

RESEARCH ARTICLE

Inositol 1,4,5-trisphosphate (IP₃)-dependent Ca²⁺ signaling mediates delayed myogenesis in Duchenne muscular dystrophy fetal muscle

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a progressive neuromuscular disorder characterized by muscle wasting and premature death. The defective gene is dystrophin, a structural protein, absence of which causes membrane fragility and myofiber necrosis. Several lines of evidence showed that in adult DMD patients dystrophin is involved in signaling pathways that regulate calcium homeostasis and differentiation programs. However, secondary aspects of the disease, such as inflammation and fibrosis development, might represent a bias in the analysis. Because fetal muscle is not influenced by gravity and does not suffer from mechanical load and/or inflammation, we investigated 12-week-old fetal DMD skeletal muscles, highlighting for the first time early alterations in signaling pathways mediated by the absence of dystrophin itself. We found that PLC/IP₃/IP₃R/Ryr1/Ca²⁺ signaling is widely active in fetal DMD skeletal muscles and, through the calcium-dependent PKC α protein, exerts a fundamental regulatory role in delaying myogenesis and in myofiber commitment. These data provide new insights into the origin of DMD pathology during muscle development.

KEY WORDS: DMD fetus, Dystrophin signaling, Calcium channels, IP₃/IP₃R pathway, Myosin isoforms

INTRODUCTION

Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy. In the early phase of the disease, the skeletal muscle of DMD patients is characterized by an ongoing process of degeneration and regeneration followed by exhaustion of its regenerative capacity, that leads to infiltration of inflammatory cells, fibrosis and finally to the disruption of the muscle tissue architecture (Emery, 2002). Although the dystrophin gene was discovered almost 30 years ago, an effective therapy has not yet been found. The membrane fragility, consequent to dystrophin absence, does not fully explain the divergent expression signature of both dystrophic mice (mdx) and DMD patients at different stages of the disease versus healthy muscles in terms of muscle wasting, inflammation and fibrosis (Chen and Ende, 2000; Boer et al., 2002;

Porter et al., 2002). Transcriptomic analysis of dystrophic fibers revealed modification or upregulation of genes involved in calcium handling, probably related to altered stability of dystrophin-associated proteins (DAPs), in metabolism, extracellular matrix genes and genes implicated in inflammatory response (Lapidos et al., 2004). It was demonstrated that dystrophin could interact with nitric oxide synthase and syntrophin [regulating other proteins involved in calcium homeostasis (Brennan et al., 1996; Taghli-Lamalle et al., 2014)] and with calmodulin in a calcium-dependent manner (Anderson et al., 1996). These studies suggested that dystrophin interactions were required for signal transduction at the membrane but, more importantly, that dystrophin itself modulated different signaling pathways. The inflammation, macrophage infiltration, degenerating and regenerating fibers, and stretch-induced damage are commonly described in DMD as secondary pathology and might interfere with analysis of dystrophin-related pathways (Boer et al., 2002).

Gharamani et al. partially resolved this issue by silencing the dystrophin gene, through stable RNA interference, in C2C12 cells: they demonstrated a multitude of de-regulated pathways involving calcium homeostasis, ion channels, metabolism and muscle contractile unit organization (Gharamani Seno et al., 2010). However, this *in vitro* model does not recapitulate the complex architecture of mechanically and electrically stimulated muscle. Fetal muscle is not influenced by gravity and does not suffer from mechanical load and/or inflammation; it therefore represents a valuable model in which to elucidate the primary role of dystrophin in regulating not only the structure of membranes but also Ca²⁺ homeostasis and modulation of signaling/metabolic pathways. Taking into account different studies describing how dystrophin absence causes an undifferentiated state of myofibers or, at least, an altered or delayed differentiation program (Chen and Ende, 2000), which is evident in mdx embryos (Merrick et al., 2009) and DMD patients (Pescatori et al., 2007), we also investigated whether its absence eventually impaired myogenesis.

Deval et al. suggested that increased calcium release from the sarcoplasmic reticulum (SR) is involved in calcium dysregulation in DMD myotubes (Deval et al., 2002). The high sensitivity to calcium of dystrophic muscle cells is dependent on two specialized intracellular Ca²⁺-releasing channels located at the SR membrane: the ryanodine receptor 1 (Ryr1) and inositol 1,4,5-trisphosphate (IP₃) receptors (IP₃Rs) (Carrasco and Figueroa, 1995; Imbert et al., 1996). Recently, Bellinger et al. identified a structural and functional defect in Ryr1 in mdx muscles (Bellinger et al., 2009), and Morel and others suggested that *Ryr1* expression could be altered also in vascular myocytes of mdx mice (Morel et al., 2009). Finally, Andersson et al. hypothesized that leaky Ryr1 channels could underlie multiple forms of muscular dystrophy linked to

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mutations in genes encoding components of the dystrophin-glycoprotein complex (Andersson et al., 2012). Moreover, a previous study showed that basal levels of IP3 were increased two- to three-fold in dystrophic human and murine cell lines compared with normal cell lines (Liberona et al., 1998). As IP3 production is induced by phospholipase C (PLC) activation, it was reported that dystrophin absence determines increased PLC activity and IP3 production (Mondin et al., 2009; Sabourin et al., 2012).

The calcium signaling cascade could activate a proteolytic pathway leading to myofiber necrosis, as demonstrated by delayed onset of dystrophic symptoms in mdx mice after chronically blocking calcium channels *in utero* (Jørgensen et al., 2011), or modulate myogenic expression (Bassel-Duby and Olson, 2006). Cell fate after intracellular calcium accumulation depends on the ability of calcium decoders to promote gene expression (Tavi and Westerblad, 2011). Multiple calcium decoders are known and among them calmodulin, calcineurin and protein kinase C (PKC) α exert a fundamental role in skeletal myofibers (Gundersen, 2011) through the activity of nuclear factor of activated T cells (NFAT) and histone deacetylases (HDACs) on myogenic regulating factors (MRFs) (Liu et al., 2005; Shen et al., 2006). Extending these findings to pathological cardiac growth, several papers demonstrated that Ca²⁺ released from IP3-dependent pathways activates nuclear calmodulin-dependent protein kinase-II (CaMKII) (Kawaguchi et al., 1993; Nakayama et al., 2010) and, consequently, the cardiac gene program (Anderson et al., 2011). Calcium transients can induce both fast and slow changes, according to the specific calcium sensor involved. This phenomenon, known as muscle plasticity, is important in muscle architecture remodeling both in physiological and pathological conditions, such as in muscular dystrophies.

In our work, we found that in fetal DMD skeletal muscles IP3/Ca²⁺ signaling is widely expressed and, through PKC α -calcium sensors, exerts a fundamental regulatory role in delaying myogenesis and in myofiber commitment. As this pathway is different from those described in adult muscle, we highlighted the difference underlying primary and secondary pathologies that characterize DMD skeletal muscles. Furthermore, these data might explain the contradictory results observed when other groups tried to compare different models such as myoblasts/myotubes or whole muscles. Our evidence suggests the calcium-related pathway is activated differently in fetal and adult DMD skeletal muscle fibers.

RESULTS

Characterization of 12-week-old human fetal normal and dystrophic muscles

We performed histological evaluation of muscular biopsies from 12-week-old DMD and healthy (CTR) fetuses. Hematoxylin and Eosin (H&E) analysis and Azan Mallory (AM) staining did not reveal any difference in fiber morphology or in development of connective tissues: in particular, no sign of necrosis or muscular damage was observed (Fig. 1A). However, in DMD muscular biopsies we found a reduction in fiber density, as seen by a significant increase in the proportion of extracellular matrix (percentage of extracellular matrix \pm s.d.: CTR 16.65 \pm 1.084 and DMD 26.99 \pm 1.444, P <0.0001; Fig. 1B), and a reduction, although not significant, of the proportion of myofibers per area (Fig. 1C). Interestingly, we calculated the difference between total section area and myofiber area and we confirmed the reduction of myofiber-filled space in DMD biopsies (total section area–myofiber area: CTR 82,463 \pm 5914 μ m² and DMD 135,498 \pm 7946 μ m², P <0.0001). To analyze further the developmental stage of DMD and CTR

