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Effects of omega-3 on matrix metalloproteinase-9, myoblast transplantation and satellite cell activation in dystrophin-deficient muscle fibers

Samara Camaçari de Carvalho[1] · Sajedah M. Hindi[2] · Ashok Kumar[2] · Maria Julia Marques[1]

Abstract In Duchenne muscular dystrophy (DMD), lack of dystrophin leads to progressive muscle degeneration, with DMD patients suffering from cardiorespiratory failure. Cell therapy is an alternative to life-long corticoid therapy. Satellite cells, the stem cells of skeletal muscles, do not completely compensate for the muscle damage in dystrophic muscles. Elevated levels of proinflammatory and profibrotic factors, such as metalloproteinase 9 (MMP-9), impair muscle regeneration, leading to extensive fibrosis and poor results with myoblast transplantation therapies. Omega-3 is an anti-inflammatory drug that protects against muscle degeneration in the *mdx* mouse model of DMD. In the present study, we test our hypothesis that omega-3 affects MMP-9 and thereby benefits muscle regeneration and myoblast transplantation in the *mdx* mouse. We observe that omega-3 reduces MMP-9 gene expression and improves myoblast engraftment, satellite cell activation, and muscle regeneration by mechanisms involving, at least in part, the regulation of macrophages, as shown here with the fluorescence-activated cell sorting technique. The present study demonstrates the benefits of omega-3 on satellite cell survival and muscle regeneration, further supporting its use in clinical trials and cell therapies in DMD.

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✉ Maria Julia Marques
marques@unicamp.br

1 Department of Structural and Functional Biology, Institute of Biology, University of Campinas, UNICAMP, Campinas, SP CEP 1083-970, Brazil

2 Department of Anatomical Sciences and Neurobiology, University of Louisville School of Medicine, Louisville, KY, USA

Introduction

Duchenne muscular dystrophy (DMD), a fatal genetic muscle disease, is one of the most severe and common forms of muscular dystrophy in childhood (Mendell et al. 2012). It is estimated that 1 in 3500–6000 live-born males have DMD mutations (Mendell et al. 2012). In DMD patients and in the *mdx* mouse model of DMD (Bulfield et al. 1984), mutations in the DMD gene lead to the absence of dystrophin (Bulfield et al. 1984; Davies 1997). Myofibers lacking dystrophin display muscle damage, inflammation and chronic muscle degeneration and regeneration (Pastoret and Sebille 1995). Cycles of degeneration and regeneration lead to the exhaustion of the satellite cell (SC) pool and defects in muscular regeneration (Morgan and Zammit 2010). When SCs are exhausted, damaged muscle fibers are replaced by fibrotic and fatty tissues, compromising normal muscle function (Morgan and Zammit 2010).

No effective treatment is available for muscular dystrophy (Leung and Wagner 2013) and patients die from respiratory or cardiac failure in the second or third decade of life (Emery 2002). The only intervention shown to retard the disease progression is corticosteroid treatment but with the potentially limiting side effects of weight gain, loss of bone density and behavioral problems, such as hyperactivity with long-term use (McDonald et al. 2013; Ricotti et al. 2013). Therefore, new pharmacological, cell and genetic therapies are being extensively sought for DMD (Negroni et al. 2011; Fairclough et al. 2013).

Cell transplantation is a promising therapeutic approach for DMD (Mendell et al. 1995; Negroni et al. 2011; Péault et al. 2007). Ideally, normal donor myoblasts fuse with host myoblasts and myofibers and restore dystrophin expression, as

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demonstrated in *mdx* mice (Partridge et al. 1989; Collins et al. 2007; Sacco et al. 2008). However, extensive studies have demonstrated several limitations to the success of this therapy, such as the limited migratory capacity of the transplanted cells and the lack of immune suppression leading to graft rejection (Bentzinger et al. 2010; Negroni et al. 2011). Nevertheless, stem-cell-based therapeutic strategies are under intense investigation and the functional enhancement of endogenous muscle stem cells is one of the approaches with regard to cell-based therapy (Bentzinger et al. 2010; Quattrocelli et al. 2010).

Metalloproteinases (MMP) are elevated in *mdx* mice and associated with muscle injury and the exacerbation of myopathy (Li et al. 2009; Kumar et al. 2010; Alameddine 2012). Reduced levels of MMP-9 ameliorate dystrophic skeletal muscle structure (Li et al. 2009) and improve muscle regeneration, as demonstrated in the mdx;Mmp-9$^{+/-}$ mouse (Kumar et al. 2010). Genetic inhibition of MMP-9 facilitates exogenous myoblast engraftment and improves satellite cell activation and muscle regeneration with the involvement of the Notch and Wnt signaling pathways (Brack et al. 2008; Kitamoto and Hanaoka 2010; Wen et al. 2012), which are altered in DMD patients and in the *mdx* mouse model of DMD (Pescatori et al. 2007; von Maltzahn et al. 2012).

We have previously demonstrated that treatment with the polyunsaturated fatty acid omega-3 reduces inflammation and myonecrosis in *mdx* mice, at earlier and later stages of the disease (Carvalho et al. 2013; Fogagnolo Mauricio et al. 2013; Apolinário et al. 2015). Furthermore, omega-3 increases M2 macrophages (Carvalho et al. 2013) and improves muscle regeneration, possibly by increasing interleukin 10 (IL-10) levels (Carvalho et al. 2013). Omega-3 has been shown to possess anti-inflammatory activity in many diseases (Kuroki et al. 1997; Yu and Bjorksten 1998; Oddy et al. 2004; Zamaria 2004; Lai et al. 2006; Ordovas 2006) and some reports have demonstrated that omega-3 treatment decreases the levels of MMP in several tissues (Derosa et al. 2012; Kavazos et al. 2015).

In the present study, we test our hypothesis that omega-3 can modulate MMP-9 levels in the dystrophic muscle and thereby improve myoblast transplantation in the quadriceps muscle of *mdx* mice and satellite cell activation, similar to the reports of studies of mdx;Mmp-9 heterozygous mice (Hindi et al. 2013).

