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Strategies to improve the muscle repair and the outcome of cell
mediated therapies in muscular dystrophy

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THE THESIS EXPLAINED

The biological issue

Duchenne muscular dystrophy (DMD) is a genetic disease characterized by muscle wasting and chronic inflammation. Moreover, the continuous cycles of degeneration and regeneration and the hostile microenvironment affect the satellite cells function and exhaust their regenerative capacity. Previous work done in our laboratory showed that lack of PKCΘ in mdx mice, the mouse model of DMD, improves muscle maintenance, regeneration and performance and reduces muscle inflammation. The observed phenotype was primarily due to lack of PKCΘ in hemopoietic cells, as demonstrated by BM transplantation experiments. Indeed, PKCΘ plays a unique role in T cell activation, and represents an attractive molecular target for the treatment of immune disorders. The aims of this project were to establish whether lack of PKCΘ in dystrophic muscle: (1) preserves regenerative ability of resident cell populations and (2) improves the survival and the ability of transplanted stem cells to generate new muscle fibers and correct the genetic defect of the recipient.

Results

In the present study, I show that the lack of PKCΘ in mdx mouse boosts muscle regeneration even at advanced stages of the disease and prevents fibrosis. This phenotype is associated to a higher number of Pax7 positive cells in mdxΘ-/- muscle compared with mdx muscle, during the progression of the disease. Moreover, I found that the Pax7 and Notch signalling, the crucial regulators of satellite cell self-renewal, are up-regulated in mdxΘ-/- muscle compared mdx muscle. These results suggest that lack of PKCΘ in mdx mice preserves the muscle regenerative ability improving the satellite cells pool maintenance. Beside satellite cells, lack of PKCΘ in dystrophic muscle affects also the activity of the fibro-adipogenic progenitors (FAPs), preventing FAPs conversion into fibro-adipocytes and increasing their pro-regenerative activity.

Moreover, I show in this study that lack of PKCΘ in mdx muscle prolongs survival of transplanted mesoangioblasts (MABs) within muscle, compared to mdx, and favours their ability to form myofibers.

Conclusions

These results demonstrate that lack of PKCΘ in dystrophic muscle, improves the efficacy of the resident cell populations to repair damaged muscle and the engraftment of transplanted stem cells. These effects are probably the results of reduced muscle inflammation and fibrosis, which may make the microenvironment less hostile for the formation of new myofibers from both endogenous and exogenous stem cell populations. However, the possibility that lack of PKCΘ may directly modify muscle cells behaviour cannot be ruled out. The underlying mechanisms are under investigation and may contribute to the identification of additional molecular/ cellular target to ameliorate the disease and to improve cell mediated therapeutic strategies.

1. INTRODUCTION

1.1 Muscle regeneration

Skeletal muscle is a dynamic tissue, able to modify its size and regenerate following injury. As many other postnatal tissues, the regenerative ability of skeletal muscle is due to the pool of resident stem cells, which in muscle are called satellite cells. Satellite cells were initially identified by Mauro for their location between the sarcolemma and the basal lamina of myofibers [1]. Satellite cells are quiescent in healthy, grown muscle. However, when muscle is damaged, satellite cells are activated, proliferate, and differentiate into myofibers while a minor subset self-renew. The muscle regenerative process occurs in three main phases: I) inflammatory response , II) activation and differentiation of satellite cells and III) growth and remodeling of the regenerated muscle tissue [2].

- I) *inflammatory response*: necrotis fibers induce inflammatory response that recruit circulating leukocytes. Neutrophils are the first inflammatory myeloid cells to infiltrate the damaged muscle [3]. Then, two distinct subpopulations of macrophages invade the site of the injury: M1 ($CD68^+$/$CD163^-$) and M2 ($CD68^-$/$CD163^-$) (Chazaud et al., 2009). M1 rapidly increase within 24 hours after injury and secrete proinflammatory cytokines such as TNFα and IL1. M1 are responsible for the phagocytosis of cellular debris and for inducing satellite cell proliferation. M2 increase at 2-4 days after injury and secrete anti-inflammatory cytokines such as IL-10, IL4 and IL13. M2 promote satellite cells differentiation and fusion, but also increase collagen production by fibroblasts [4-6].
- II) *activation and differentiation of satellite cells:* satellite cell activation is governed by several environment factors. Upon muscle damage, satellite cells can go through several round of amplification and then exit the cell cycle to generate a committed population of myoblasts. The committed myoblasts fuse to each other to form the new myofibers [7].