muscles, we characterized myosin heavy chain (MyHC) expression (Fig. 1D). Immunofluorescence analysis showed significant differences in expression of myosin isoforms in DMD versus healthy fetal muscles as demonstrated by a reduced percentage of developmental (MyHC-dev: CTR 90.76 \pm 1327% and DMD 82.92 \pm 2.032%, P =0.0052; Fig. 1E) and neonatal (MyHC-neo: CTR 90.43 \pm 1.413% and DMD 84.79 \pm 1.783%, P =0.0247; Fig. 1F) positive myofibers. Also the percentage of mature fibers (MyHC-slow and -fast positive) was altered (MyHC-fast: CTR 55.11 \pm 4.077% and DMD 67.2 \pm 3.113%, P =0.0315; MyHC-slow: CTR 63.15 \pm 2.634% and DMD 29.16 \pm 0.867%, P <0.0001; Fig. 1G,H). To understand these data better, we performed co-immunostaining of fast and slow MyHC to analyze the proportion of these fiber types in human biopsies. Morphometric analysis revealed a reduction in the cross-sectional area (CSA) of fast myofibers in DMD muscles (mean \pm s.e.m.; CSA: CTR 5.967 \pm 0.393 pixels and DMD 4.269 \pm 0.121 pixels, P <0.0001), whereas the CSA of slow myofibers was comparable to that of slow myofibers of CTR muscles (mean \pm s.e.m.; CTR 7.306 \pm 0.356 pixels and DMD 6.838 \pm 0.2392 pixels) (Fig. 2A,B). More interestingly, differences arose regarding the number of myofibers expressing fast and slow MyHC isoforms. In particular, the number of myofibers expressing slow MyHC was significantly higher in control than in dystrophic fetal muscle (percentage of total fibers \pm s.d.: CTR 57.4 \pm 7.2% and DMD 23.5 \pm 6.2%). Accordingly, fast MyHC was detected in 62 \pm 7.9% of dystrophic myofibers, whereas only 37.5 \pm 6.4% of control myofibers expressed fast myosin. The differences in expression of each MyHC isoform were statistically significant (Fisher's exact test: P <0.0001 for fast and slow myofibers). This tendency was confirmed by western blot (WB) experiments (Fig. 2D). Hybrid myofibers co-expressing fast and slow MyHC were also detected, accounting for 5.1 \pm 1.9% and 14.5 \pm 5.2% of total myofibers in control and DMD fetal muscles, respectively (Fig. 2C). Interestingly, we demonstrated by real-time quantitative PCR (RT-qPCR) that myofiber gene expression is altered in dystrophic fetal muscles, as they expressed a significantly higher level of fast-type genes, such as sarcoplasmic reticulum calcium ATPase-1 (*ATP2A1*) (P =0.092), troponin T type 3 (skeletal, fast) (*TNNT3*) (P =0.0678) and, although not significant, troponin fast C type 2 (*TNNC2*). Conversely, they expressed significantly lower levels of slow-type genes, such as slow myosin heavy chain 2 (*MYHC-SL2*) (P =0.0136), the troponin T type 1 (skeletal, slow) (*TNNT1*) (P =0.03) and, although not significant, creatine kinase mitochondrial 2 (*CKMT2*) (Fig. 3A). As it has been demonstrated that SIX genes are the master regulators for the differential activation of specific muscle programs, and in particular the central switch to drive the skeletal muscle fast phenotype during fetal development, we investigated the expression of SIX genes and also of EYA genes, which interact synergistically with them, by RT-qPCR. However, we did not find any appreciable difference between human healthy and dystrophic fetal muscles (Fig. 3B). It has been shown that in mammals, embryonic skeletal muscle differentiation arose from two different myogenic waves, which generate firstly primary and, subsequently, secondary myotubes, from which newly formed muscle arises (Cossu et al., 1996; Merrick et al., 2009). At this stage of development, different myosin isoforms are co-expressed depending on the specificity of function of individual skeletal muscle groups (Cho et al., 1994; Merrick et al., 2007). According to our data, the abnormal expression of fast and slow isoforms that we described in DMD fetal muscles could be attributed to an uncontrolled fiber type switch or to a developmental delay between primary and secondary myogenesis. Nevertheless,

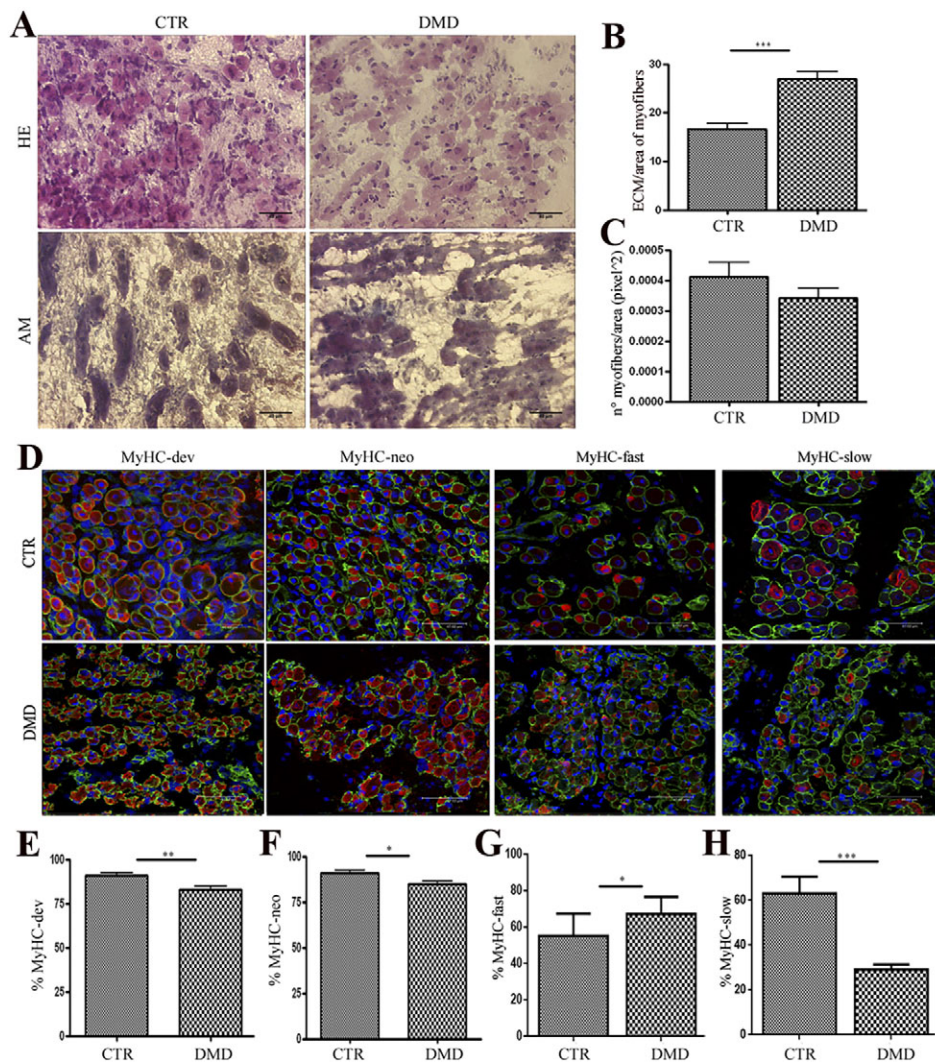


Fig. 1. Altered muscular architecture in DMD fetal myofibers. (A) H&E staining revealed similar myofiber morphology between CTR and DMD muscles. No signs of necrosis or sarcolemma damage were visible. AM staining revealed a similar level of collagen deposition between CTR and DMD muscle sections. However, increased extracellular matrix deposition was observed in DMD tissue. Scale bars: 40 μ m. (B, C) Histograms representing the myofiber density of CTR and DMD fetal muscles, calculated as the extracellular matrix (ECM; i.e. the white space between the myofibers) versus myofiber area (B) and as the number of myofibers presented in a fixed grid area (C). (D) Immunofluorescence analysis of muscle sections stained with antibodies against MyHC isoforms (red) and laminin (green). Nuclei were identified with DAPI (blue). (E–H) CTR fetal muscles show a higher percentage of MyHC-developmental, -neonatal and -slow positive myofibers (E, F, H) and a lower percentage of MyHC-fast positive myofibers (G) than DMD fetal muscles (percentage of laminin-positive myofibers that are MyHC positive). Data are expressed as mean \pm s.d.; * P <0.05, ** P <0.005, *** P <0.001.

taken together with the reduction of the CSA of fast myofibers (Fig. 2A,B) and the significantly increased proportion of extracellular matrix (Fig. 1B) in dystrophic muscles, we suggest a delay in maturation of DMD fetal muscles.