Materials and methods

Animals

Male and female *mdx* (strain: C57BL/10 ScSnDMDmdx), Mmp9-knockout (strain: FVB.Cg-Mmp9tm1Tvu/J) and mT/mG (strain: Gt(ROSA)26Sortm4(ACTB-tdTomato,-

EGFP)Luo/ J) mice were obtained from Jackson Laboratory (Bar Harbor, Me., USA). Mmp9-knockout mice were first crossed with C57BL10/ScSn mice for seven generations and then with *mdx* mice to generate littermate wild-type, mdx;Mmp9 $^{+/+}$ and mdx;Mmp9 $^{+/-}$ mice as previously described (Li et al. 2009). Polymerase chain reaction (PCR) analysis from tail DNA determined all genotypes. Mice were housed in the animal facility of the University of Louisville under conventional conditions with constant temperature and humidity and were fed a standard diet. All experiments with animals were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Experimental design

Male and female *mdx* mice ($n = 16$; 14 days of age, mdx;O-3) received, 3 times a week by gavage, omega-3 oil capsules (FDC Vitamins, Inc., Miami, FL, USA; Omega-3 EPA 1000 mg; Fogagnolo Mauricio et al. 2013) containing 0.4 g EPA, 0.2 g DHA, 2 mg vitamin E, 0.9 g proteins, 2.0 g total fat, 0.4 g saturated fat, 0.0 g trans fat, 0.0 g monosaturated fat and 1.0 g polyunsaturated fat, at a dose of 300 mg/kg body weight, for 16 days as previously reported (Machado et al. 2011; Carvalho et al. 2013). Untreated *mdx* and *mdx*;Mmp9 mice ($n = 16$; 14 days of age) received an equivalent amount of mineral oil (liquid petrolatum for oral human use; Nujol, Mantecorp, Sao Paulo, Brazil) by gavage for 16 days, as previously described (Carvalho et al. 2013; Fogagnolo Mauricio et al. 2013; Fig. 1). The quadriceps femoris (QDR) muscle was studied, since this muscle demonstrated a higher capacity of regeneration (Carvalho et al. 2013; Apolinário et al. 2015). Omega-3 therapy did not alter body mass (untreated *mdx*: 19.7 ± 2.4 g; *mdx*;O3: 20.8 ± 3.0 g; *mdx*;Mmp9: 22.8 ± 2.6 g; $P < 0.05$, analysis of variance [ANOVA]) or quadriceps weight (normalized to body mass: untreated *mdx*: 6.26 ± 0.8 mg; *mdx*;O3: 6.6 ± 0.2 mg; *mdx*;Mmp9: 6.0 ± 0.3 mg; $P < 0.05$, ANOVA).
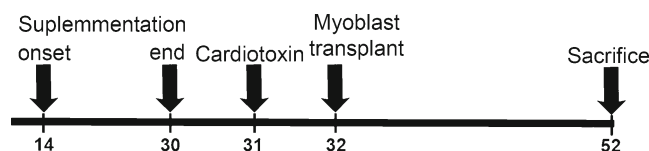

Fig. 1 Experimental timeline. Supplementation with omega-3 (mdx;O3 mice) or with Nujol (untreated *mdx* and mdx;Mmp9$^{+/-}$ mice) started at 14 days of age and ended at 30 days of age (16 days of supplementation). Injury with cardiotoxin (31 days of age) and myoblast transplant (32 days of age) were performed in the right quadriceps muscle of all mice (untreated *mdx*, mdx;Mmp9$^{+/-}$ and mdx;O3 mice). Quadriceps muscles were harvested at 20 days post-myoblast transplantation (52 days of age)

Primary myoblast cultures

Primary myoblasts were isolated from the hind limb muscles of mTmG mice that expressed membrane-targeted tandem dimer Tomato (mT, a red fluorescent protein) and were cultured following a similar protocol as that previously described (Hindi et al. 2013). Briefly, hind limb skeletal muscles from mice were aseptically isolated, minced into a coarse slurry and enzymatically digested at 37 °C for 1 h by adding 400 IU/ml collagenase II (Worthington, cat. no. LS004196). Digested slurry was serially filtered through a 70-µm and then a 30-µm filter and then spun and isolated cells were resuspended and seeded in F-10 medium (containing 20% fetal bovine serum [FBS] and supplemented with 10 ng/ml basic fibroblast growth factor) on culture dishes coated with 10% matrigel (BD Biosciences). After 3 days, cells were pre-plated to eliminate fibroblasts after which time cells were expanded in culture and utilized for transplantation experiments.

Myoblast transplantation into quadriceps femoris

Muscle regeneration in untreated and omega-3-treated *mdx* mice and mdx;Mmp9 $^{+/-}$ mice was induced by injecting cardiotoxin (50 µl of a 1.2% $BaCl_2$ solution, Sigma) into the mid-belly of the right quadriceps femoris (QDR) muscles ($n = 4$–6; mice at 31 days of age) at 24 h before cell transplantation. Approximately 10×10^5 mTmG myoblasts were transplanted into the mid-belly of CTX-injected QDR muscle (mice at 32 days of age). Muscles were harvested at 20 days post-myoblast transplantation and analyzed for mT fluorescence and dystrophin expression (Fig. 1).

Immunohistochemistry and hematoxylin-eosin staining

Right quadriceps muscles (following myoblast transplantation) were removed ($n = 4$–6), frozen in isopentane cooled in liquid nitrogen and sectioned in a microtome cryostat. Muscle sections were blocked in 1% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h and incubated with anti-embryonic myosin heavy chain (eMyHC; 1:150; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, USA) or anti-dystrophin (1:50; Abcam) in blocking solution at 4 °C overnight under humidified conditions. The sections were washed briefly with PBS before incubation with Alexa-Fluor-H-488- or -594-conjugated secondary antibody (1:3000; Invitrogen) for 1 h at room temperature and then washed 3 times for 5 min with PBS. The slides were mounted in fluorescence medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and examined at room temperature on a Nikon Eclipse TE 2000-U microscope (Nikon) equipped with a digital camera

(Nikon Digital Sight DS-Fi1) and Nikon NIS Elements BR 3.00 software (Nikon).

Left quadriceps muscles were removed ($n = 4$–6) and cryostat cross-sections were stained with hematoxylin and eosin to quantify myofiber area. Slides were examined with a Nikon Eclipse TE 2000-U microscope (Nikon) connected to a digital camera (Nikon Digital Sight DS-Fi1) and a personal computer. The area of 400 myofibers/muscle was measured with Image J software. Necrotic fibers were identified by immunostaining with Cy3-labeled goat anti-mouse IgG (1:3000; Invitrogen).

RNA isolation and quantitative real-time PCR

RNA isolation and quantitative real-time PCR (QRT-PCR) were performed by using the method previously described (Paul et al. 2012). The left QDR muscles ($n = 4$–6) were used and samples were run in triplicate. Briefly, cDNA was generated from total RNA by the ImPromega-II Reverse Transcription System from Promega. The first-strand cDNA reaction (0.5 ml) was subjected to real-time PCR amplification by using gene-specific primers (Electronic Supplementary Material, Table S1).

Fluorescence-activated cell sorting

Activated satellite cells and M1 and M2 macrophages were analyzed by fluorescence-activated cell sorting (FACS) as previously described (Hindi et al. 2012). Left QDR muscle cells were incubated in DMEM (Dulbecco's modified Eagle Medium) supplemented with 2% FBS and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and any dead cells (positive for propidium iodide staining: approximately 0.1%) were excluded from all FACS analyses. For satellite cell identification and quantification by FACS from a heterogeneous cell population, cells were immunostained with antibodies against CD45, CD31 and Ter-119 for negative selection (all phycoerythrin [PE]-conjugated, eBiosciences) and with α7-integrin (MBL International) for positive selection. An Alexa-Fluor-488-conjugated antibody (Invitrogen) was used as a secondary antibody against α7-integrin.

