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

Running title: GPx3 mediates the antioxidant effects of RA.

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Summary

Protection of satellite cells from cytotoxic damages is a critical issue 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 these cytotoxic damages *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, a gene encoding an antioxidant enzyme, as a potential retinoic acid target gene in human myoblasts. Knockdown of glutathione peroxidase 3 via siRNA 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.

Keywords: GPx3, vitamin A, retinoic acid, myoblasts, 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 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 muscles apparently

healthy has allowed to propose that myoblasts from unaffected muscles can be purified and implanted in 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. (Vauchez et al., 2009) and Vella et al. (Vella et al., 2011) have isolated ALDH^{high} muscle progenitors cells that exhibit increased stress resistance and regenerative capacity. 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, 2011). 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; Maden et al., 2000; Reijntjes et al., 2009). However, the effect of RA on oxidative stress and survival of skeletal muscle cells was not determined.

The cellular redox potential is maintained by a balanced regulation of prooxidative 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 back free radical-mediated attacks (Silva and Coutinho, 2010). GPx are 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 *in vivo* transplantation assays. In this study, we demonstrated that RA protected healthy and dystrophic (FSDH) human myoblasts from cytotoxic damage and improved cell survival in transplantation assays. In addition, we identified 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 signalling 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, *via* western blotting and RT-qPCR of proliferative (P) and differentiated human myoblasts (D) (Fig. 1A, B, 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 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 of their expressions was detected during differentiation (Fig. 1A, B, 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, 2011). 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 a 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 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, observed *via* staining with ethidium homodimer-1, a compound that only crosses the damaged membranes of dead/necrotic cells and produces bright-red fluorescent nucleic acid staining. We demonstrated that cells died from apoptosis since myoblasts presented 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 of 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 myoblast treated with H₂O₂ whereas RA reduced significantly H₂O₂-induced accumulation of ROS in human myoblasts (Fig. 2D). Therefore, we determined that RA significantly protects adherent myoblasts from H₂O₂-induced oxidative stress damage.

RA restores myoblast adhesion impaired by H₂O₂

Since H₂O₂ induced detachment of cells from the substratum and apoptosis (Grossmann, 2002; Song et al., 2010), we thus examined the effects of H₂O₂ on myoblast adhesion in 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 H_2O_2 . 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 H_2O_2 concentration led to a decrease in myoblast adhesion (Fig. 3A). 20% of the cells were already unable to adhere to the matrix at $50 \mu M H_2O_2$. At $250 \mu M H_2O_2$, only 10% of total cells remained attached to the matrix (Fig. 3B). Conversely, myoblasts that were pre-treated with RA exhibited adhesion percentages that were significantly greater than those of control (ctrl) cells at all tested doses of H_2O_2 excepted at $250 \mu M$ (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 H_2O_2 concentration led to a decrease in myoblast adhesion; in contrast, pre-treatment with RA restored the adhesion of FSHD myoblasts for all concentrations of H_2O_2 , with the exception of $250 \mu M$ (Fig. 3C). We confirmed those results by determining the concentration of H_2O_2 at which half of the cells adhere to the dish (IC50) for healthy and FSHD cells (Table 1 for myoblast characteristics). After RA treatment, the IC50 significantly increased from $156.4 \mu M H_2O_2$ (± 2.28 SEM) to $214.7 \mu M H_2O_2$ (± 1.464 SEM) ($p=0.0336$ SEM) for “healthy” cells and from $126.6 \mu M H_2O_2$ (± 9.42 SEM) to $193.3 \mu M H_2O_2$ (± 3.75 SEM) ($p=0.0437$) 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 deleterious effects of H_2O_2 -induced oxidative stress both in myoblasts derived from healthy and dystrophic (FSHD) patients.

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 increased cell survival after implantation into the muscle of an adult recipient. We assessed the survival of myoblasts during the first 48 h 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 *via* FACS. As shown in Figs 4A and 4B, a distinct population of CD56^{positive} cells was detectable by FACS (middle panel), whereas no fluorescent cells were detected in muscle-derived cells of control non-injected *scid* mice (left panel). Pre-treatment with RA significantly enhanced the number of healthy and FSHD human myoblasts recovered after transplantation (right panels) (Fig. 4A, B).