Expression of myogenic markers in 12-week-old human fetal healthy and dystrophic muscles

As myofibers develop from Pax7⁺ muscle progenitors that migrate from the central dermomyotome (Relaix et al., 2005), we investigated the expression of PAX7 and we showed a significantly higher number of PAX7⁺ cells per myofiber in DMD samples compared with healthy samples (CTR 2.313 \pm 0.246 and DMD 3.446 \pm 0.0254, P =0.0445) (Fig. 4A,B), and we demonstrated by WB analysis a significantly higher expression of PAX7 protein (CTR 0.393 \pm 0.0518 and DMD 1.032 \pm 0.1362, P =0.0183) (Fig. 4C). As it is known that Pax3 is an important determinant of embryo myogenesis and Pax7⁺ cells derive from Pax3⁺ cells during and after somitogenesis, we investigated its expression in murine and human dystrophic fetal muscles. Pax3 expression was found in neither fetal murine mdx (E18.5) nor DMD 12-week-old fetal muscles (data not shown). The RT-qPCR analysis and, more interestingly, the WB analysis, demonstrated the downregulation of MYOD expression in DMD muscles (Fig. 4D,E) (P =0.0206 and P =0.0057, respectively).

Calcium measurement in fetal muscles

As calcium overload is one of the major features in DMD pathogenesis, we analyzed the calcium content of muscles from 12-week-old DMD and CTR fetuses. Previous works reported that fetal DMD muscles were histologically normal except for occasional eosinophilic hypercontracted fibers (Bertorini et al., 1984; Lotz and Engel, 1987). Therefore, we performed Alizarin Red (AR) staining and observed a consistently higher accumulation of Ca²⁺ in DMD fetal muscles than in healthy samples (Fig. 5A). Staining quantification demonstrated a fourfold increase of intracellular calcium in DMD versus CTR myofibers (DMD 43.01 \pm 6.055% versus CTR 14.8 \pm 2.752%, P =0.0008) (Fig. 5A'). Calcium accumulation was also confirmed by Fluo-4 staining (Fig. 5B,B'). As this event in dystrophic muscle is thought to be crucial for caspase activation, we performed immunofluorescence analysis of active caspase 3 and we demonstrated that calcium levels were not related to either necrosis or caspase 3 activation (Fig. 5C). Furthermore, we performed alkaline phosphatase (AP) to stain regenerating fibers in adult muscles and we found that the majority of DMD and healthy fetal myofibers were AP positive without differences in the number of stained myofibers between specimens, consistent with the fact that regenerating fibers recapitulate maturative steps of developing fibers during myogenesis. However, the AP staining intensity was increased in fetal DMD myofibers, suggesting a more immature myofiber

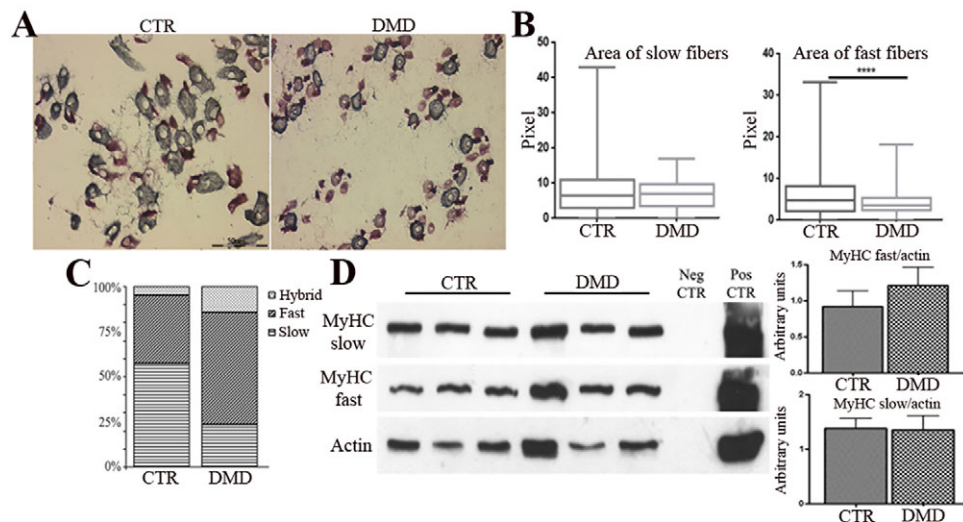


Fig. 2. Fast and slow fiber-type specification is altered in DMD fetal muscles. (A) Immunostaining in 12-week-old DMD and CTR fetal muscles that allowed us to measure the proportion of fast fibers (pink) and slow fibers (gray) per section. (B) Slow-myofiber area was similar between CTR and DMD but fast-myofiber area was significantly higher in CTR versus DMD tissue (slow: 25th percentile CTR 3.0, DMD 3.6; median, CTR 6.45 DMD 6.975; 75th percentile CTR 10.95, DMD 9.78) (fast: 25th percentile CTR 2.25, DMD 2.43; median CTR 4.8, DMD 3.6; 75th percentile CTR 8.13, DMD 5.4) ($P < 0.0001$). (C) Histogram showing the proportion of each MyHC isoform in a fixed grid area in DMD and CTR fetal muscles. (D) WB analysis of the expression of MyHC slow and fast demonstrating a higher expression of MyHC fast in dystrophic samples. Adult human healthy muscle was used as positive control (Pos CTR) and water was used as negative control (Neg CTR) to avoid false positives deriving from buffer contamination. Data are expressed as mean \pm s.d.; **** $P < 0.0001$.

phenotype. Interestingly, we showed that higher content of calcium was common to slow/fast and hybrid myofibers (Fig. 5C,D).

Characterization of C57BL/6J and mdx fetal muscles

To identify the source of calcium overload it was necessary to investigate various membrane ion channels. We focused on the major Ca^{2+} channels and those identified as altered in dystrophic

muscles (both in murine and in human muscles). Thus, we decided to perform an initial screening in mouse fetal muscle tissues and, in a second step, we investigated in human fetal muscles only those channels that were altered in dystrophic murine muscles. This selection process allowed the best use of the human fetal tissue available. First of all, we demonstrated that calcium overload was also present in mdx fetuses. Fluo-4 staining confirmed calcium

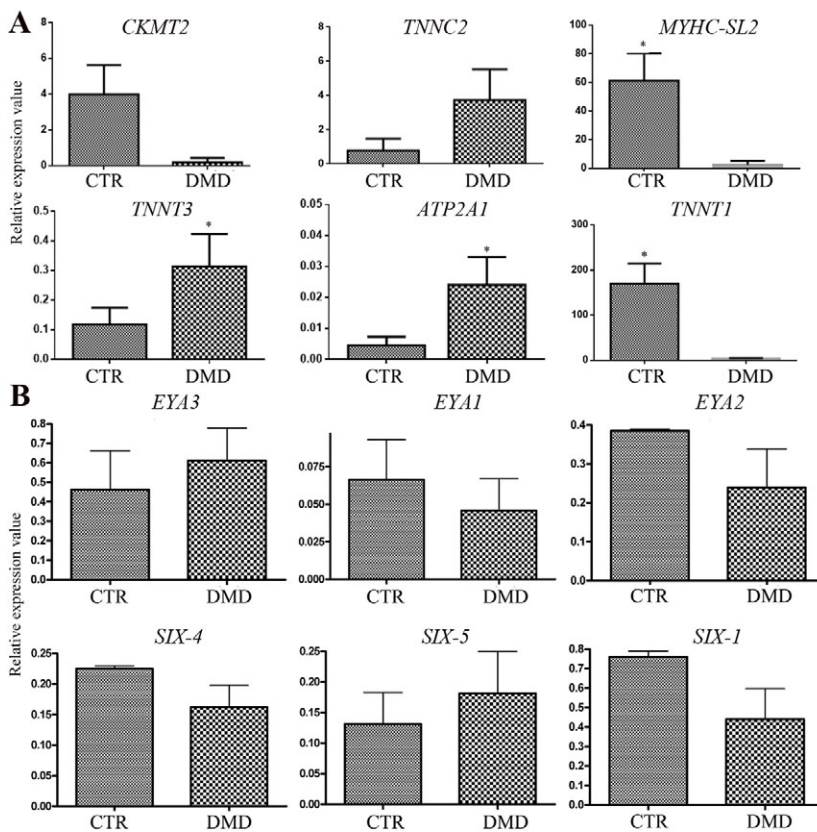


Fig. 3. Evaluation of gene expression in CTR and DMD fetal muscles. (A,B) Quantitative PCR experiments performed on CTR and DMD fetal muscles. Relative expression values revealed significant differences in the expression of fast- (*ATP2A1*, *TNNC2*, *TNNT3*) and slow- (*MYHC-SL2*, *CKMT2*, *TNNT1*) type genes (A) and no significant differences in the expression of SIX and EYA genes (B). Data are expressed as mean \pm s.d. * $P < 0.05$.