Macrophages were quantified from heterogeneous cell population by selection of F4/80 $^+$ (PerCP Cy5.5-conjugated, eBiosciences) cells against negative selection by CD56/Sca-1 and Ter-119 (all PE-conjugated, eBiosciences). From the F4/80 $^+$ cells, we isolated CD11c $^+$ (allophycocyanin-conjugated, eBiosciences) M1 and CD206 $^+$ (fluorescein-isothiocyanate-conjugated, Biolegend) M2 macrophages. FACS analysis was performed on a C6 Accuri cytometer equipped with three lasers. The output data were processed and plots were prepared by using FCS Express 4 RUO software (De Novo Software).

Statistical analysis

Results are expressed as means ± standard deviation (SD). Statistical analysis was performed by using Student's *t*-test (two tailed) to compare quantitative data populations with normal distribution and equal variance. A value of $P < 0.05$ was considered statistically significant, unless otherwise specified.

Results

Omega-3 treatment reduces MMP-9, myonecrosis and markers of muscle atrophy in *mdx* mice

We first examined whether omega-3 treatment could reduce MMP-9 gene expression in the quadriceps muscle at an early stage (52 days of age) of the disease. We observed that omega-3 significantly decreased MMP-9 gene expression (Fig. 2a; 35% decrease) in the dystrophic quadriceps muscle. A trend towards a decrease in the gene expression of the transforming growth factor-beta (TGF-β1), a marker of fibrosis, was noted in the omega-3-treated *mdx* mice but this was not statistically significant from untreated mice (Fig. 2b). The same result was observed in the *mdx*-MMP-9$^{+/-}$ mouse (Fig. 2b). To asses overall structural integrity, we evaluated sarcolemma injury and quadriceps muscle atrophy. Sarcolemma injury, an indicator of muscle necrosis, was analyzed by performing IgG staining (Fig. 3a–c). The number of IgG-filled fibers was reduced by about 41% by omega-3, similar to the reduction observed in the *mdx*-MMP-9$^{+/-}$ mouse (Fig. 3d). Muscle atrophy was analyzed by measuring myofibers sizes (Fig. 3h) and the mRNA levels of Atrogin1 (Fig. 3i) and MUSA1 (Fig. 3j). A trend was noted for omega-3 to increase fiber area (Fig. 3h) and to reduce the transcript levels of Atrogin-1 and

MUSA1 (Atrogin: 25% reduction; MUSA1: 21% reduction) but these effects were not statistically significant. Our results suggest that omega-3 treatment reduces MMP-9 gene expression. The inhibition of MMP-9 by omega-3 ameliorated the overall conditions of dystrophic quadriceps muscles, comparable with the conditions observed in the *mdx*-MMP-9 heterozygous mouse (demonstrated by a decrease in injured fibers and protection against atrophy).

Omega-3 improves myoblast engraftment in dystrophic quadriceps muscle of *mdx* mice

Since MMP-9 inhibition improved myoblast engraftment in the tibialis anterior of the MMP-9$^{+/-}$ mouse (Hindi et al. 2013), we were interested to determine whether the suppression of MMP-9 by omega-3 could also affect myoblast engraftment in the quadriceps muscle of *mdx* mice. In *mdx* mice, engrafted myoblasts were grouped closely together at the site of the injection, forming clusters of mTmG-positive myoblasts (Fig. 4a). In omega-3-treated and in *mdx*-MMP-9 heterozygous mice, engrafted myoblasts were also grouped together at the site of injection but some cells could be seen extending beyond the transplantation site, compared with untreated *mdx* mice in which myoblast grafts were restricted to the site of injection (Fig. 4a–c). In the transplanted quadriceps of omega-3-treated mice, mT/mG fibers were significantly increased (four-fold increase) when compared with untreated *mdx* mice (Fig. 4g); a similar result was obtained in mdx;Mmp9$^{+/-}$ mice (Fig. 4g). An apparent increase in dystrophin-positive fibers was also evident in omega-3-treated muscles (Fig. 4d–f). These results suggest that omega-3 provides a suitable muscle environment to support myoblast engraftment and dystrophin expression in the quadriceps muscle.
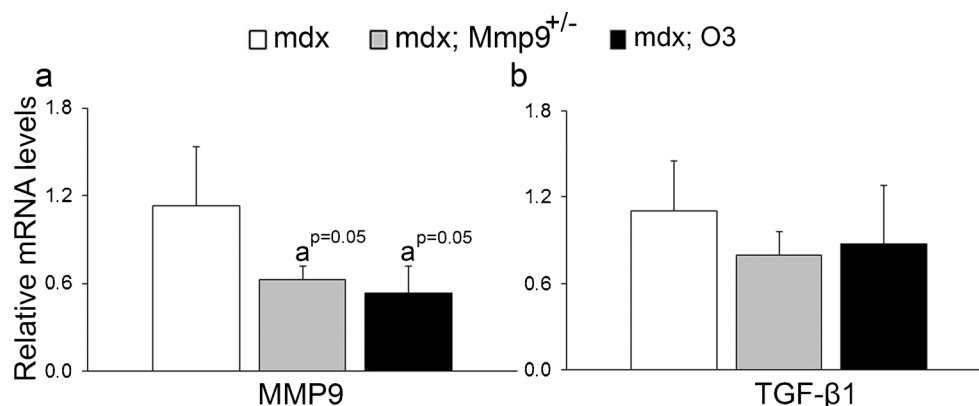

