whether the RA signaling pathway was functional in human myoblasts ($n=5$; see Table 1), we first characterized the endogenous expression of the three main RA receptor isotypes, namely, RAR alpha, RAR beta and RXR alpha, using western blotting and RT-qPCR of proliferative (P) and differentiated (D) human myoblasts (Fig. 1A–C). As expected, the differentiation marker, myogenin, was enriched in myotubes, whereas the proliferative marker, cyclin A, was mainly detected in proliferative myoblasts both at the protein (Fig. 1A,B) and mRNA (Fig. 1C) levels. RAR alpha, RAR beta and RXR alpha were expressed in human myoblasts, although no notable change in their expressions was detected during differentiation (Fig. 1A–C). RA induces RAR-beta transcription through retinoid receptors that bind to the RA-responsive element in the RAR-beta promoter region (beta RARE) (Samarut and Rochette-Egly, 2012). Thus, the transcriptional activity of endogenous RAR/RXR heterodimers after RA treatment can be evaluated using a RARE β -tk-luciferase reporter gene that contains an RA response element from the RAR beta promoter and by determining RAR beta mRNA levels. Treatment with RA induced a 10-fold increase in RARE beta-tk-luciferase activity (Fig. 1D). Cells were also treated with VA, which cannot bind on its own to RA receptors but can be converted to RA by endogenous aldehyde dehydrogenases. Similar to RA, treatment with VA induced a 10-fold increase in RARE beta-tk-luciferase activity (Fig. 1D). In addition, we determined the endogenous expression of RAR beta mRNA after addition of RA or VA via RT-qPCR. Treatment with RA or VA induced a 12- to 15-fold increase in RAR beta mRNA

Table 1. Human myoblast characteristics of healthy individuals and FSHD patients

Individuals	Age (years)	Sex ^a	Muscle ^b	No. of D4Z4 units	Myogenicity ^c (%)
Control					
NO36	41	M	Quadriceps	ND	>99
N042	24	F	Quadriceps	ND	>99
N045	35	F	Quadriceps	ND	>99
N046	31	M	Quadriceps	ND	>99
N048	30	M	Quadriceps	ND	>99
FSHD					
M038	36	M	Quadriceps	7	>99
M041	23	F	Quadriceps	8	>99
M051	32	F	Quadriceps	7	>99
M052	20	F	Quadriceps	4	>99
M054	25	M	Quadriceps	4	>99

^aM, male; F, female.

^bSite of the muscle biopsy.

^cPercentage of desmin-positive cells versus the total number of cells.

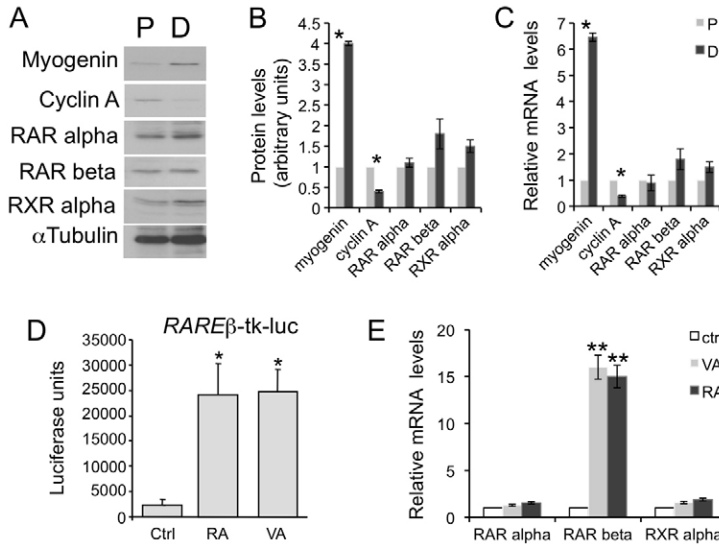


Fig. 1. RA receptors are expressed and transcriptionally active in human myoblasts. (A–C) Human myoblasts were harvested at proliferative myoblast (P) and differentiated myotube (D) stages and were analyzed for myogenin, cyclin A, RAR alpha, RAR beta and RXR alpha (A) at the protein levels by western blot analysis, quantified (B) and (C) at the mRNA levels by RT-qPCR. Levels of protein and mRNA were expressed relative to those in proliferative myoblasts arbitrarily set as 1. (D) Luciferase activities after transfection of myoblasts with RARE β -tk-luc and treatment with 5×10^{-6} M VA or 10^{-7} M RA. (E) RT-qPCR analysis of relative expression of RAR alpha, RAR beta and RXR alpha in human myoblasts treated with 5×10^{-6} M VA or 10^{-7} M RA for 24 hours. Levels of RAR alpha, RAR beta and RXR alpha mRNA were expressed relative to those in untreated cells (ctrl; arbitrarily set as 1). * $P \leq 0.05$; ** $P \leq 0.01$.

expression. As expected, RA did not induce RAR alpha or RXR alpha expression (Fig. 1E). These data indicated that RAR receptors were present and were transcriptionally active in human myoblasts.

RA protects human myoblasts from H₂O₂-induced apoptosis

Because we demonstrated that high ALDH activity is associated with increased cell viability, we postulated that the final metabolite of this enzyme, RA, could efficiently protect human myoblasts from oxidative stress damage. We previously showed that hydrogen peroxide (H₂O₂) increased the percentage of dead cells in adherent myoblast culture, which were characterized by identification of damaged membranes after staining with ethidium homodimer-1, a compound that only crosses the damaged membranes of dead and necrotic cells and produces bright-red fluorescent nucleic acid staining. We demonstrated that cells died from apoptosis because myoblasts had depolarized mitochondria, which were visualized by JC1-green staining and strong caspase 3/7 activities. We tested the effect of RA on myoblast survival by treating human myoblasts ($n=5$) with 10^{-7} M RA for 48 hours prior to incubation with H₂O₂. As expected, H₂O₂ enhanced the percentage of ethidium homodimer-1- (Fig. 2A), JC1-green- (Fig. 2B) and caspase 3/7-positive cells (Fig. 2C). Pre-treatment with RA efficiently reduced the number of apoptotic cells that were positive for ethidium homodimer-1 staining (Fig. 2A), JC1-green staining (Fig. 2B) and caspase 3/7 activity (Fig. 2C). To confirm that exogenous treatment with H₂O₂ induced reactive oxygen species (ROS) accumulation and to determine the effect of RA on ROS level, we used a ROS indicator probe loaded into myoblast. When oxidized by various active oxygen species, carboxy-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) is converted to a fluorescent form (Fig. 2D). Flow cytometric comparison of the ROS indicator carboxy-H2DCFDA fluorescence revealed a strong increase in ROS levels in myoblasts treated with H₂O₂ whereas RA reduced significantly H₂O₂-induced accumulation of ROS in human myoblasts (Fig. 2D; $P \leq 0.05$).