GPx3 is a RA target gene.

Our results showed that RA protects human myoblasts from H₂O₂-induced cytotoxicity when treated at least 48h 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 Table s1 in supplementary materials). 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 2-fold 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 h (Fig. 5B). This kinetic was compared to that of RAR beta mRNA, a 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 h. In contrast, GPx3 mRNA expression peaked between 24 and 48 h after RA treatment. The kinetic of GPx3 expression was consistent with those observed for the anti-cytotoxic effects of RA. We did not obtain a GPx3 signal *via* 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 retinoic acid signalling 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 three days and then treated two days with retinoic acid. As control, myoblasts were treated with RA at proliferative stage. As expected GPx3 and RAR beta mRNA levels 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 signalling 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 (Figure 5E). Myoblasts were treated for two 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).

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 interference 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, 6D). We detected changes in cell phenotype after transfection with siGPx3. We noticed that there were always fewer cells in siGPx3 condition with a significant 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, 6E). RA treatment did not prevent ROS accumulation (Fig. 6C) and cell death in siGPx3 myoblasts (Fig. 6E). In contrast, the antioxidant Tempol restored a level of ROS comparable to that of 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 can be 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 GPx3 as a potential RA target gene 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* (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; Froeschle et al., 1998; Zhu et al., 2009). Therefore, RA controls specific signalling 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 describe 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 8- to 10-fold increase) in human muscle cells, whereas the expression of antioxidant enzymes, such as others GPx family members, catalase GSS, Trx-1, Trxrd-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% for irradiated keratinocytes and endothelial cells (Sorg et al., 2005; Vorotnikova et al., 2004). RA also plays a role in maintaining the integrity of hematopoietic cells (Oritani et al., 1992), 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 antiapoptotic genes have been identified as retinoid-responsive, such as SOD-2 in neuroblastoma and acute myeloblastic leukemia cells (Kiningham 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 element, known to be essential 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 » characterised 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 selenoprotein N (SelN) gene (SEPN1 gene) have been shown to directly cause skeletal muscle disorders (Castets et al., 2012). According to our data, since GPx3 could protect adult muscle precursor cells from oxidative damages, it is therefore interesting to evaluate the regenerative capacity of GPx3 ^{-/-} mice and whether selenium deficiency is associated with impaired muscle repair and regeneration in human.

Our study shows that GPx3 is a target gene of RA and plays a role in both the anti-apoptotic signalling pathway of RA and normal muscle cell survival. In skeletal muscle, several signalling 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 signalling pathway is more active in undifferentiated myoblasts than differentiated myotubes (this manuscript).

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 expressed 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., 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 determine whether GPx3 is involved in protection of satellite cells from cytotoxic damages. 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 same cells. Treatment with the antioxidant Tempol in GPx3-inactivated myoblasts, 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 (Herault et al., 2012). In yeast, GPx-3 interacts directly with several proteins (Lee et al., 2008). Some functional consequences of these interactions are known as 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 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 three 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. 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 colonisation into the host tissue (Riederer et al., 2011). Thus, improving culture media and/or recipient's microenvironment, could enhance proliferation and cell survival during this critical time window of three 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 signalling pathway can also protect dystrophic cells before their potential use in clinical trials. We showed in this manuscript 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 cells 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 critical role that could ensure efficient adult skeletal muscle regeneration.

Material and Methods

Chemicals and reagents

All-trans retinoic acid (RA), vitamin A (VA), vitamin C (VC), 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. Hydrogen peroxide solution, Sigma Aldrich, St Quentin Fallavier, France. 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 adults (mean age 32.2 +/- 6.30 S.D.) and five facioscapulohumeral muscular dystrophy (FSHD; between 4 to 8 D4Z4 repeats; mean age 27.2 +/- 6.60 S.D.) patients at the Centre Hospitalier Universitaire Lapeyronie (Montpellier, France) (Table). 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 Medium nutrient mixture F-12 HAM (DMEM/F12; Sigma-Aldrich, St Quentin Fallavier, France), 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 (β FGF) 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 is 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 to 5 days.