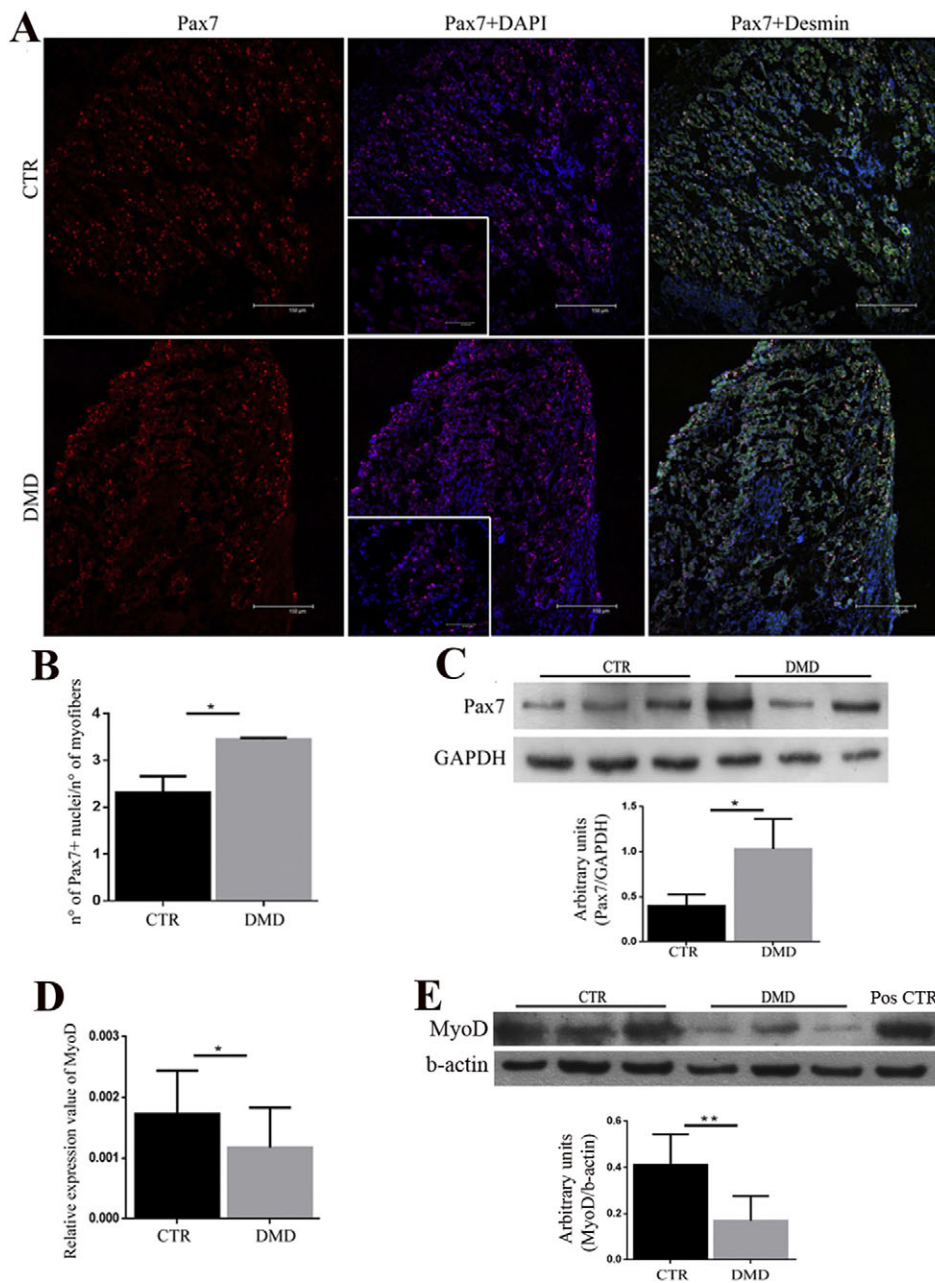


Fig. 4. Increased PAX7 expression in DMD fetal muscles. (A,B) Human dystrophic (DMD) and healthy (CTR) muscles were stained with antibodies against PAX7 (red) and desmin (green) (A) and we demonstrated an increase of the ratio PAX7⁺ nuclei/total number of myofibers in DMD samples (B). Insets show higher magnification images. Scale bars: 150 μ m. (C) Analysis of Pax7 expression by WB revealed a higher expression of PAX7 in dystrophic samples as shown by densitometric analysis. (D,E) RT-qPCR (D) and WB (E) analyses showed downregulation of MYOD expression in DMD fetal muscles. RH30 rhabdomyosarcoma cells were used as positive control (Pos CTR). Data are expressed as mean \pm s.d.; * P <0.05, ** P <0.005.

accumulation in myofibers from embryonic day (E) 18 mdx fetuses while no Ca²⁺ accumulation was observed in C57BL/6J fetal muscles (Fig. 6). Desmin staining of serial sections confirmed that the calcium accumulation was relative to myofibers (Fig. 6).

Expression of Ca²⁺ channels in murine fetal muscle tissues

To analyze the possible origin of the calcium overexpression in dystrophic muscles, we investigated the expression level of *Orail*, the pore-forming unit of store-operating Ca²⁺ channels (SOCE), and of another component of the SOCE complex, the stromal interaction molecule 1 (*Stim1*). RT-qPCR showed that there was an increase in mRNA expression of these genes in the mdx fetuses, but only *Orail* was statistically significant (C57BL/6J 0.1733 \pm 0.037 and mdx 4.75 \pm 1.15, P =0.0129) (Fig. 7A). Moreover, we evaluated through WB the expression of other proteins, such as Ryr1 and Trpc1, and we found that Ryr1 was significantly overexpressed in mdx mice (C57BL/6J 0.2232 \pm 0.041 and mdx 0.5216 \pm 0.057, P =0.0133)

(Fig. 7B), but Trpc1 expression did not change significantly (Fig. 7C). As Ryr1 and IP3R belong to the same class of calcium channels and intracellular calcium increase can be mediated by IP3/IP3R binding, we firstly investigated the expression of IP3R in murine fetal muscles by WB analysis. We observed a significant higher concentration of IP3R in dystrophic fetal muscles (C57BL/6J 0.3299 \pm 0.051 and mdx 0.9917 \pm 0.1897, P =0.0281) (Fig. 7D). Moreover, we demonstrated by ELISA that mdx muscles expressed higher levels of IP3 related to C57BL/6J samples (C57BL/6J 96.58 \pm 3.449 pg/ml and mdx 141.2 \pm 8.283 pg/ml, P =0.0001) (Fig. 7E), suggesting an over-activity of PLC in dystrophic fetal muscle tissues.

Expression of Ca²⁺-dependent protein in murine fetal muscle tissues

Among the plethora of proteins that are regulated by calcium concentration, Ca²⁺-calmodulin-dependent protein kinase II (CaMKII) regulates gene expression in cardiac and skeletal