Fig. 2 Omega-3 decreased MMP-9 gene expression in muscle cells. Untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous (*mdx;Mmp9*$^{+/-}$) and omega-3-treated *mdx* (*mdx;O-3*) mice of 52 days of age. **a** Relative mRNA levels of metalloproteinase 9 (*MMP-9*). **b** Transforming growth factor beta (*TGF-β1*) measured by quantitative real-time polymerase chain reaction (QRT-PCR). *Error bars* represent SD. Student's *t*-test; $p < 0.05$, values significantly different from untreated *mdx* mice (*a*); $n = 5$ in each group
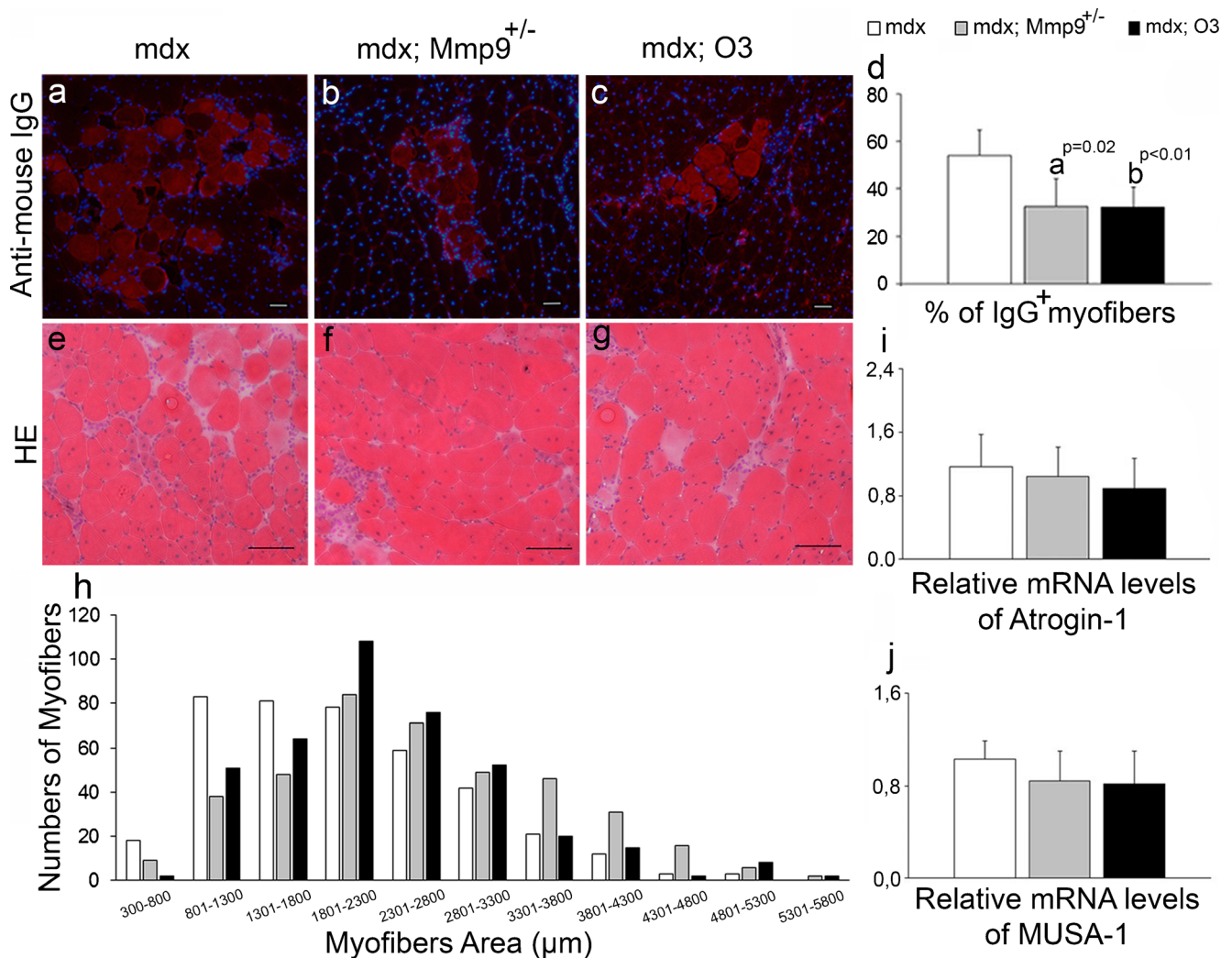
Fig. 3 Omega-3 reduced myonecrosis and tended to decrease markers of atrophy. Muscle sections of quadriceps femoris of 52-day-old untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous (*mdx;Mmp9*$^{+/-}$) and omega-3-treated *mdx* (*mdx;O-3*) mice. **a–c** Muscle sections immunostained with Cy3-labeled goat anti-mouse IgG and 4,6-diamidino-2-phenylindole (DAPI). *Bars* 30 μm. **d** Quantification (%) of IgG-positive fibers. **e–g** Muscle sections stained with hematoxylin and eosin (*HE*). *Bars* 100 μm. **h** Numbers of myofibers per area (μm); 400 myofibers/muscle were measured. **i**, **j** Relative mRNA levels of Atrogin-1 (**i**) and MUSA-1 (**j**) measured by QRT-PCR assay. $n = 4$ or 6 in each group. *Error bars* represent SD. Student's *t*-test; $P < 0.05$, values significantly different from untreated *mdx* mice (*a*, *b*)

Omega-3 increased satellite cells activation and M2 macrophages in dystrophic quadriceps

Previously, we demonstrated that omega-3 treatment improved quadriceps regeneration in *mdx* mice by increasing the number of centronucleated fibers and MyoD levels (Apolinário et al. 2015). Here, we observed a three-fold increase in the number of embryonic myosin heavy chain (eMyHC)-positive fibers (Fig. 5g). We next examined whether the omega-3 effects on muscle regeneration were related to satellite cell activation. The number of activated satellite cells quantified by the FACS technique (Burkin and Kaufman 1999; Dahiya et al. 2011; Hindi et al. 2012; Paul et al. 2012) was higher in the mdx;O-3 muscle (13.8% increase in relation to untreated mice) and in the mdx;Mmp9$^{+/-}$ (17.9% increase

compared with untreated mice; Fig. 6g) compared with untreated *mdx* mice.

On the basis that macrophages play important roles in fiber degeneration and regeneration (Villalta et al. 2011; Deng et al. 2012), we examined the relative amounts of M1 by the FACS technique. The amount of M1 macrophages (CD11c$^+$) detected by the FACS technique was significantly higher in the quadriceps muscle of untreated *mdx* mice compared with mdx;O-3 treated and mdx;Mmp9$^{+/-}$ mice (Fig. 7d). M2 macrophages (CD206$^+$) were significantly elevated in the dystrophic muscle of mdx;O-3-treated (58%) and mdx;Mmp9$^{+/-}$ (52%) compared with untreated mdx mice (Fig. 7h). Taken together, these results demonstrate that omega-3 improves muscle regeneration by activating satellite cells, increasing the amount of pro-regenerative M2 macrophages and
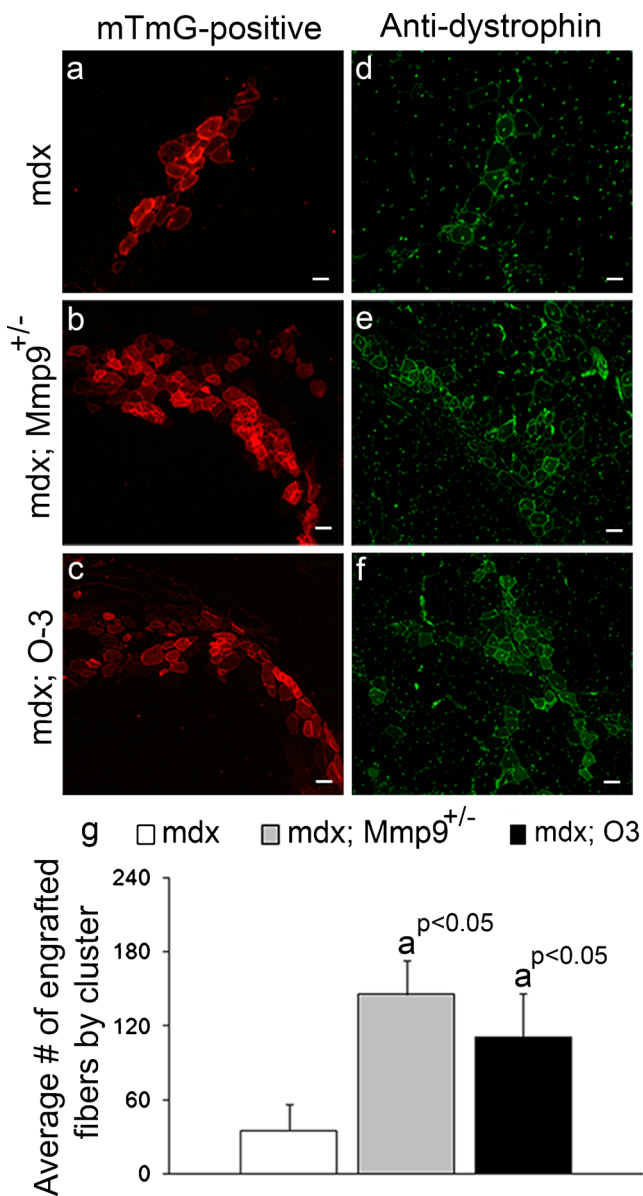
mTmG-positive Anti-dystrophin