Apoptosis studies

Myoblasts were seeded onto 60 mm collagen-coated dishes, cultured in growth medium, untreated or pre-treated twice with 10^{-7} M RA for 48 hours (each 24 hours), and treated with 150 μ M H_2O_2 overnight. Dead myoblasts were revealed *via* staining with ethidium homodimer-1 and were analyzed by either by microscope analysis or by Fluorescence Activated Cell Sorting (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 for 48 hours (each 24 hours). 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). Cell counting was performed 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, Saint Aubin, France). 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, Saint Aubin, France) using 1 µg of total DNA; this included 100 ng of the reporter construct (RAREβ-tk-luc), 800 ng of pcDNA3 vector and 100 ng of RL-tk-luc (Promega, Charbonnières, France). To evaluate RAREβ-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 h 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, Charbonnières, France). 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 three days (N042; see Table 2 and Table s1 in supplementary material) 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, Hoerdtt, France), and cDNA was prepared using a Verso cDNA kit (Thermo Scientific, Ilkirch, France).

The expression of various genes was analyzed via reverse transcription polymerase chain reaction (RT-qPCR) with a LightCycler (Roche Diagnostics, Meylan, France) and the following procedure: 95° C for 10 sec, 60° C for 10 sec and 72° C for 15 sec. Primers were designed using Light Cycler software design2 probe5 (Roche Diagnostics, Meylan, France) and were tested for homology with other sequences in the NCBI BLAST database. We performed relative quantification. 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 normalised target values in experimental control conditions (expressed in proliferative cells or control non-treated cells for example) is set up at one. Each of the target values is expressed as n-fold differences relative to the experimental control.

gene	forward	reverse
catalase	CTGACTACGGGAGCCAC	TGATGAGCGGGTTACACG
GSS	AAGACACTCGTGATGAACAAG	AGAGGAATGACAAATACAGAGGAT
SOD1	CCTCTATCCAGAAAAACACGG	CCAAACGACTTCCAGCG
SOD2	GGAGATGTTACAGCCCAGATAG	CAAAGGAACCAAAGTCACG
Thioredoxin reductase 1	TGTTGGAGCATCCTATGTCG	TCAAATCCTCTAAGAAGAATGGACC

Thioredoxin	GCCTTTCTTTTCATTCCCTCTC	GCTTTTCCTTATTGGCTCCAG
GPx1	GCAACCAGTTTGGGCATC	CTTGGGGTTCGGTCATAAGC
GPx2	ACAGTCTCAAGTATGTCCGT	TGTCCTTCAGGTAGGCGA
GPx3	CGGGGACAAGAGAAGTCG	CCCAGAATGACCAGACCG
GPx4	CTTCACCAAGTTCTCATCG	GGGCAGGTCTTCTCTATC
RAR alpha	GCTGGGCAAATACACTACGA	TGGCGAACTCCACAGTC
RAR beta	GATGTAAGGGCTTTTCCGC	TCATTCTGACAGATTCTTTGGAC
RXR alpha	CGCTGAGGGAGAAGGTC	GAAGAGATGTTCCAGGCATTTGA
myogenin	ACCCCGCTTCTATGATGG	ACACCGACTTCCTCTTACACA
RPLP0	TCATCCAGCAGGTGTTCG	AGCAAGTGGGAAGGTGTAA

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, St Quentin Fallavier, France; diluted 1/10000 (v/v)); monoclonal anti-myogenin (Becton Dickinson, Le pont de Claix, France; 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, Heidelberg, Germany; diluted 1/500 (v/v)); monoclonal anti-RXR beta (Euromedex, Souffelweyersheim, France; diluted 1/1000 (v/v)); and monoclonal anti-cyclinA (Sigma-Aldrich, St Quentin Fallavier, France; 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 blot were normalised 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-Cancéreux (Val d'Aurelle-Paul

Lamarque, Montpellier, France). *SCID* immunodeficient mice (Charles River, L'arbresle cedex, France) were anesthetized at 5 weeks *via* 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 two 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, Le Pont-De-Claix, France) and analyzed *via* 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. * $P \leq 0.05$, ** $P \leq 0.01$ mean values $\pm$ SEM ($n = 3$ different cultures derived from 5 healthy human adults and 5 FSHD patients; see Table1). For myoblast transplantation, $n = 6$ mice injected.