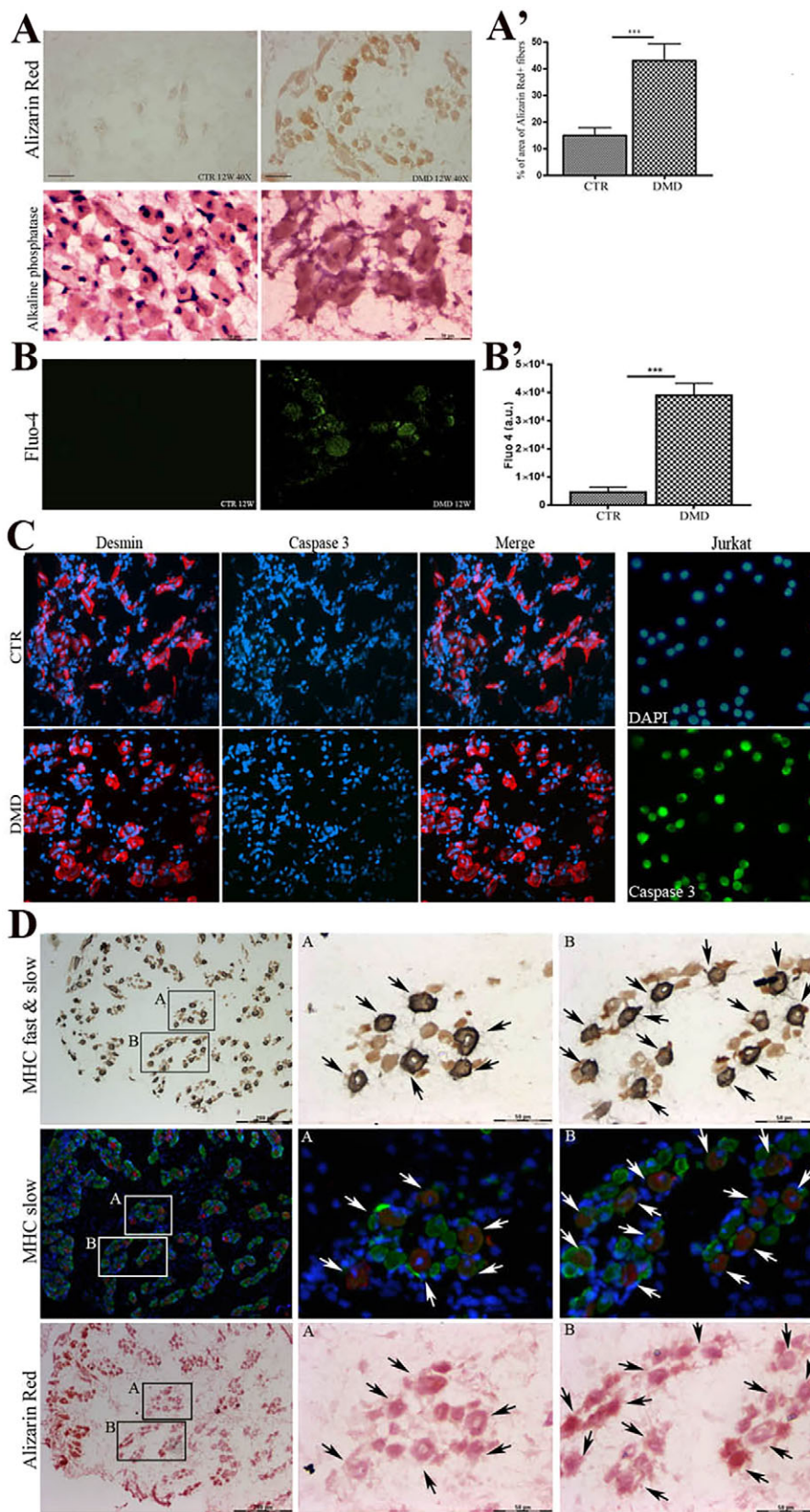


Fig. 5. Ca²⁺ accumulation in DMD fetal biopsies. (A) AR staining demonstrated an increased calcium deposition in DMD versus healthy CTR myofibers (where no positive myofibers were observed). Scale bars: 40 μ m. AP staining shows myofibers in skeletal muscle. Scale bars: 50 μ m. (B) Fluo-4 staining confirmed calcium accumulation in DMD fetal muscles. Histograms representing the quantification of AR and Fluo-4 staining in CTR and DMD fetal muscles are shown in A' and B', respectively. Data are expressed as mean \pm s.d.; *** P <0.001. Scale bar: 50 μ m. (C) Immunofluorescence staining of active Caspase-3 (green). Jurkat cell line treated with 2 μ M camptothecin was used as positive control. Myofibers were stained with Desmin (red); no sign of caspase-3 activation was observed in dystrophic or healthy myofibers, suggesting that calcium overload did not promote apoptotic cascade. The majority of DMD and healthy fetal myofibers were AP positive without differences in the number of stained myofibers between specimens. It is interesting that AP staining is more intense in DMD myofibers, suggesting a delay in their myogenesis. (D) Immunohistochemistry (top panels) on DMD muscle serial sections for fast (pink) and slow (gray) myofibers and immunofluorescence (middle panels) for slow MyHC (red) and desmin (green) expression confirm that both fast and slow MyHC myofibers are calcium enriched as visualized with AR staining (bottom panels). Boxed areas labeled A and B are shown at higher magnification (40 \times magnification vs. 10 \times) in the corresponding panels on the right. Arrows indicate the same cluster of slow MyHC myofibers in each panel. Arrows indicate the same cluster of slow MyHC myofibers in each panel. Scale bars: 200 μ m (50 μ m for enlargements).

muscle. As suggested by Kramerova et al. (2012), we evaluated the expression of this protein in our samples and we showed that there is a little but not significant increase of CaMKII in mdx fetal muscle tissues (Fig. 7F). Considering the evidence obtained by Wang et al.

(2009), Madaro et al. (2012) and the work of Kawasaki, demonstrating the role of PKC in the phosphorylation of Orail (Kawasaki et al., 2010), we decided to investigate the expression of PKC α in murine fetal muscle tissues. In skeletal muscle, PKC α

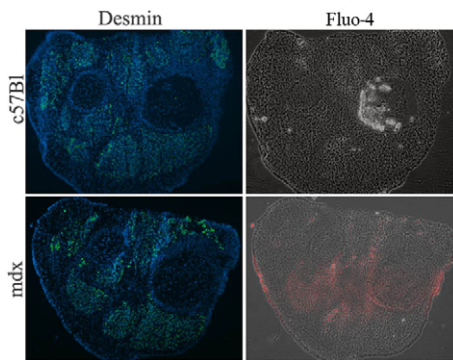


Fig. 6. Ca^{2+} accumulation in myofibers of E18 lower limbs of mdx fetuses. Fluo-4 staining demonstrated increased calcium deposition in fetal mdx versus C57BL/6J myofibers (where no Fluo-4-positive myofibers were observed). Immunofluorescence of desmin (green) was performed on serial sections to confirm that Ca^{2+} deposition was inside skeletal myofibers (4 \times magnification).

protein is regulated by Ca^{2+} concentration and plays a fundamental role in muscle development and plasticity (Gundersen, 2011). Because PKC α exerts its function when phosphorylated, we measured the phospho-PKC α /total PKC α ratio for each sample to avoid any bias deriving from differences in muscle content between samples. Interestingly, we found that the phospho-PKC α /total PKC α ratio was significantly higher in dystrophic than healthy samples ($P=0.0025$; Fig. 7G).

Overexpression of PKC α in human fetal dystrophic muscle

In accordance with the results obtained in murine fetuses, we demonstrated a significant increase of RYR1 expression in human fetal dystrophic samples (CTR 0.466 ± 0.07423 and DMD 1.143 ± 0.2332 , $P=0.0199$) (Fig. 8A) and, more interestingly, of IP3R by WB (CTR 0.3826 ± 0.03879 and DMD 0.9122 ± 0.096 , $P=0.0015$) (Fig. 8B) and IP3 by ELISA analysis (Fig. 8C). Furthermore, we observed that the phospho-PKC α /total PKC α ratio was significantly higher in human dystrophic fetal muscles than in healthy ones (Fig. 8D,E; $P=0.008$). A more sensitive approach was required to improve analysis regarding PKC α activation. The most common assay for kinases, measuring the transfer of radioactive phosphate to a substrate protein or peptide, is effective but inconvenient and hazardous owing to the large quantity of ^{32}P used. To circumvent these difficulties, we turned to the PepTag Assay, which is sensitive to a variety of different PKC isoforms (α , β , γ , μ). Although only PKC α is known to be muscle specific, recent evidence suggests that PKC ϵ might also act in this tissue (Niu et al., 2011). To avoid biases related to the presence of multiple PKC isoforms, the expression of *PRKCE* (the gene encoding PKC ϵ) in human healthy and dystrophic fetal muscles was assessed using RT-qPCR. *PRKCE* transcript was barely detectable in all samples tested (data not shown). Performing the PepTag Assay, we confirmed that the ratio of phosphorylated PKC α to total PKC α was significantly higher in DMD fetal muscle samples than in healthy ones ($P=0.0115$; Fig. 8F).

DISCUSSION

Thirty years after the discovery of the dystrophin gene, the molecular bases of DMD pathophysiology are still unknown. In particular, understanding whether the absence of only one protein results in such a complicated phenotype remains a challenge. The mechanical susceptibility of dystrophic myofibers is a well-known phenomenon leading to myofiber damage and necrosis. The scaffold of DAPs, through the assembly of multiprotein signaling

complexes, regulates suppressors of cytokine signaling (SOCs) and IP3Rs, with important consequences in maintaining normal calcium homeostasis in the skeletal muscle cell. The lack of dystrophin and the consequent disorganization of the membrane-associated cytoskeleton could induce a dysfunction of calcium influxes and releases, with cumulative consequences on fetal DMD myogenesis, and adult muscle damage. However, dystrophin absence might be more crucial, and might not simply unbalance the mechanical structure of plasma membrane but deeply modify gene expression, as suggested by previously published transcriptomic analysis of dystrophic muscles (Haslett et al., 2003; Ghahramani Seno et al., 2010).