- III) *growth and remodeling of the regenerated tissue*: Myogenesis and tissue remodeling phases proceed simultaneously. The remodeling phase includes the production and re-organization of extracellular matrix (ECM), revascularization and reinnervation of regenerating myofibers.

1.1.1 Satellite cell

Satellite cells are the principal myogenic stem cell and are normally quiescent in adult muscle. Satellite cells self-renewal guarantees the muscle ability to regenerate following multiple injuries. Once activated, satellite cells can generate functionally different daughter cells by asymmetric division: one daughter will go on to differentiate after a variable number of symmetric cell divisions, while the other will return to the quiescent state, locating between the basal lamina end the plasmalemma of the repaired myofibers. As shown in Figure 2, the different progenies can be distinguished by the expression of Paired box 7 (Pax7) and Myogenic Differentiation 1 (MyoD): the Pax7+MyoD− expressing cells as the self-renewal population, the Pax7+MyoD+ as proliferating and the Pax7−MyoD+ as differentiating (Zammit et al., 2004). Pax7 is a transcription factor considered as a satellite cell marker. It is required for satellite cell specification, maintenance and muscle regeneration. Pax7 plays a crucial role in maintaining satellite cell quiescence and self-renewal, and is expressed by both quiescent and activated satellite cells. In activated satellite cells, Pax7 promotes cell cycle exit and return to quiescence, preventing the differentiation and ensuring the replenishment of the satellite cell compartment during skeletal muscle regeneration[8]. Pax7 null mice exhibit normal muscle formation, but reduced postnatal muscle growth [9]. The number of satellite cells in Pax7 mutant mice strongly declines during postnatal growth and muscle regeneration is impaired [10]. Pax-7 overexpression in myoblasts down-regulates MyoD expression and transcriptional activity [11]. MyoD is considered the myogenic master regulator of the skeletal muscle

gene expression program [12]. MyoD activity induces Myogenin (MyoG) expression promoting terminal differentiation. The ratio of Pax7 and MyoD activities are critical for satellite cell self-renewal and myogenic differentiation processes [11].
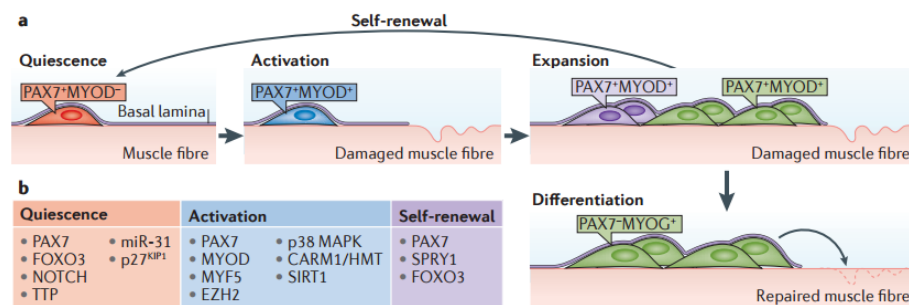

Figure 2. a. Satellite cell activation upon muscle injury **b** Cell-intrinsic factors that have roles in satellite cell quiescence, activation and self-renewal [13].

Satellite cell fate is mostly determined by the microenvironment. Multiple factors within the niche can regulate satellite cell activation, differentiation and self-renewal. One of the pathways known to be important for the maintenance of diverse types of adult stem cells is the Notch signalling[14-16]. Notch is a family of transmembrane receptors including Notch1,-2,-3 and -4. The interaction with its ligands (Delta-like 1, Delta-like 4, Jagged 1 and Jagged 2) induces protease cleavage of the intracellular domain of Notch (NICD). NICD is a transcription coactivator that interacts with RBPJ and induces its binding to DNA to regulate the expression of Notch targets (Hey1, HeyL, Hes) [17]. Also in skeletal muscle, Notch signalling appears to be one of the pathway involved in regulating satellite cell activation/self- renewal. Notch 1,2 and 3 are expressed in quiescent satellite cell, while muscle fibres are the major source of Notch ligand [18]. Several resident cells, including PICs, FAPs and mesenchymal cell, could also be source of Notch-ligand [16]. It is known that Notch activation prevents myogenic differentiation and promotes satellite cell self-renewal, by