anti-eMyHC Merged

Fig. 5 Omega-3 improved muscle regeneration. Muscle sections of the right quadriceps femoris injected with cardiotoxin at 52-days of age from untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous (*mdx;Mmp9*^{+/−}), and omega-3-treated *mdx* (*mdx;O-3*) mice were immunostained for embryonic myosin heavy chain (*eMyHC*) antibody (**a–c**). Nuclei stained with DAPI (**d–f**). Images were merged by using Nikon NIS Elements BR 3.00 software. *Bars* 30 μm. **g** Number of eMyHC-positive fibers per field. *n* = 6 in each group. *Error bars* represent SD. Student's *t*-test; *P* < 0.05, values significantly different from untreated *mdx* mice (*a*)

Fig. 4 Omega-3 improved muscle engraftment. Muscle sections of the quadriceps femoris injected with cardiotoxin of 52-day-old untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous (*mdx;Mmp9*^{+/−}) and omega-3-treated *mdx* (*mdx;O-3*) mice show mT protein fluorescence (**a–c**) and dystrophin expression (**d–f**) following transplantation. *Bar* 30 μm. **g** Average number of engrafted cells per cluster. *n* = 6 in each group. *Error bars* represent SD. Student's *t*-test; *P* < 0.05 values significantly different from untreated *mdx* mice (*a*)

decreasing the amount of M1 macrophages in *mdx* quadriceps muscle.

Omega-3 treatment affects Notch and Wnt signaling in skeletal muscle of mdx mice

Notch and Wnt pathways are required for satellite cell activation and differentiation and have been investigated in stem cells (Brack et al. 2008; Kitamoto and Hanaoka 2010; Wen
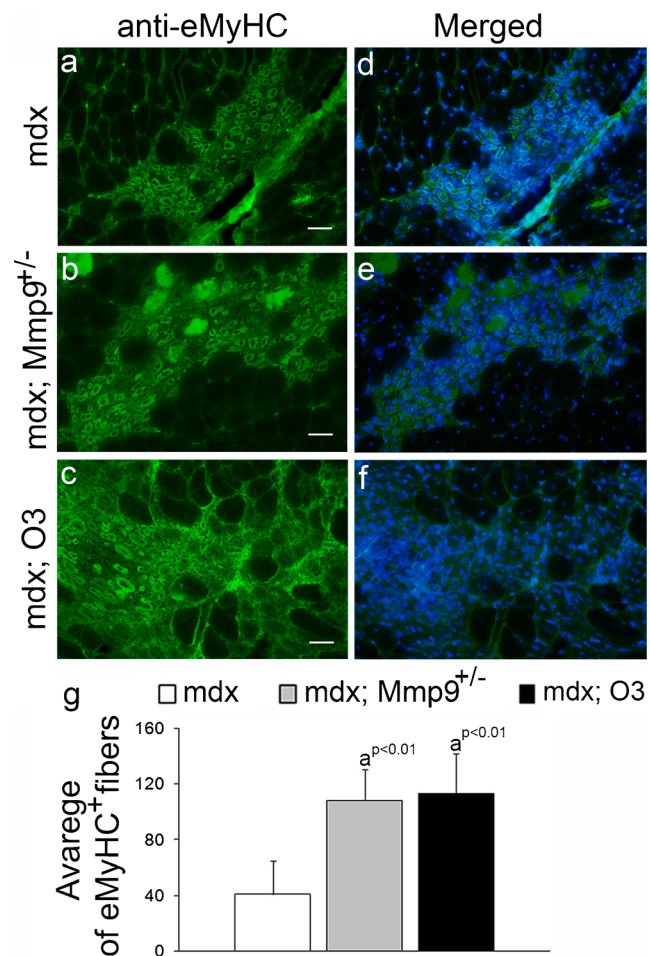
et al. 2012) and muscle fibers (Hindi et al. 2013; Church et al. 2014). To study Notch signaling, we quantified the transcript levels of Notch ligands (Jagged1, DLL1, and 4), receptors (1, 2, and 3), intracellular domain (NICD) and Hes1 (a direct transcriptional target of Notch signaling; Dhanesh et al. 2016). The transcript levels of Notch1, Notch 3, NICD and Hes1 were significantly increased and Jagged1 was significantly decreased in the omega-3-treated and *mdx*-MMP-9 heterozygous mice (Fig. 8a–c). DLL1 and DLL4 levels were not changed by omega-3 (Fig. 8b). Transcript levels of the canonical Wnt pathway components (Wnt3a and Axin2) were significantly increased by omega-3 treatment (Fig. 9a, c). Omega-3 also significantly increased the mRNA levels of Wnt5a and Wnt7a, compared with untreated *mdx* mice (Fig. 9a). The transcript levels of Wnt11 were increased only