In this work, we investigated fetal DMD skeletal muscle development in order to improve our understanding of the primary pathology in the absence of inflammation or fibrosis and under reduced mechanical load. In adult muscles, dystrophin and α -syntrophin regulate PLC activity by stabilizing the sarcolemma; thus, dystrophin absence leads to increased PLC activity (Jordan et al., 2004; Mondin et al., 2009; Sabourin et al., 2012). Furthermore, previous studies showed that dystrophic human samples and murine cell lines expressed high levels of IP3 compared with normal cell lines (Liberona et al., 1998; Mondin et al., 2009) and IP3-induced Ca^{2+} release altered gene expression regulation (Powell et al., 2001; Araya et al., 2003). In addition, it was determined that modifications in the metabolism of IP3 were fundamental at different stages of muscle development (Carrasco et al., 1997), and others suggested a role for IP3/IP3R during skeletal myocyte development (Roseblit et al., 1999) and in cardiomyocyte hypertrophy (Arantes et al., 2012). Therefore, we investigated IP3R and IP3 pathway, demonstrating their overactivity in fetal DMD muscle. IP3R belongs to the same class of receptors as RYR1, and IP3/IP3R binding determines calcium cell accumulation as we observed in DMD fetal biopsies (Fig. 9). Calcium entry could also be regulated through a multitude of SOCs: α 1-syntrophin and PKC α could balance the activity of *Stim1/Orai1/Trp1* genes (Sabourin et al., 2012; Harsseeh et al., 2013). In addition to altered expression of *Orai1*, we observed an overexpression of *Trp1* and *Ryr1*, in accordance with previously reported data (Carrasco and Figueroa, 1995; Imbert et al., 1996; Bellinger et al., 2009; Morel et al., 2009; Andersson et al., 2012). Calcium concentration has a well-known importance in the development of DMD pathogenesis. However, some controversies arose regarding the elevation of steady-state cytosolic calcium levels in DMD muscles, as it was observed in muscles and myofibers (Bakker et al., 1993; Denetclaw et al., 1994) even though it was not definitely confirmed in cultured mdx or DMD myotubes (Gailly et al., 1993; Head, 1993; Pressmar et al., 1994; Collet et al., 1999). Imbert and colleagues suggested that elevated steady-state calcium levels observed in well-differentiated DMD myotubes cocultured with neural explants were due to specific calcium channels during muscle spontaneous activity (Imbert et al., 1995). By contrast, DMD myotubes in primary culture did not develop a significant increase in calcium (Rivet-Bastide et al., 1993; Pressmar et al., 1994), suggesting involvement of a different Ca^{2+} pathway. Calcium signaling is involved in a multitude of muscular pathways that lead to modification of gene expression and, especially in DMD, to myofiber injury (Alderton and Steinhardt, 2000). In particular, it was suggested that a persistent intake of Ca^{2+} activates Ca^{2+} -sensitive proteolytic and phospholipolytic activities, resulting in the degradation of dystrophic muscle fibers (Alderton and Steinhardt, 2000). However, we did not observe any evidence of fiber degeneration, apoptotic activation, or alteration in the number

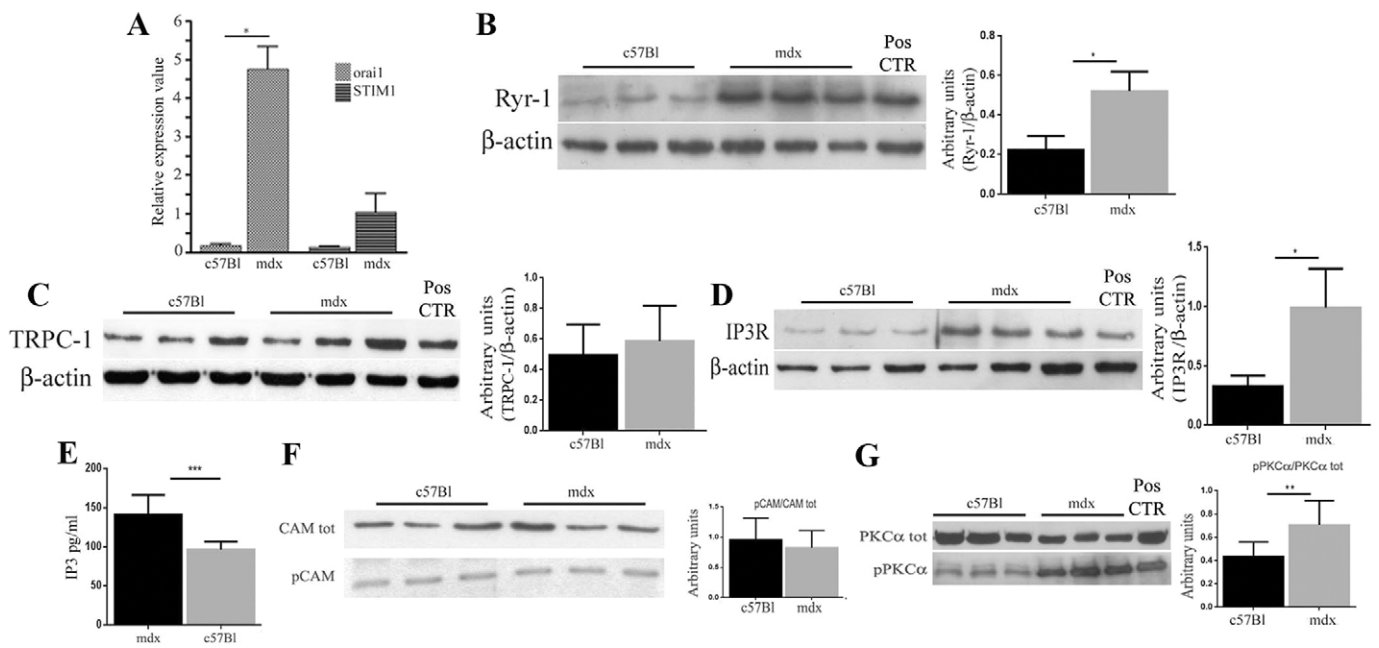


Fig. 7. Modulation of protein expression in mdx fetal muscles. (A) RT-qPCR evaluation of the expression of *Orai1* and *Stim1* revealed an increase these genes in mdx fetal muscles. (B–D) WB immunoblot and densitometric quantification analysis of C57BL/6J and mdx E18 fetuses for the expression of Ryr1 (rat skeletal muscle was used as positive control, Pos CTR) (B), *Trpc1* (C57BL/6J testis was used as positive control) (C) and IP3R (rat cerebellum was used as positive control) (D). (E) ELISA quantification of IP3 showed higher expression in mdx fetal muscles related to C57BL/6J ones. (F,G) WB analysis of C57BL/6J and mdx E18 fetal muscles for the expression of total and phosphorylated CaMKII (F) and PKC α (rat cerebellum lysate was used as positive control) (G). Data are expressed as mean \pm s.d.; * P <0.05, ** P <0.005, *** P <0.001.

of regenerating myofibers. These data suggest that the pathways involved in fetal muscles are different from those underlying the adult dystrophic muscle phenotype (Fig. 9). Calcineurin and CaMKII are Ca²⁺ decoders that are dependent on calmodulin-Ca²⁺ binding (Klee et al., 1998; Sakuma and Yamaguchi, 2010) and are able to alter gene transcription by inducing nucleo-cytoplasmic shuttling of transcription factors (e.g. NFAT and myocyte enhancer factor-2, MEF2) (Chen et al., 2001; Liu et al., 2001) and transcription modulators (histone deacetylases, HDACs) (Liu et al., 2005; Shen et al., 2006). These enzymes are involved in muscle plasticity induced by changes in calcium concentration in response to environmental factors (i.e. aerobic/anaerobic exercise training or muscular disorders). Once activated by prolonged and slow electric stimuli determining calcium accumulation, they promote gene expression towards a slow, oxidative and resistant phenotype (Chin et al., 1998). Similarly, in adult DMD muscles the proportion of slow myosin-expressing fibers is higher than in healthy muscles. Moreover, it was demonstrated that fast myofibers in human skeletal muscles are subjected to great stress by the fast-contraction-relaxation cycle and are most susceptible to mechanical damage (Bottinelli et al., 1996). This is probably a consequence of an adaptive response of the body to the muscle disease (Campbell, 1995; Pedemonte et al., 1999). This way, DMD fast myofibers seem to be the first to degenerate and to be replaced by slow fibers. Fetal muscle does not require a switch of fiber phenotype into fatigue and mechanical stress resistance, as it grows in conditions of reduced gravity and stress. In line with this fetal environment, we demonstrated inactivity of the Ca²⁺/calmodulin/CAMKII pathway. Taken together with the work of Oh and collaborators, demonstrating that slow fiber expression during embryogenesis and early postnatal growth is under the control of a developmental program in a calcineurin-independent manner (Oh et al., 2005), we investigated the expression level of another calcium-dependent