Therefore, we determined that RA protects adherent myoblasts from H₂O₂-induced oxidative stress damage.

RA restores myoblast adhesion impaired by H₂O₂

Because H₂O₂ induced detachment of cells from the substratum and apoptosis (Grossmann, 2002; Song et al., 2010), we examined the effects of H₂O₂ on myoblast adhesion in the presence of RA. We performed a quantitative adhesion assay initially developed to mimic the poor adhesion of mesenchymal stem cells in oxidative stress condition, a phenomenon observed during transplantation (Song et al., 2010). ‘Healthy’ myoblasts ($n=5$) were pre-treated for 2 days with RA before being trypsinized, plated and immediately exposed to increasing concentrations of H₂O₂. Non-adherent cells were removed after 2 hours by successive washes and the percentage of adhesive cells was quantified by staining cells with Hoechst (a fluorescent DNA stain).

An increase in the H₂O₂ concentration led to a decrease in myoblast adhesion (Fig. 3A): 20% of the cells were unable to adhere to the matrix when exposed to 50 μ M H₂O₂ and at 250 μ M H₂O₂, only 10% of the total cells remained attached to the matrix (Fig. 3B). Conversely, significantly more RA-pre-treated myoblasts that control cells were able to remain attached at all tested doses of H₂O₂ excepted at 250 μ M (Fig. 3B). We conducted the same experiment on human myoblasts derived from patients with FSHD ($n=5$; see Table 1). As with the ‘healthy’ control cells, an increase in H₂O₂ concentration led to a decrease in myoblast adhesion; in contrast, pre-treatment with RA restored the adhesion of FSHD myoblasts at all concentrations of H₂O₂, with the exception of 250 μ M (Fig. 3C). We confirmed those results by determining the concentration of H₂O₂ at which half of the cells adhere to the dish (IC₅₀) for healthy and FSHD cells (see Table 1 for myoblast characteristics). After RA treatment, the IC₅₀ significantly increased from $156.4 \pm 2.28 \mu$ M H₂O₂ (\pm s.e.m.) to $214.7 \pm 1.464 \mu$ M H₂O₂ ($P=0.0336$) for healthy cells and from $126.6 \pm 9.42 \mu$ M H₂O₂ to $193.3 \pm 3.75 \mu$ M H₂O₂ ($P=0.0437$) for FSHD cells. We noticed that IC₅₀ values (before and after RA treatment) were higher for healthy than FSHD myoblasts but not statistically significant.

In these experiments, we showed that RA pre-treatment prevents the deleterious effects of H₂O₂-induced oxidative stress in myoblasts derived from both healthy and dystrophic (FSHD) patients.

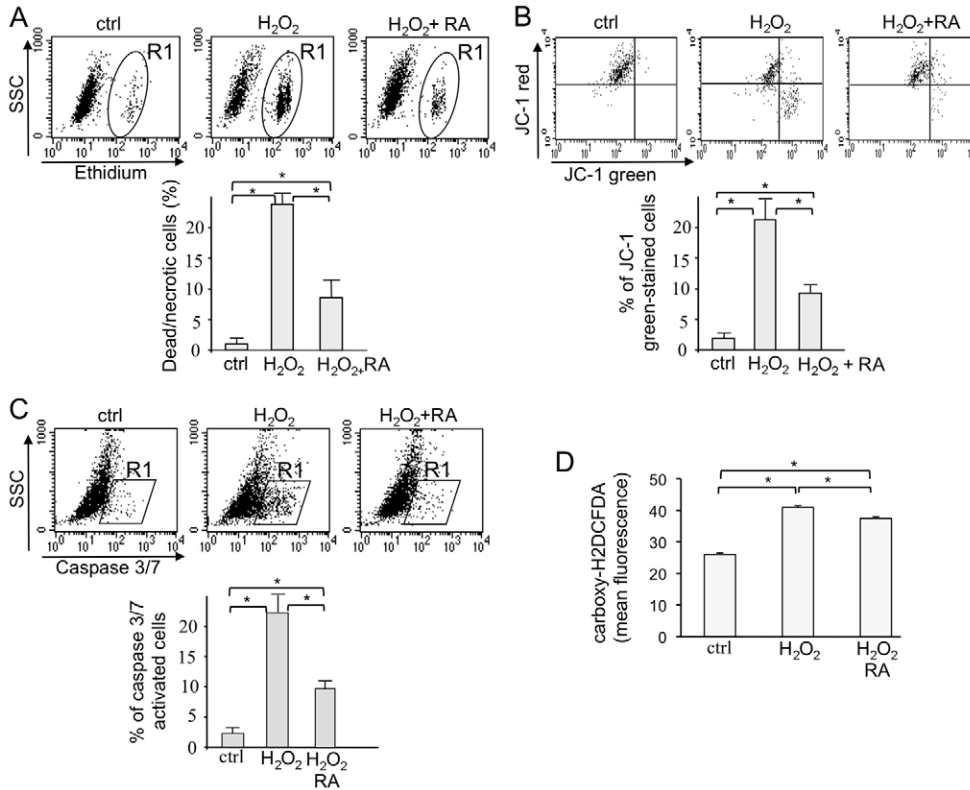


Fig. 2. RA counteracts H₂O₂-induced apoptosis in human myoblasts.