Fig. 6 Omega-3 enhanced satellite cell population. **a–c** Muscle sections of quadriceps femoris of 52-day-old mice were immunostained with DAPI and for Pax7 (*arrows* activated satellite cells). *Bars* 30 μm. **d–f** Representative fluorescence-activated cell sorting (FACS) dot plots showing the percentage of satellite cells in quadriceps femoris muscle of 52-day-old mice. Negative selection antibodies (CD31, CD45, Ter119) are in *upper box*, whereas positive selection antibody (α7-integrin) is in *lower box*. **g** Quantification (%) of satellite cells by FACS. Student's *t*-test; $P < 0.05$ values significantly different from untreated *mdx* mice (a)
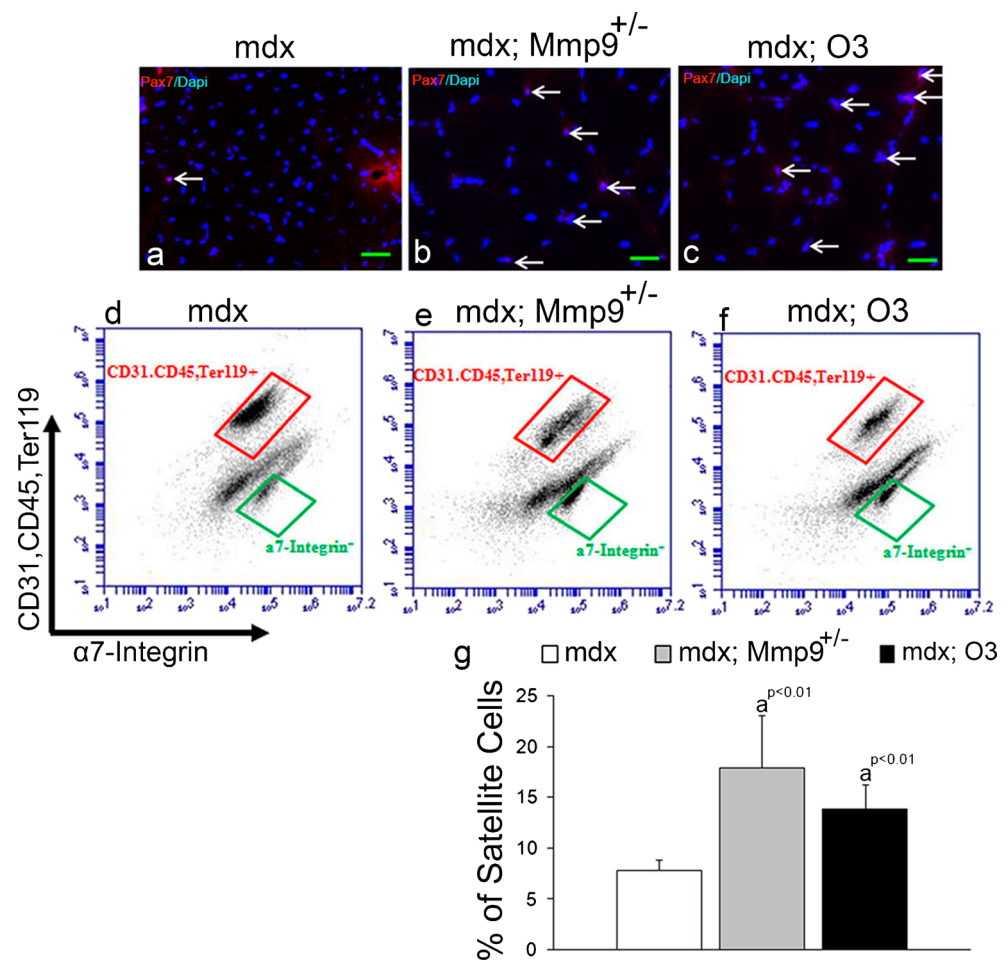
in the mdx;Mmp9$^{+/−}$ mice. Wnt receptor frizzled 2 was increased, whereas frizzled 1 was decreased by omega-3 treatment (Fig. 9b). These results show that both Notch and Wnt signaling are expressed in the quadriceps muscle at the age studied and are affected by omega-3, with similar results being detected in the MMP-9 heterozygous mice.

Discussion

We have previously demonstrated that omega-3 decreases myonecrosis and inflammation in various skeletal muscles of the *mdx* mouse (Carvalho et al. 2013; Fogagnolo Mauricio et al. 2013). In the present study, we added further information by showing that omega-3 improves myoblast engraftment in the quadriceps muscle as demonstrated by a fourfold increase in the graft area (in comparison with the untreated *mdx* case), the number of engrafted fibers per clusters and a concomitant increase in the number of fibers positive for dystrophin. Cell transplantation is considered a promising strategy to restore dystrophin in skeletal muscles of DMD patients

(Tedesco et al. 2010; Negroni et al. 2011). The restoration occurs by the fusion of donor cells that express dystrophin (Wang and Rudnicki 2012). However, many studies have shown that transplanted cells normally fail to repopulate the dystrophic muscle possibly because of fibrosis that impedes cell migration and proliferation (Qu et al. 1998; Brack et al. 2007; Darabi et al. 2008; Murphy et al. 2011). In dystrophic muscles, the inflammatory cells at the site of injury produce proinflammatory and profibrotic factors, including tumor necrosis factor-alpha (TNF-α), MMP-9 and TGF-β, which are involved in dystrophy progression (Li et al. 2009; Nadarajah et al. 2011; Mauricio et al. 2016). A decrease in inflammation improves myoblast survival in dystrophic muscles (Qu et al. 1998; Darabi et al. 2008). MMP-9, which is involved in the reorganization of the extracellular matrix (Mott and Werb 2014; Page-McCaw et al. 2007) and is considered a proinflammatory factor, is normally increased in dystrophic muscle (Li et al. 2009; Alameddine 2012). Previously, we have shown that omega-3 decreases the levels of TNF-α, factor nuclear kappa B (NFkB) and TGF-β in various dystrophic muscles (Apolinário et al. 2015; Mauricio et al. 2016). In the present
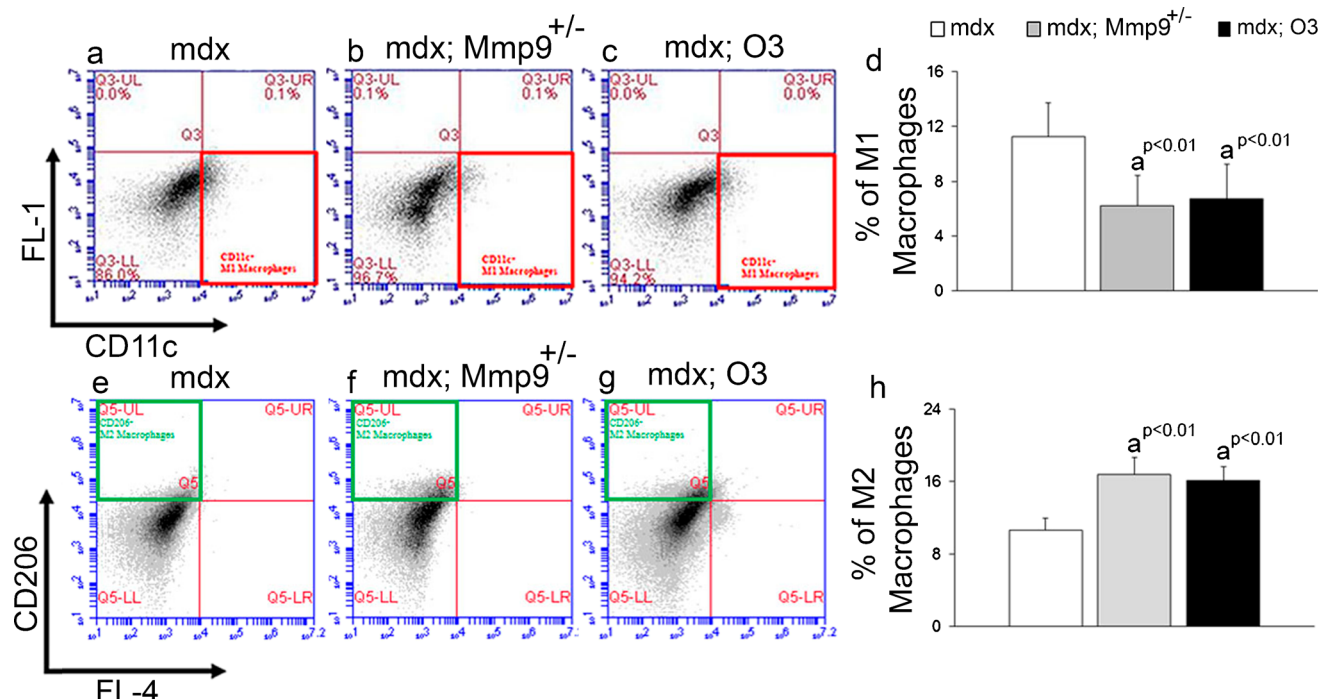
Fig. 7 Omega-3 reduced M1 phenotype and increased M2 macrophage. Representative FACS dot plots displaying the percentage of CD11c^{+} M1 macrophages (**a–c**) and CD206^{+} M2 macrophages (**e–g**) of quadriceps femorais of 52-day-old untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous *mdx* (*mdx;Mmp9*^{+/−}) and omega-3-treated *mdx* (*mdx;O-3*) mice. Quantification of M1 (**d**) and M2 macrophages (**h**) by the FACS technique. *n* = 4 in each group. *Error bars* represent SD. Student's *t*-test; *P* < 0.05, values significantly different from untreated *mdx* mice (*a*)