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Figure legends

Fig. 1. RA receptors are expressed and transcriptionally active in human myoblasts

(A)(B)(C) Human myoblasts were harvested at proliferative myoblast (P) and differentiated myotube (D) stages and were analysed for myogenin, cyclin A, RAR alpha, RAR beta and RXR alpha (A) at protein levels *via* western blot analysis, quantified and quoted in (B); and (C) at mRNA levels via quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). Levels of protein and mRNA were expressed relative to those in proliferative myoblasts arbitrarily set as 1. (D) Data plotted onto the graph represent 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).

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. Those cells were quantified and expressed as the percentage of dead cells (Lower panels). (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. Data represent the percentage of JC-1 green-positive cells (lower panel). (C) (Upper panels) Example of cytometric analysis of human myoblasts treated with H₂O₂ or H₂O₂ + RA and stained for

caspase 3/7 activity. Data represent the percentage of caspase 3/7-positive cells gated in R1 (lower panel). (D) Data plotted onto the graph represent quantification of intracellular ROS levels in H_2O_2 or H_2O_2 + RA treated myoblasts evaluated by flow cytometry using H2DCFDA probe.

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 H_2O_2 for 2 hours. Human myoblasts were subsequently stained with Hoescht, fixed and photographed. (B) Cell pictures (5 fields for each condition) were quantified using Histolab software. Data plotted onto the graph represent the percentage of adherent cells relative to the percentage of control (H_2O_2 -untreated) cells fixed at 100 (left axis). (C) Human “FSHD” myoblasts, either untreated or pre-treated with RA for 48 hours, were trypsinized and exposed to increasing concentrations of H_2O_2 for 2 hours before being stained with Hoescht, fixed and photographed. Cell pictures were quantified using Histolab software. Data plotted onto the graph represent the percentage of adherent cells relative to the percentage of control (H_2O_2 -untreated) cells fixed at 100 (left axis). Bar = 50 μ M.

Fig. 4. RA increased the number of myoblasts recovered after transplantation

We injected human myoblasts 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. We determined the number of human “healthy” cells

(A) and human FSHD cells (B) present in host quadriceps based on the percentage of human cells (as determined by FACS) and the total number of cells.

Fig. 5. GPx3 expression is enhanced by RA and VA

Human myoblasts 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 quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) analysis of the relative expression levels of 9 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 each 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 two days with RA at myoblast (proliferative stage: P) or myotube (differentiated stage: D) stages. Total RNA were extracted and analysed 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). In 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 each 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) and NAC (5×10^{-3} M). Total RNA was isolated and subjected to RT-qPCR analysis of the relative expression levels of GPx3.

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 analysed 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 H2DCFDA probe. (C) siCtrl and siGPx3 myoblasts were stained with ethidium homodimer (red) and Hoechst (blue), fixed and photographed. Bar: 50µM. (D) Quantification of intracellular ROS levels in siCtrl and siGPx3 treated or not with RA or Tempol for 48 hours, evaluated by flow cytometry using H2DCFDA probe. (E) Quantification of ethidium homodimer staining in siCtrl and siGPx3 treated or not with RA or Tempol for 48hours 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 Hoechst, fixed and photographed. Cell pictures were quantified with Histolab software. Data plotted onto the graph represent the percentage of adherent cells relative to the percentage of control (H₂O₂-untreated) cells fixed at 100.

Table legends

Table 1: Human myoblast characteristics.

Recapitulative table of the data about healthy patients and patients with FSHD: name of the cell line, age and sex of the patient (M: male; F: female), site of the muscle biopsy, number of D4Z4 units and myogenicity of the cell line (percentage of desmin positive cells versus the total number of cells).

Table 2: Microarray analysis of main antioxidant genes.

Total RNA was prepared from one culture of human healthy myoblasts (N042; see table 1) treated or not for 72 hours with RA or VA. Microarrays were processed in the Microarray Core Facility of the Institute for Research in Biotherapy of Montpellier (<http://irb.chu-montpellier.fr>).

Figure 1

Figure 2

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Figure 3

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Figure 4

Figure 5

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Figure 6

	Age (years)	Sex	Muscle	D4Z4 units	Myogenicity (%)
Controles					
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

Table 1

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Probe Set ID	Gene Title	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

Table 2

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