(A) (Upper panels) Example of cytometric analysis of human myoblasts treated with H₂O₂ or H₂O₂+RA and stained with ethidium homodimer-1. Cells gated in R1 represent the subpopulation of cells with bright-red fluorescence resulting from staining of nucleic acids by ethidium homodimer-1 in dead cells. (Lower panel) The red fluorescent cells were quantified and expressed as a percentage of the dead cells. (B) (Upper panels) Example of cytometric analysis of human myoblasts treated with H₂O₂ or H₂O₂+RA and stained with JC-1. In non-apoptotic cells, JC-1 fluoresces red, whereas apoptotic cells are stained green. (Lower panel) The percentage of JC-1-green-positive cells. (C) (Upper panels) Example of cytometric analysis of human myoblasts treated with H₂O₂ or H₂O₂+RA and stained for caspase 3/7 activity. (Lower panel) The percentage of caspase 3/7-positive cells gated in R1. (D) Quantification of intracellular ROS levels in H₂O₂- or H₂O₂+RA-treated myoblasts evaluated by flow cytometry using the H2DCFDA probe. **P*≤0.05.

The potential benefits of retinoid treatment of human myoblasts in transplantation assays

Because RA can efficiently protect human myoblasts from oxidative-stress-induced damage, we examined whether RA could counteract the adverse effects of cell transplantation and increase cell survival after implantation into the muscle of an adult recipient. We assessed the survival of myoblasts during the first 48 hours in host skeletal muscle tissues because more than 90% of implanted myoblasts die during this period (Beauchamp et al., 1999). The healthy and FSHD human myoblasts were pre-treated with RA and were implanted into the muscles of *scid* mice. Two days after implantation, muscle-derived cells were harvested and analyzed for human CD56 expression using fluorescence-activated cell sorting (FACS). As shown in Fig. 4A,B, a distinct population of CD56-positive cells was detectable by FACS (middle panels) in control cells, whereas no fluorescent cells were detected in muscle-derived cells of control non-injected *scid* mice (left panels). Pre-treatment with RA significantly enhanced the number of healthy and FSHD human myoblasts recovered after transplantation (right panels).

GPx3 is an RA target gene

Our results showed that RA protects human myoblasts from H₂O₂-induced cytotoxicity when treated at least 48 hours prior to H₂O₂ treatment. Concomitant treatment with H₂O₂ and RA did not prevent H₂O₂-induced apoptosis or modify cell adhesion (data not shown). Therefore, these data suggested that RA regulated the expression of genes associated with increased cell viability and antioxidant function. We performed a microarray analysis to identify the retinoid-responsive genes that mediate the anti-cytotoxic effects of RA in human myoblasts. The expression of a number of genes encoding antioxidant enzymes in

RA-treated myoblasts was largely unchanged. Interestingly, the expression of glutathione peroxidase 3 (GPx3) was dramatically increased by the treatment with RA or VA (Table 2; see also supplementary material Table S1). To confirm the microarray data, RT-qPCR was performed (Fig. 5A). The level of GPx3 mRNA increased dramatically with RA or VA treatment. In contrast, catalase (cat.), glutathione synthetase (GSS), thioredoxin 1 (Thx1), thioredoxin reductase 1 (Thx-r1), superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1), glutathione peroxidase 2 (GPx2) and glutathione peroxidase 4 (GPx4) levels were unchanged. We observed a modest but significant twofold increase in SOD2 mRNA (Fig. 5A). Next, we performed a kinetic analysis to characterize the GPx3 mRNA profile in human myoblasts exposed to RA for up to 96 hours (Fig. 5B). This kinetics were compared to that of RAR beta mRNA, an RA target gene classified as an 'immediate early gene'. As expected, RA rapidly induced the expression of RAR beta mRNA with a peak of expression between 8 and 24 hours. In contrast, GPx3 mRNA expression peaked between 24 and 48 hours after RA treatment. The kinetics of GPx3 expression were consistent with those observed for the anti-cytotoxic effects of RA. We did not obtain a GPx3 signal using immunocytochemistry with several sources of GPx3 antibodies (data not shown). Therefore, we measured GPx activity in human myoblasts treated with RA; as shown in Fig. 5C, RA treatment led to increased GPx activity. We then asked whether the retinoic acid signaling pathway was activated in differentiated cells. First, we showed that GPx3 mRNA levels remained relatively stable during differentiation (Fig. 5D). Then, myoblasts were induced to differentiate for 3 days and then treated for 2 days with retinoic acid. As a control, myoblasts were treated with RA at the proliferative stage. As expected GPx3 and RAR beta mRNA

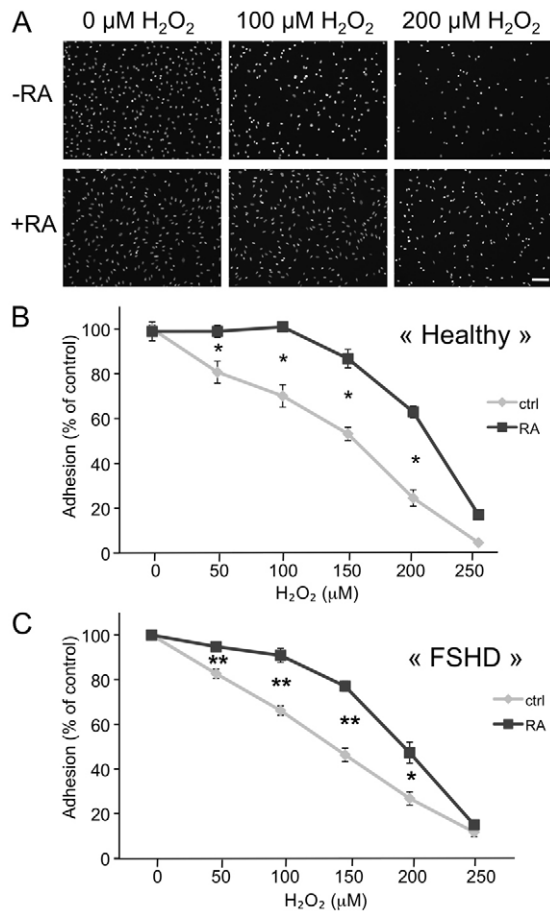


Fig. 3. RA restores human myoblast adhesion under oxidative stress conditions. (A) Human 'healthy' myoblasts, either untreated or pre-treated with RA for 48 hours, were trypsinized and exposed to increasing concentration of H₂O₂ for 2 hours. Human myoblasts were subsequently stained with Hoescht, fixed and photographed. (B) Data plotted onto the graph represent the percentage of adherent cells relative to the percentage of control (H₂O₂-untreated) cells (set as 100%). Quantified using Histolab software using data are from five fields for each condition. (C) Human FSHD myoblasts, either untreated or pre-treated with RA for 48 hours, were trypsinized and exposed to increasing concentrations of H₂O₂ for 2 hours before being stained with Hoescht, fixed and photographed. The percentage of adherent cells relative to the percentage of control (H₂O₂-untreated) cells (set as 100%) was quantified using Histolab software. Scale bar: 50 μM. **P*≤0.05; ***P*≤0.01.