protein, PKC α . In accordance with previously published data that demonstrated that PKC α exerted a primary role in pathogenesis of DMD (Wang et al., 2009; Madaro et al., 2012), we showed its early overactivation in DMD fetal muscle tissues. PKC α is involved in signal transduction cascades regulating the proliferation of myoblasts (Goel and Dey, 2002). Furthermore, PKC α is engaged in repressing myogenic factors, in particular MyoD (Vaidya et al., 1991), suggesting its role as a negative modulator of myogenesis (Capiati et al., 1999). Interestingly, Ryr1 activity had been related to both PKC α activation and MyHC-slow gene suppression. In addition, muscle PKC α once activated translocates to the nucleus and interacts with MRFs (Cleland et al., 1989; Huang et al., 1992), and it was demonstrated in the avian models that PKC α activity was higher in fast than in slow myofibers (DiMario and Funk, 1999; DiMario, 2001; Jordan et al., 2004). In agreement with these data, we showed that enhanced PKC α activation resulted in altered myogenesis, characterized by a delay in fiber maturation and modification of fiber type composition. We observed a reduction in the total number of myofibers and a reduction in the proportion of MyHC-expressing myofibers, and an increase in the proportion of extracellular matrix. Interestingly, we showed in fetal DMD muscle tissues an overexpression of PAX7 and a reduction of MyoD expression; therefore, we suggest that the differentiation program of myofibers is delayed. Furthermore, DMD muscles presented a greater proportion of hybrid (expressing both fast and slow myosin isoforms) and fast myofibers, and a concurrent reduction of myofiber size of fast myofibers. These data were confirmed by the RT-qPCR analysis of relative expression values of several fast- (*ATP2A1*, *TNNC2*, *TNNT3*) and slow- (*MYHC-SL2*, *CKMT2*, *TNNT1*) specific genes. Of note, similar findings were reported in developing fetuses suffering from other neuromuscular disorders such as spinal muscular atrophy (SMA) (Martínez-Hernández et al., 2014). However, these observations are in contrast with data

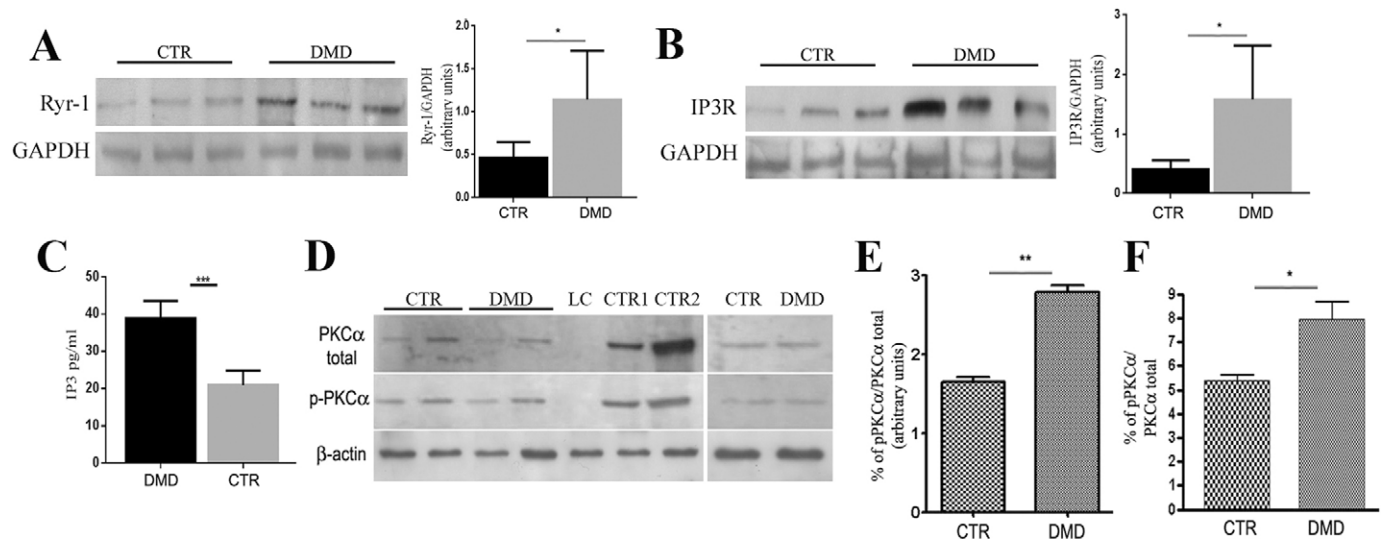


Fig. 8. Modulation of protein expression in DMD fetal muscles. (A,B) Histogram representing the ratio between RYR1 and GAPDH (A) and between IP3R and GAPDH (B), measured by WB analysis. (C) ELISA quantification of IP3 showed higher expression in fetal DMD compared with healthy muscles. (D) WB analysis of human fetal healthy and dystrophic samples for the expression of β -actin, phosphorylated PKC α and total PKC α . HeLa cells (CTR1) and brain tissue from C57BL/6J (CTR2) were used as positive controls. LC indicates loading control. (E) Histogram representing the ratio between phosphorylated and total PKC α , measured by WB analysis. (F) Histogram of emission obtained using the PepTag Assay shows that the proportion of phosphorylated PKC α was greater in DMD fetal muscle, indicating that the PKC α pathway is more activated in DMD than in healthy fetal muscles. Data are expressed as mean \pm s.d.; * P <0.05, ** P <0.005, *** P <0.001.

obtained in DMD patients, considering that in adult dystrophic muscles the number of fibers expressing slow myosin is higher than those expressing fast myosin. As discussed above, we supposed that in adult DMD muscles the sustained and prolonged calcium accumulation, which derived from membrane tears, membrane mechanical stretching and inflammation, leads to the activation of different signaling pathways, resulting in the expression of a more resistant slow-type phenotype (Higginson et al., 2002; Parsons et al., 2003, 2004).

Owing to the shortage of human samples from DMD fetuses and the difficulties in handling such fragile tissue samples, DMD fetal development was commonly considered to be unaltered. Here, we demonstrated early pathological pathways in DMD fetal muscles that differ from those previously described in adult DMD muscles. According to our data and to the demonstration that PKC α expression decreases to allow differentiation (Capiati et al., 1999, 2000), we propose that dystrophin absence causes the activation of a PLC/IP3R/calcium/PKC α pathway during development of DMD muscle, when no mechanical stress or fiber degeneration are present. This activation leads to a delay in myofiber maturation and a rearrangement of the expression of fast/slow-specific genes. From our knowledge, this is the first evidence that the myo-pathology of DMD begins with a significant delay of fetal muscle differentiation at an early fetal stage. Modulation of the IP3/IP3R pathway and fiber type specification might thus offer a powerful method to limit the muscle damage of DMD. Further studies exploring the molecular links between IP3/PKC α and proteins involved in calcium signaling will help us to understand the interplay between DAPs and calcium homeostasis, raising the hope for pharmacologically or genetically modulating each step of the involved pathways.

MATERIALS AND METHODS

Tissues

Informed consent to use human fetal tissues was obtained according to the guidelines of the Committee on the Use of Human and

Murine Subjects in Research of Ospedale Maggiore Policlinico di Milano (Milan, Italy). Twelve-week-old human fetal tissues ($n=3$) were obtained anonymously after spontaneous, voluntary or therapeutic pregnancy terminations. DMD fetuses ($n=3$) were genetically characterized by the 'Centro di Diagnosi Prenatale (Prenatal Diagnostic Center) Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico'. DMD fetuses presented different frameshift mutations of the dystrophin gene: $\Delta 47-52$, $\Delta 12$, $\Delta 19$. We analyzed the muscles of the lower limb, selecting the quadriceps for analyses. Mdx (C57BL/10 background, C57BL/10-*mdx*) and C57BL/6J mice were maintained following the authorization of the National Institute of Health and Local Committee (10/13-2014/2015); E18 fetuses were collected from both *mdx* and C57BL/6J mice. Muscle samples were frozen in liquid nitrogen, cooled in isopentane, and sectioned using a cryostat. The analysis was performed on lower limb muscles. For murine morphological analysis, the entire limb was cryosectioned at the leg level.