study, we demonstrated that omega-3 decreases MMP-9 expression in the *mdx* quadriceps. MMP-9 inhibition increases myoblast engraftment in the tibialis anterior (Hindi et al. 2013) and quadriceps muscles, as seen here. Therefore, we consider it reasonable to suggest that omega-3 treatment, by attenuating inflammation and decreasing MMP-9, provides a better environment in the quadriceps muscle for transplanted myoblasts to thrive and proliferate and that this treatment is thus potentially useful in clinical studies of cell therapy for DMD.

Dystrophic muscles have a poor capacity to regenerate (Webster and Blau 1990) possibly because of a decrease in satellite cell function (Blake et al. 2002; Morgan and Zammit 2010; Hindi and Kumar 2016). We have previously reported that omega-3 can improve muscle regeneration in *mdx* quadriceps muscle (De Carvalho et al. 2013; Apolinário et al. 2015) and the increased number of eMyHC-positive fibers in the dystrophic quadriceps seen here further supports this regenerative ability of omega-3. Furthermore, by using FACS analysis, we demonstrated that omega-3 improves the number of activated satellite cells. While the way that omega-3 improves satellite-cell activation remains unclear, a possible explanation may be related to the effects of omega-3 on macrophage populations. M1 macrophages are generally accepted to promote a cytotoxic environment in dystrophic skeletal muscles (Villalta et al. 2009). We demonstrated that omega-3, by acting on IL-10, promotes a shift from an M1

macrophage population to an M2 macrophage population in the *mdx* quadriceps (Carvalho et al. 2013). The decrease in M1 macrophages seen here with FACS may also help to explain the reduced expression of MMP-9 by omega-3, since omega-3 modulates the binding activity of NF-κB and activator protein-1 (AP-1), which could lead to decreased MMP-9 protein levels (St-Pierre et al. 2003; Zhao and Chen 2005). Further, the increase in M2 by omega-3 may help to stimulate satellite cell proliferation (Villalta et al. 2009; Tidball 2011; Hindi et al. 2013), as seen here.