were strongly induced by RA in myoblasts. Surprisingly, RA treatment in differentiated cells slightly induced both GPx3 and RAR beta mRNA, suggesting that the retinoic acid signaling pathway is partially impaired in myotubes (Fig. 5D). Consistent with this hypothesis, RA was unable to counteract the deleterious effect of an H₂O₂-induced oxidative stress in myotubes (data not shown). We then determined whether GPx3 could be the target of others molecules with antioxidant activity (Fig. 5E). Myoblasts were treated for 2 days with RA, VA, ascorbic acid (vitamin C), Tempol (a long-lasting water soluble nitroxide), mito-Tempo (a mitochondria-targeted antioxidant), NAC (N-acetyl cysteine, a precursor in the formation of glutathione) and GPx3 mRNA levels were determined by RT-qPCR. Only RA and VA induced a significant increase of GPx3 gene expression in myoblasts (Fig. 5E; *P*≤0.01).

GPx3 is associated with the improved viability of human myoblasts

To determine the manner in which GPx3 contributes to the antioxidant effect of RA, we knocked down GPx3 using small interfering RNA (siRNA). Negative control siRNA (sictrl), which did not target any human or mouse genes, was used as a control. Cells were transfected twice (time 0 and 48 hours later) with GPx3 siRNA (siGPx3) which reduced by 98% the expression of GPx3 mRNA (Fig. 6A). Since GPx3 is a secreted protein that scavenges radical oxygen species (ROS), we then evaluated the levels of ROS in sictrl and siGPx3 cells. Flow cytometric comparison of the ROS indicator carboxy-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) fluorescence revealed a relative induction in ROS levels in siGPx3 compared to sictrl cells (Fig. 6B,D). We detected changes in cell phenotype after transfection with siGPx3. We noticed that there were always fewer cells in siGPx3 group with a substantial number of floating cells (data not shown). Therefore, uptake of ethidium homodimer was used to assess cell viability. We observed an increase in cell death in siGPx3 myoblasts (Fig. 6C,E). RA treatment did not prevent ROS accumulation (Fig. 6D) and cell death in siGPx3 myoblasts (Fig. 6E). In contrast, the antioxidant Tempol restored ROS to a level similar to that in sictrl cells (Fig. 6D). Interestingly, Tempol significantly reduced but did not completely abolish the percentage of dead cells in siGPx3 culture (Fig. 6E). Then, sictrl and siGPx3 myoblasts were treated with H₂O₂ and cell adhesion was examined. We demonstrated that RA could not restore the adhesion of siGPx3 myoblasts, which was inhibited by H₂O₂ (Fig. 6F).

Based on these results, we conclude that GPx3 plays a major role in human myoblast viability and mediates the anti-cytotoxic effects of RA.

Discussion

Cell therapies for degenerative diseases of skeletal muscles have produced disappointing results but have also identified the main limitations of this approach. One of these limitations concerns the mortality of implanted skeletal muscle precursor cells (i.e. myoblasts). Several strategies have been developed to reduce cell mortality, including pharmacological approaches that utilize molecules with anti-apoptotic activities (Mias et al., 2008; Suzuki et al., 2004; Urish et al., 2009). Recently, we showed that aldehyde dehydrogenase activity and Aldh1a1 levels were elevated in a majority of human myoblasts, and aldehyde dehydrogenase activity was associated with increased cell survival in oxidative stress conditions (Jean et al., 2011). Because Aldh1a1 catalyzes the oxidation of VA to RA, we examined whether RA could regulate myoblast viability. In this study, we demonstrated that RA treatment prevents oxidative stress induced by H₂O₂ in human healthy and dystrophic skeletal muscle cells. Interestingly, pre-treatment of human myoblasts with RA induced higher survival rates upon transplantation into *scid* mice. We identified the gene for GPx3 as a potential RA target and demonstrated that GPx3 mediates the antioxidant effect of RA on human skeletal muscle cells.

RA appeared to be required for the maintenance of muscle cell identity during development. A moderate VA deficiency in newborn rats decreases the expression of Myf5 and myogenin, which are two transcription factors of the MyoD gene family that are involved in muscle determination and differentiation (Downie et al., 2005). RA stimulation activates MyoD and the terminal differentiation of pre-somitic and somitic mesoderm of zebrafish

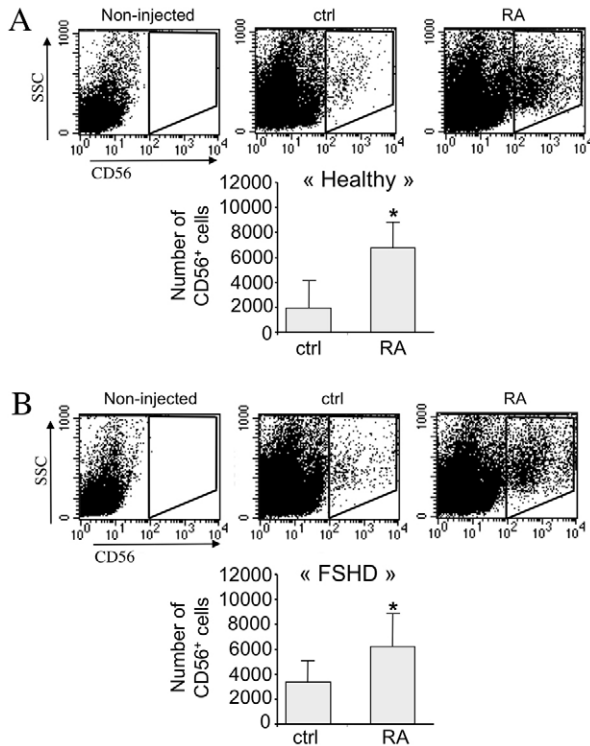


Fig. 4. RA increased the number of myoblasts recovered after transplantation. Human myoblasts were injected into the quadriceps of *scid* mice. Two days after implantation, muscle-derived cells were analyzed by FACS after incubation with a human CD56 antibody. Myoblasts gated in R1 represent bright-CD56-positive cells. Left panel: control non-injected mice; middle panel: mice injected with human myoblasts; right panel: mice injected with human myoblasts pre-treated with RA. The number of human 'healthy' cells (A) and human FSHD cells (B) present in host quadriceps were determined based on the percentage of human cells (as determined by FACS) and the total number of cells. * $P \leq 0.05$.