Immunohistochemistry

Serial 10- μ m-thick sections were stained with H&E, AM, AR (Meola, 2005) and AP (Grim and Carlson, 1990). For immunofluorescence analysis, slides were incubated with primary antibodies against the following: MyHC-developmental (NCL-MHCd), MyHC-neonatal (NCL-MHCn), MyHC-fast (NCL-MHCf) and MyHC-slow (NCL-MHCs) (1:20, Leica); laminin (1:100, L8271, Sigma); desmin (1:100, Abcam, ab8592); Pax7 and Pax3 (1:100, Developmental Studies Hybridoma Bank, AB 528428) and anti-active caspase 3 (1:50, Abcam, ab2302). Detection was performed using Alexa Fluor-conjugated secondary antibodies at 4 μ g/ml (Molecular Probes). Images were captured using the Leica TCS SP2 confocal system (Leica). For immunohistochemistry, the same primary antibodies were used with Vector SG (gray; for MyHC-slow) and Vector VIP (pink; for MyHC-fast) peroxidase kits, followed by Vectastain ABC reagent and 3,3'-diaminobenzidine (all Vector Laboratories). ImageJ software (NIH; <http://rsbweb.nih.gov/ij/>) was used for image quantification analysis. For calcium imaging, slides were incubated overnight with 5 μ M Fluo-4 (Life Technologies) and extensively washed before image capture with a fluorescence microscope (Leica DMIRE2). This protocol was modified from other published data for live tissue imaging (Ding, 2012).

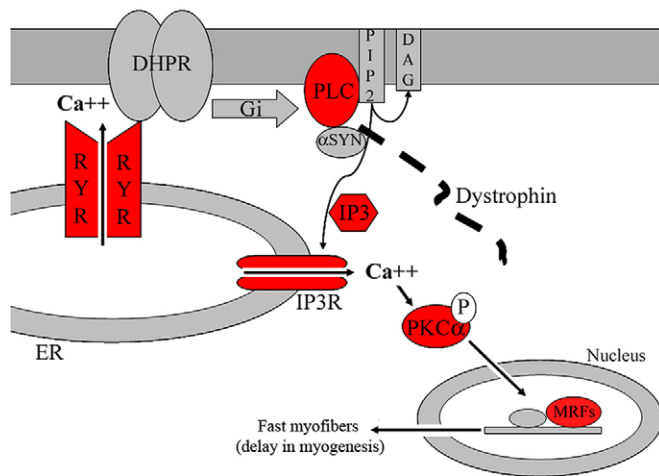


Fig. 9. A proposed model of PLC/IP3/IP3R/Ryr1/Ca²⁺/PKC α -dependent pathway in DMD fetal muscle. Normally, the dihydropyridine receptor (DHPR) is associated with RYR1: this complex is linked to a Gi protein, which in turn activates the PLC protein, regulating the levels of IP3. It is known that the binding between G proteins and α -syntrophin (α SYN) exerts modulatory effects on PLC signaling activity. As the absence of dystrophin in DMD muscles has as primary consequence the absence of α -syntrophin, this PLC-dependent pathway is disrupted and IP3 is overexpressed. This way, IP3 induces massive calcium release via IP3R activation of PKC α . When phosphorylated, PKC α is responsible for a perturbation of MRF expression, which causes the delay in myofiber maturation and the rearrangement of the expression of fast-specific genes. All the proteins that we found to be overexpressed in DMD fetal muscles are red.

Analysis of morphometrical features of DMD and CTR fetal muscle tissues

Images were captured by Leica microdissector (LMD 6500), fluorescence microscope (Leica DMIRE2) and confocal microscope (Leica TCS-SP2). For quantitative analysis, ImageJ software (NIH) was used. For quantification analysis, 30 sections from each human biopsy were analyzed. For fiber density analysis, the quantification was achieved by counting fiber number in H&E-stained samples over a fixed grid area in 30 sections from each human biopsy ($n=3$), and by counting the H&E-stained area versus the white space (extracellular space) within fibers in a fixed grid area. Manual counting of fiber area was performed using ImageJ software. Slow and fast MyHC⁺ fiber area was counted on 30 sections from each human biopsy ($n=3$). AR staining was quantified using the Threshold color Plug in of ImageJ software as the percentage of red stained area over a fixed grid area. For the analysis of MyHC fiber expression, the neonatal/developmental/fast/slow MyHC⁺ fibers were counted and expressed as a percentage of the total number of laminin⁺ myofibers over a fixed grid area. For the analysis of Pax7 expression, the Pax7⁺ cells were counted and their number was expressed relative to the number of the desmin⁺ myofibers over a fixed grid area.

Western blot analysis

Total protein concentration of muscle tissue samples from normal and DMD fetuses; brain and testis of C57BL/6J; C57BL/6J and mdx E18 fetuses; rat skeletal muscle, cerebrum and cerebellum was determined as previously described (Parolini et al., 2009). Samples were resolved on an 8% polyacrylamide gel and transferred to supported nitrocellulose membranes (Bio-Rad Laboratories). Next, the filters were incubated overnight at 4°C with the following primary antibodies: anti-phospho-PKC α (1:1000; Millipore, 06-822); anti-PKC α (1:1000; BD Biosciences, 610107); anti-CaMKII (1:500; Cell Signaling, 3362); anti-phospho-CaM Kinase II (1:1000; Thermo Scientific, MA1-047) to detect the CaMKII protein phosphorylated at threonine-286; anti-TRPC1 (1:500; Santa Cruz Biotechnology, sc-23011); anti-Ryr1 (1:500; LifeSpan Biosciences, LS-C153542); anti-Pax7 (1:500; Developmental Studies Hybridoma

Bank, AB 528428); anti-IP3R (1:500; Abcam, 155804); anti-MyoD (1:500; BD Biosciences, 554130). Then, the filters were washed, detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako) and developed by ECL (Amersham Biosciences). Densitometric analyses were performed using ImageJ software.

Quantification of phosphorylated PKC α

Phosphorylation of PKC α was analyzed using a PepTag Assay for Non-Radioactive Detection of Protein Kinase C (Promega). For each muscle, 0.2-g samples were processed according to the manufacturer's instructions. To separate phosphorylated from non-phosphorylated PKC α , samples were loaded onto a horizontal agarose gel: corresponding bands were cut and quantified by a spectrofluorometer.

RT-qPCR

Total RNA was extracted from muscles of mdx and C57BL/6J fetuses, dystrophic and normal muscle tissues, and adult normal muscle. First-strand cDNA was prepared as previously published (Benchaouir et al., 2007). Primers used are listed in Table S1, as published by Richard et al. (2011). The expression levels of each gene were measured using SYBR Green. Analyses were performed from different muscle samples in duplicate cDNA samples. Threshold cycles (Ct) of target genes were normalized against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), and relative transcript levels were calculated from the Ct values as $X=2^{-\Delta Ct}$ where X is the fold difference in amount of target gene versus *GAPDH* and $\Delta Ct=Ct_{target}-Ct_{GAPDH}$.

ELISA

The expression of IP3 was determined on fetal human and murine normal and dystrophic muscles using a mouse inositol 1,4,5,-trisphosphate (IP3) ELISA Kit (CusaBio, Wuhan, China). Frozen tissue sample homogenates were prepared as indicated by the supplier and the assay procedure was run as suggested (CusaBio). Samples and standards absorbance was read using a 'GloMax Discover' microplates reader (Promega). Results were calculated by plotting the mean absorbance for each standard using the professional soft 'Curve expert 1.3' provided by CusaBio.

Statistical analysis

Data were analyzed using Graph Pad Prism (statistical program). Data were expressed as mean \pm s.d. Fiber counts in mice were compared using Student's *t*-test. To compare multiple group means, one-way analysis of variance (ANOVA) was used. When only two groups were compared, the *t*-test was applied assuming equal variances. Fisher's Exact Test was used to compare the proportion of MyHC-expressing myofibers in CTR and DMD samples. The difference between groups was considered significant at $P\leq 0.05$.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.F., C.S., M.M. and Y.T. designed the study. A.F., C.S., L.C., D.P., U.G., F.C., S.M. performed experiments and analyzed data. A.F., C.S. and Y.T. wrote the paper with contributions from all authors. F.C. and S.M. did the immunofluorescence, D.P. the western blot analyses. A.C. provided the fetal material.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.126193/-DC1>

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