The Notch and Wnt signaling pathways are involved in satellite cell renewal and myogenesis (Brack et al. 2008; Wen et al. 2012; Jiang et al. 2014; Rudnicki and Williams 2015) and most studies have examined these pathways directly in satellite cells and derived progenitors (Brack et al. 2007, 2008; Le Grand et al. 2009; Mourikis et al. 2012). In the present investigation, Notch and Wnt pathway components were detected in muscle fibers, as in previous studies (Hindi et al. 2013; Church et al. 2014). We observed that omega-3 increases the expression of genes related to the Notch pathway (Notch1, Notch3, NICD, and Hes1) in the dystrophic quadriceps muscle, a finding possibly correlated with the increased number of activated satellite cells observed with omega-3 treatment. Omega-3 also improves the gene expression of most of the Wnt genes studied (Wnt-3a, −5a, −7a) with a concomitant increase in axin 2, the direct downstream target of Wnt signaling (Jho et al.
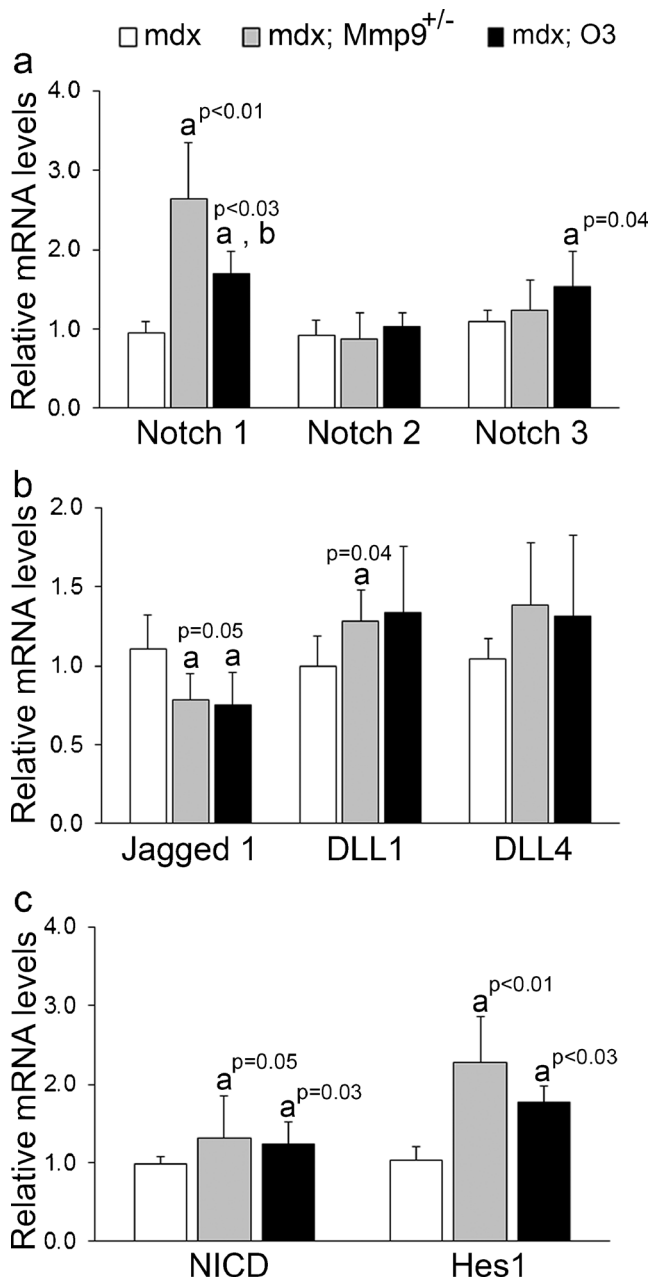
Fig. 8 Omega-3 increased Notch signaling in muscle cells. Relative mRNA levels of Notch receptors (Notch1, Notch2 and Notch3; **a**), ligands (Jagged1, DLL1, and DLL4; **b**) and Notch intercellular domain (NICD; **c**) and Notch target gene (Hes1; **c**) in the quadriceps femoris of 52-day-old untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous *mdx* (*mdx;Mmp9*^{+/−}) and omega-3-treated *mdx* (*mdx;O-3*) mice. *n* = 4 or 6 in each group. *Error bars* represent SD. Student's *t*-test; *P* < 0.05, values significantly different from littermate of untreated *mdx* (*a*) or mdx;Mmp9^{+/−} (*b*) mice
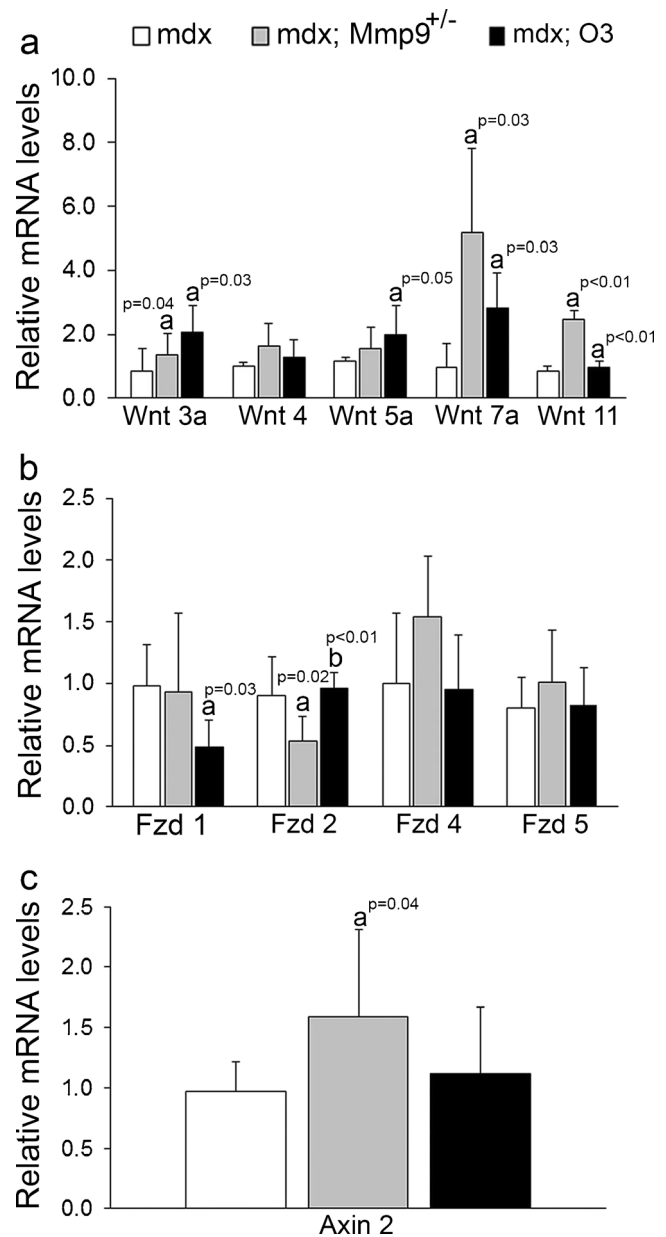
Fig. 9 Omega-3 increased Wnt signaling in muscle cells. Relative mRNA levels of Wnt3a, Wnt4, Wnt5a, Wnt7a and Wnt11 (**a**), Frizzled (Fzd)1, Fzd2, Fzd4, and Fzd6 (**b**) and Axin2 (**c**) in quadriceps femoris of 52-day-old untreated *mdx* (*mdx*), *mdx* Mmp9-heterozygous (*mdx;Mmp9*^{+/−}) and omega-3-treated *mdx* (*mdx;O-3*) mice. *n* = 4 or 6 in each group. *Error bars* represent SD. Student's *t*-test; *P* < 0.05, values significantly different from littermate of untreated *mdx* (*a*) or mdx;Mmp9^{+/−} (*b*) mice

2002). Wnt3a has been related to fibrosis formation in aged wild-type skeletal muscles (Brack et al. 2007). Here, the mice were young (about 7 weeks of age) and the activation of Wnt signaling in muscle by omega-3 at this period did not change the expression of TGF-β, a pro-fibrotic factor. Gene expression profile studies in the muscles of golden

retriever muscular dystrophy (GRMD) dogs have revealed that the mildly affected GRMD dog presents an increased expression of the Jagged1 gene, a regulator of the Notch signaling pathway (Lindsell et al. 1995), suggesting that Jagged1 represents a target for DMD therapy (Vieira et al. 2015). In the present study, omega-3 treatment was correlated with the decreased expression of the Jagged1 gene in the *mdx* quadriceps muscle but

this is not accompanied by a worsened phenotype of this muscle, at least in the *mdx* mice model of dystrophy and at the age studied. Whereas studies of satellite cells in vitro would be necessary to elucidate the effects of omega-3 on these pathways in dystrophic muscles, the present results may help to explain, at least in part, the positive effects of omega-3 on muscle regeneration and satellite cell activation.

The present results are comparable with those reported in mdx-MMP-9 heterozygous mice (Hindi et al. 2013). In the previous report, the inhibition of MMP-9 resulted in the activation of endogenous satellite cells and an improvement in myoblast engraftment through the activation of the Notch and Wnt signaling pathways in the tibialis anterior muscle. In the present study, we demonstrated that omega-3 treatment reproduces the phenotypic outcomes observed by genetic ablation of MMP-9 in the dystrophic quadriceps muscle, during the early stage of dystrophy. In addition, a trend was seen for omega-3 therapy to reduce the mRNA levels of Atrogin-1 and MUSA, key mediators of muscle atrophy (Murton et al. 2008; Usui et al. 2011; Sartori et al. 2013). Therefore, omega-3 treatment might be useful for preserving muscle mass in cell and genetic therapy strategies.

Overall, the present results suggest that omega-3, in addition to protecting dystrophic muscle fibers from degeneration (Carvalho et al. 2013; Fogagnolo Mauricio et al. 2013), benefits myoblast transplantation (increased dystrophin-positive fibers), promotes the activation of endogenous satellite cells and improves the muscle environment for repair by increasing pro-regenerative M2 macrophages and decreasing M1 macrophages and MMP-9 gene expression. In conclusion, our results provide a possible strategy for improving cell therapy in DMD with a ready-to-use drug that has been employed to treat several other human diseases (Kuroki et al. 1997; Yu and Bjorksten 1998; Oddy et al. 2004; Zamaria 2004; Kompauer et al. 2005; Ordovas 2006; Lai et al. 2006).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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