(Hamade et al., 2006) and the expression of transcription factors (Pax3, Meox2, and both MyoD and Myf5) that specify muscle progenitors of chicken limb buds (Reijntjes et al., 2009). The ability of RA to regulate muscle differentiation depends on the

presence of RA receptors (Carnac et al., 1993; Froeschlé et al., 1998; Zhu et al., 2009). Therefore, RA controls specific signaling pathways that reinforce muscle determination and could explain why RA treatment enhances myogenic conversion in ES and embryonic carcinoma cells (Le May et al., 2011).

In this study we have identified a new function for RA in muscle cells: its ability to protect cells from induced oxidative stress. Our results showed that RA induced the strong expression of GPx3 (approximately eight- to tenfold increase) in human muscle cells, whereas the expression of antioxidant enzymes, such as others GPx family members, catalase GSS, Trx-1, Trxr-1 and SOD1, were marginally affected. Although we also observed a modest induction of SOD2, we were not able to detect an increase in SOD activity (data not shown). Therefore, we postulate that the antioxidant effects of RA are predominantly mediated by the induction of GPx3. This hypothesis was confirmed by gene knockdown experiments: siRNA-mediated knockdown of GPx3 in human muscle cells counteracted the anti-cytotoxic effects of RA. However, we cannot exclude the possibility that another mechanism is also involved in mediating the antioxidant effect of RA. The anti-apoptotic properties of RA have been characterized in different cell lines and tissues. RA prevented DNA damage and decreased death by greater than 50% in irradiated keratinocytes and endothelial cells (Sorg et al., 2005; Vortnikova et al., 2004). RA also plays a role in maintaining the integrity of hematopoietic cells (Oritani et al., 1992) and retinal precursors (Kholodenko et al., 2007). RA may prevent cardiac remodeling and ventricular hypertrophy by inhibiting the production of angiotensin II and ROS formation and by increasing antioxidant defenses (Choudhary et al., 2008). Different anti-apoptotic genes have been identified as retinoid responsive, such as SOD-2 in neuroblastoma and acute myeloblastic leukemia cells (Kinningham et al., 2008), B-cl2 in leukemic cells (Yin et al., 2005), Trx in airway epithelial cells (Chang et al., 2002) and GPx-2 in human breast cancer cells (Chu et al., 1999). These previous results, together with our findings indicate that RA mediates its pro-survival effects by inducing several anti-apoptotic regulatory processes in a cell-specific manner.

GPx3 is a selenoprotein and deficiency in selenium has been shown to affect skeletal muscle. Selenium is an essential trace

Table 2. Microarray analysis of the main antioxidant genes

Probe set ID	Gene name	Gene symbol	Ratio RA/CTRL	Ratio VA/CTRL
200642_at	Superoxide dismutase 1, soluble	SOD1	1.06	1.13
221477_s_at	Superoxide dismutase 2, mitochondrial	SOD2	1.52	1.66
211922_s_at	Catalase	CAT	1.59	1.57
200736_s_at	Glutathione peroxidase 1	GPX1	1.18	1.23
214091_s_at	Glutathione peroxidase 3	GPX3	12.75	18.86
201106_at	Glutathione peroxidase 4	GPX4	2.16	2.01
213170_at	Glutathione peroxidase 7	GPX7	0.84	0.92
228141_at	Glutathione peroxidase 8 (putative)	GPX8	1.03	1.05
211630_s_at	Glutathione synthetase	GSS	1.05	1.11
205770_at	Glutathione reductase	GSR	1.22	1.37
201266_at	Thioredoxin reductase 1	TXNRD1	1.69	1.75
211177_s_at	Thioredoxin reductase 2	TXNRD2	0.70	0.58
221906_at	Thioredoxin reductase 3	TXNRD3	1.08	0.86
208864_s_at	Thioredoxin	TXN	1.04	1.06
209078_s_at	Thioredoxin 2	TXN2	0.95	0.90

Total RNA was prepared from one culture of human healthy myoblasts (N042; see Table 1) treated for 72 hours with RA or VA, or left untreated.

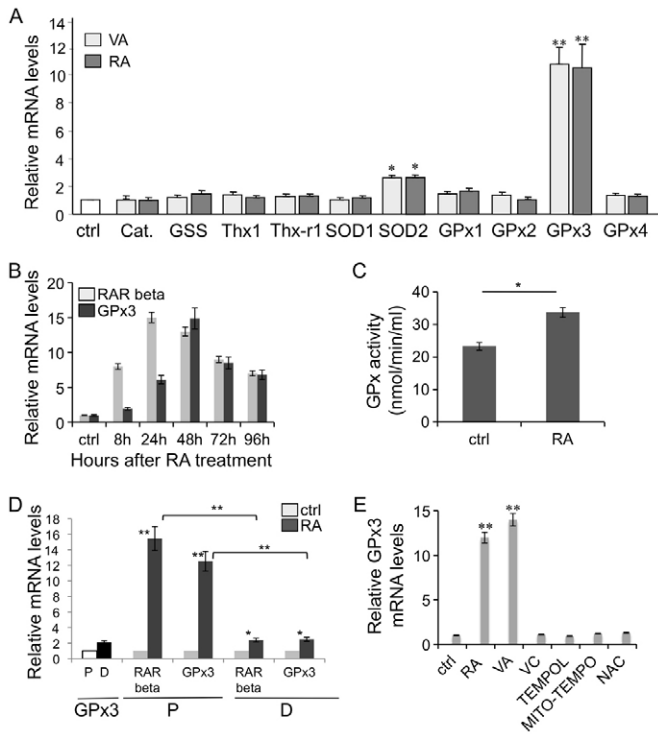


Fig. 5. GPx3 expression is enhanced by RA and VA. Human myoblasts were treated with DMSO (control) or VA (5×10^{-6} M) or RA (10^{-7} M) for 48 hours. (A) Total RNA was isolated and subjected to RT-qPCR analysis of the relative expression levels of nine primary 'antioxidant' genes, catalase (cat), glutathione synthetase (GSS), thioredoxin 1 (Thx1), thioredoxin reductase 1 (Thx-r1), superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (GPx1), glutathione peroxidase 2 (GPx2), glutathione peroxidase 3 (GPx3) and glutathione peroxidase 4 (GPx4). (B) RT-qPCR analysis of relative expression levels of RAR β and GPx3 mRNA in human myoblasts treated with 10^{-7} M RA every 24 hours for 96 hours. RAR β and GPx3 mRNA levels are expressed relative to those in untreated cells (arbitrarily set as 1). (C) Glutathione peroxidase activity was measured, with 30 μ g of protein being extracted from myoblasts that were either untreated or treated with RA. (D) Muscle cells were treated or not for 2 days with RA at myoblast (proliferative stage: P) or myotube (differentiated stage: D) stages. Total RNA were extracted and analyzed for RAR beta and GPx3 mRNA levels by RT-qPCR. GPx3 levels were determined at proliferative and differentiated stage, and expressed relative to proliferative stage (arbitrarily set as 1). For RA-treated cells, RAR beta and GPx3 mRNA levels are expressed relative to those in untreated cells (arbitrarily set as 1) either at the proliferative (P) or differentiated (D) stage. (E) Myoblasts were treated every 24 hours for 48 hours with either VA (5×10^{-6} M), RA (10^{-7} M), ascorbic acid (50×10^{-6} M), Tempol (100×10^{-6} M), Mito-Tempo (100×10^{-6} M) or NAC (5×10^{-3} M). Total RNA was isolated and subjected to RT-qPCR analysis of the relative expression levels of GPx3. * $P \leq 0.05$; ** $P \leq 0.01$.

element, known to be required and potentially toxic for human health. Selenium deprivation has been associated with different pathologies in human and livestock including the so-called 'white muscle or nutritional muscle dystrophies' characterized by fatigue, muscle weakness, impaired movement and heart problems (Rayman, 2012). Since selenium is required for the biosynthesis and activity of selenoproteins, numerous epidemiological studies investigated whether selenoprotein genes could be involved in human disease associated with selenium deficiency (Bellinger et al., 2009). To date, only

mutations in the selenoprotein N (SEPN1) have been shown to directly cause skeletal muscle disorders (Castets et al., 2012). Because we found that GPx3 could protect adult muscle precursor cells from oxidative damage, we were interested to evaluate the regenerative capacity of *Gpx3*^{-/-} mice and whether selenium deficiency is associated with impaired muscle repair and regeneration in humans.

Our study shows that the *Gpx3* gene is a target of RA and plays a role in both the anti-apoptotic signaling pathway of RA and normal muscle cell survival. In skeletal muscle, several signaling pathways can regulate GPx3 expression. GPx3 expression is very sensitive to circulating estrogen levels (Baltgalvis et al., 2010). GPx3 mediates the antioxidant effects of agonists of peroxisome-proliferator activated receptor (PPAR), a class of molecules that improves insulin sensibility in skeletal muscle and is used to treat type II diabetes. PPAR induced GPx3 expression by binding to peroxisome-proliferator response element (PPRE) in the GPx3 promoter (Chung et al., 2009). We believe that RA-mediated regulation of the GPx3 gene is indirect because the kinetics of GPx3 induction was slow, peaking at 48 hours. Furthermore, we were unable to identify putative binding sites (RARE) for RA receptors in the GPx3 promoter (unpublished observations). The molecular targets that mediate the effects of RA on GPx3 gene expression remain to be determined but may be preferentially expressed or active in immature skeletal muscle cells since the retinoic signaling pathway is more active in undifferentiated myoblasts than differentiated myotubes.

An interesting feature of the present study was the positive association between GPx3 and muscle cell viability. Recent transcriptome analysis showed that mice satellite cells express genes that confer resistance to toxic molecules (Pallafacchina et al., 2010). Among these genes, GPx3 is highly expressed in satellite cells and at lower levels in proliferative myoblasts (Pallafacchina et al., 2010) [see also additional table for transcriptomic analysis in Fukada et al. (Fukada et al., 2007)]. Interestingly, satellite cells offered a better resistance to hydrogen peroxide treatment and a higher regenerative capacity than proliferative myoblasts (Montarras et al., 2005; Pallafacchina et al., 2010). However, it remains to be determined whether GPx3 is involved in protection of satellite cells from cytotoxic damage. The capacity of GPx3 to control the level of ROS may explain the improved muscle cell viability. However, RA reduces by more than 50% the number of dead cells after a stress induced by H₂O₂, an event dependent on RA-induced GPx3 expression, but decreases by only 10% the accumulation of ROS in the same cells. Treatment of GPx3-inactivated myoblasts with the antioxidant Tempol, although effective in inhibiting the overproduction of ROS, only partially improves the survival of these cells. These results might suggest that ROS are not the only targets of GPx3. This hypothesis has been proposed in a recent manuscript describing the role of GPx3 in the activity of normal and leukemic hematopoietic stem cells (Herauld et al., 2012). In yeast, GPx-3 interacts directly with several proteins (Lee et al., 2008). Some of the functional consequences of these interactions are protection from protein inactivation and degradation, and reduction of advanced glycation endproducts (Lee et al., 2009; Lee et al., 2007). These studies suggested that Gpx3 could maintain cell homeostasis in oxidative stress condition independently of its known scavenging activity. Further studies are required to identify Gpx3 targets. We hypothesized that adhesion molecules would be one of those targets. We have

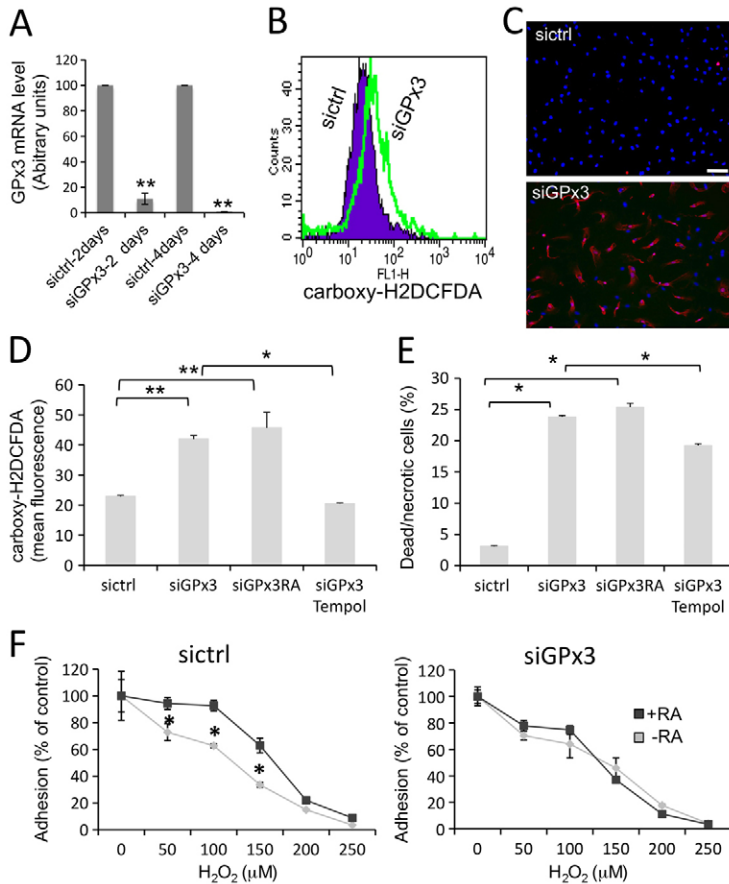


Fig. 6. Knockdown of GPx3 altered the anti-cytotoxic effects of RA. (A) Human myoblasts were transfected twice (time 0 and 48 hours later) with sictrl and siGPx3 and analyzed for GPx3 expression by RT-qPCR 48 hours and 96 hours after transfection; GPx3 mRNA levels were expressed relatively to those in sictrl myoblasts. (B) Intracellular ROS levels in sictrl (violet) and siGPx3 (green) evaluated by flow cytometry using the H2DCFDA probe. (C) sictrl and siGPx3 myoblasts were stained with ethidium homodimer (red) and Hoescht (blue), fixed and photographed. Scale bar: 50 μ m. (D) Quantification of intracellular ROS levels in sictrl and siGPx3 myoblasts treated or not with RA or Tempol for 48 hours, and evaluated by flow cytometry using the H2DCFDA probe. (E) Quantification of ethidium homodimer staining in sictrl and siGPx3 myoblasts treated or not with RA or Tempol for 48 hours evaluated by flow cytometry. (F) sictrl (left panel) and siGPx3 (right panel) myoblasts, either untreated or pre-treated with RA for 48 hours, were trypsinized and exposed to increasing concentrations of H₂O₂ for 2 hours. The myoblasts were subsequently stained with Hoescht, fixed and photographed. The percentage of adherent cells relative to the percentage of control (H₂O₂-untreated) cells (set at 100%) were quantified with Histolab software. * $P \leq 0.05$; ** $P \leq 0.01$.

started to explore this hypothesis using transcriptomic and proteomic approaches.

Myoblast transplantation is a potential treatment for degenerative muscle disease or myopathy but, unfortunately, the first clinical trials were disappointing (Skuk and Tremblay, 2011). Several studies have shown that there is a time period of 3 days after transplantation that will have a major impact on the ability of grafted cells to regenerate muscle tissue. Riederer et al. reported that implanted myoblasts started to differentiate by day 3 after transplantation, limiting their ability to proliferate and to expand into the host tissue (Riederer et al., 2012). Interestingly, if myoblasts were stimulated to proliferate *in vitro* and then transplanted, the authors observed an increase in cell number, delay of differentiation and larger area of colonization into the host tissue (Riederer et al., 2012). Thus, improving culture media and/or the recipient's microenvironment, could enhance proliferation and cell survival during this critical time window of 3 days post-implantation, allowing efficient skeletal muscle regeneration. Because autologous myoblast therapy has been proposed to treat facioscapulohumeral dystrophy (Vilquin et al., 2005), it was important to determine whether the RA signaling pathway can also protect dystrophic cells before their potential use in clinical trials. We showed in this paper that RA prevents oxidative stress attack in FSHD myoblasts. Therefore, pre-treatments with adequate levels of retinoid and growth-promoting factors could enhance muscle precursor cell expansion after transplantation and improve the efficacy of future clinical trials.

In conclusion, the results of our study support the hypothesis that GPx3 and RA play an important function in muscle stem cells survival, a crucial role that could ensure efficient adult skeletal muscle regeneration.

Materials and Methods

Chemicals and reagents

All-*trans* retinoic acid (RA), vitamin A (VA), vitamin C (VC) and N acetyl cysteine (NAC) were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Tempol and Mito-Tempo were purchased from Enzo Life Science, Villeurbanne, France, and hydrogen peroxide solution from Sigma-Aldrich. GPx activity was determined using the glutathione peroxidase assay kit (Cayman Chemical, Ann Arbor, USA).

Primary culture of human myoblasts

Quadriceps muscle biopsies were performed on five healthy adult humans (mean age 32.2 ± 6.30 years; \pm s.d.) and five facioscapulohumeral muscular dystrophy (FSHD) patients with between four and eight D4Z4 repeats (mean age 27.2 ± 6.60 years) at the Centre Hospitalier Universitaire Lapeyronie (Montpellier, France) (Table 1). Informed and written consent was obtained from all subjects after explanation of the protocol. Myoblasts were purified from muscle biopsies as previously described (Barro et al., 2010; Jean et al., 2011). Myoblasts were grown on collagen-coated dishes in growth medium consisting of Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F12; Sigma-Aldrich), 5% fetal calf serum (FCS; Thermo Scientific, Brebières, France), 0.1% ultrosor G (Pall, Saint-Germain-en-Laye, France) and 1 ng/ml basic fibroblast growth factor (bFGF) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Myogenicity was determined for each culture (percentage of desmin-positive cells versus the total number of cells). The time spent in culture was always the same for all myoblast cultures: between 10 and 15 doubling time, to avoid senescence induced by proliferation. For differentiation, confluent cells were cultured in DMEM/F12 plus 5% serum without basic FGF and ultrosor G for 3–5 days.

Apoptosis studies

Myoblasts were seeded onto 60 mm collagen-coated dishes, cultured in growth medium, and left untreated or pre-treated twice with 10^{-7} M RA, once at 24 hours and again at 48 hours, and treated with 150 μ M H_2O_2 overnight. Dead myoblasts were revealed by staining with ethidium homodimer-1 and were analyzed either by microscope analysis or by FACS. Apoptosis was assessed with JC-1, caspase 3/7 and annexin labeling as previously described (Jean et al., 2011). Staining with H2DCFDA was performed according to the manufacturer's instructions (Life Technologies, Saint Aubin, France). Flow cytometry analyses were performed using a FACSCALIBUR (Becton Dickinson, Le Pont-De-Claix, France).

Cell adhesion assay

Myoblasts were either left untreated or pre-treated twice with 10^{-7} M RA as described above. Myoblasts were subsequently trypsinized, seeded on collagen-coated dishes and stimulated with various concentrations of H_2O_2 . After 2 hours, non-adherent cells were first removed by washing with culture medium. Next, to assess the cell adhesion, the adherent cells were stained with Hoechst, a DNA intercalating dye that stains nuclei, and fixed with 4% paraformaldehyde. Five separate fields were photographed using a fluorescence microscope (Zeiss Imager M1 AX10). Cells were counted using Histolab software.

Transient transfections

siRNA transfection

GPx3 (siGPx3) and negative-control (siCtrl) Silencer Select pre-designed siRNAs were purchased from Life Technologies. Human myoblasts were transfected (using a reverse transfection protocol) with Lipofectamine RNAiMAX reagent according to the manufacturer's recommendation (Life Technologies). Cells were transfected twice (time 0 and 48 hours later) and harvested 96 hours after transfection.

Luciferase assay

Lipofectamine was used to transfect human myoblasts in accordance with the supplier's recommendations (Life Technologies) using 1 μ g of total DNA; this included 100 ng of the reporter construct (RARE β -tk-luc), 800 ng of pcDNA3 vector and 100 ng of RL-tk-luc (Promega, Charbonnières, France). To evaluate RARE β -luciferase activity, the cells were provided with additional proliferation medium at 24 hours after transfection. Treatment with VA (5×10^{-6} M) and RA (10^{-7} M) were performed at 24 hours after transfection. The cells were harvested for use in the luciferase assay at 72 hours after transfection. The activities of firefly and *Renilla* luciferase were measured sequentially using a luciferase assay kit in accordance with the supplier's recommendation (Promega). The luciferase activity (i.e. reporter activity) was measured and normalized to the pRL-tk activity.

Microarray analysis

Total RNA was prepared from one culture of human healthy myoblasts treated (or not) with RA for 3 days (N042; see Table 2; supplementary material Table S1) using a NucleoSpin RNA II system (Macherey-Nagel, Hoerd, France). A double amplification of total RNA was used to generate a suitable quantity of labeled cRNA for hybridization to GeneChip[®] Human Genome U133 Plus 2.0 arrays according to the manufacturer's protocol. Microarrays were processed in the Microarray Core Facility of the Institute for Research in Biotherapy of Montpellier (<http://irb.chu-montpellier.fr>).

RT-qPCR

Total RNA was prepared from cultured cells using a NucleoSpin RNA II system (Macherey-Nagel, Hoerd, France), and cDNA was prepared using a Verso cDNA kit (Thermo Scientific, Ilkirch, France).

The expression of various genes was analyzed by quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) with a LightCycler (Roche Diagnostics, Meylan, France) and the following procedure: 95°C for 10 seconds, 60°C for 10 seconds and 72°C for 15 seconds. Primers were designed using Light Cycler software design2 probe5 (Roche Diagnostics) and were tested for homology with other sequences in the NCBI BLAST database. We performed relative quantifications. The calculation method used was the standard curve method (for each experimental sample, the amount of target and endogenous reference RPP0 is determined from the appropriate standard curve; target and RPP0, respectively). Then, the target amount is divided by the RPP0 amount to obtain a normalized target value. One of the normalized target values in experimental control conditions (expressed in proliferative cells or control non-treated cells for example) is set as one. Each of the target values is expressed as *n*-fold differences relative to the experimental control. See supplementary material Table S2 for details of the primers used.

Western blotting

Protein extracted from human myoblasts was analyzed by western blotting as previously described (Jean et al., 2011). The following primary antibodies were used: monoclonal anti- α tubulin (Sigma-Aldrich; diluted 1/10,000, v/v); monoclonal anti-myogenin (Becton Dickinson; diluted 1/1000, v/v); polyclonal

anti-RAR alpha (Santa Cruz Biotechnology, Heidelberg, Germany; diluted 1/500, v/v); polyclonal anti-RAR beta (Santa Cruz Biotechnology; diluted 1/500; v/v); monoclonal anti-RXR beta (Euromedex, Souffelweyersheim, France; diluted 1/1000; v/v); and monoclonal anti-cyclinA (Sigma-Aldrich; diluted 1/1000; v/v). Membranes were washed in PBS and incubated with a horseradish peroxidase-conjugated anti-mouse or anti-goat antibody (GE Healthcare, Velizy-Villacoublay, France). Western blots were normalized against α -tubulin expression.

Cell transplantation

All surgical procedures were performed on animals and were approved by the Institutional Animal Care and Use Committee of the Centre Anti-Cancéreur (Val d'Aurelle-Paul Lamarque, Montpellier, France). SCID immunodeficient mice (Charles River, L'Arbresles cedex, France) were anesthetized at 5 weeks by intraperitoneal injection of 100 mg/kg ketamin hydrochloride and 10 mg/kg xylasin. Human FSHD myoblasts (1×10^5), either untreated or treated with RA, were implanted into the quadriceps muscle. Immediately after injection or 2 days after implantation, muscle-derived cells were isolated and plated for 24 hours to allow the implanted human cells and host mouse cells to recover. The cells were labeled with anti-human CD56 (Becton Dickinson) and analyzed by FACS. We derived the actual number of human cells that were injected into the quadriceps by determining the percentage of human cells (quantified by FACS) and the total number of cells.

Statistical analysis

The statistical significance of the differences was determined using Mann-Whitney tests, with *P*-values of 0.05 and *P* \leq 0.01 regarded as significant. Values are means \pm s.e.m. (*n* = 3 different cultures derived from five healthy human adults and five FSHD patients; see Table 1). For myoblast transplantation, six mice were injected.

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