upregulating Pax7 and inhibiting MyoD [19]. By contrast, the inhibition of Notch activity by Numb, a Notch inhibitor, promotes myoblast differentiation [20]. In the absence of Notch, satellite cells undergo accelerated terminal differentiation without self-renewal, resulting in satellite cell depletion [21]. Moreover, Notch signalling inhibition in muscle stem cells causes also several muscular dystrophic features and impaired muscle regeneration [22]. Notch activity is high in quiescent satellite cells and decrease in activated myogenic progenitors. The rapid decrease of Notch activity after injury reflects the physiological dissociation of satellite cells from the ligand expressed on myofibers. During muscle regeneration, temporary decrease of Notch signalling should be necessary to exit quiescence and allow amplification of the myogenic population. When activated satellite cells return to quiescence, by day 4-5 post-injury, Notch activity increases again. The upregulation of Notch signalling is crucial to the re-establishment of self-renewed satellite cells [18]. During muscle regeneration process, the Notch activity in satellite cells could be supported by Delta1 expressed on myofibers. Indeed, following injury, Delta1 is up-regulated in committed myogenic cells supporting Notch activation in satellite cells. Insufficient upregulation of the Notch ligand, Delta-1, following injury in old animals leads to a decrease in their regenerative potential, which can be restored by forced activation of Notch [23]. A possible model of how Notch signalling may regulate satellite cells self-renewal is summarized in Figure 3.
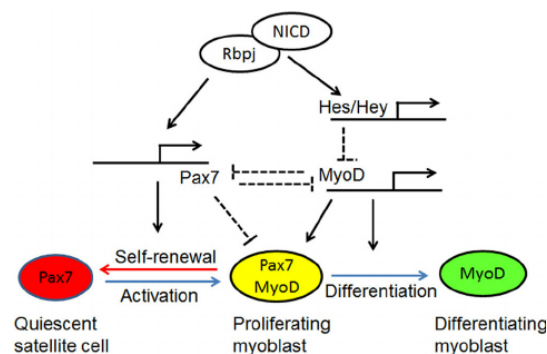
Figure 3. Notch's regulation of stem cell fate in muscle. Activated Notch (NICD) binds to RBP-J to form a transcriptional activation complex that upregulates the transcription of Pax7 and inhibits MyoD, promoting satellite cell self-renewal and blocking myogenic differentiation [19].

1.1.2 Factors and cell population involved in skeletal muscle regeneration

As summarized in Figure 1, satellite cell activity is regulated by several factors which compose their niche. including ECM and local cell populations. Alterations in any components of satellite cell niche may thus affect the satellite cell functionality.
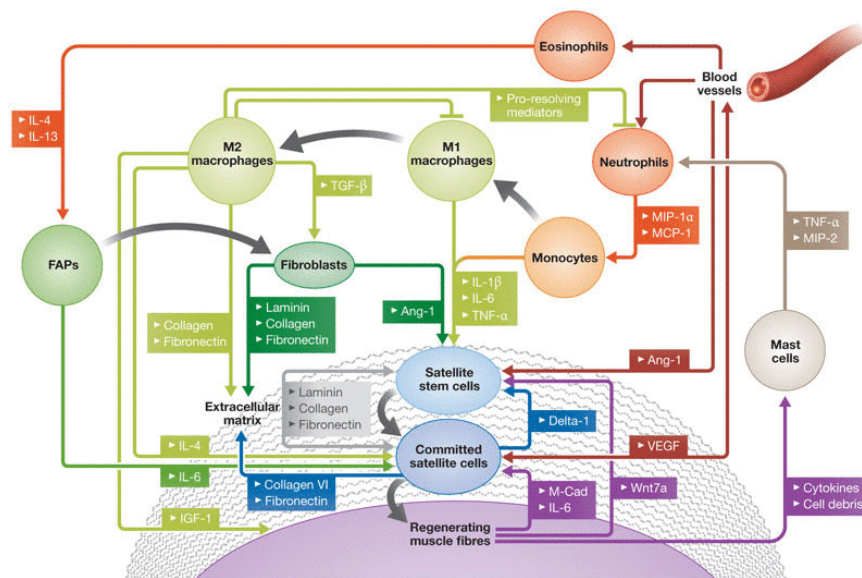
Figure 1. Signals operating in skeletal muscle stem cell niche [24]

ECM
In general, ECM provides the mechanical support, influences the cellular differentiation and operates as a scaffold for nerves, vessels, and muscle cells [25]. Moreover, ECM can modulate the satellite cells activity by either direct or indirect action. In fact, ECM components can establish biochemical gradients by binding growth factors, such as FGF and HGF, thus regulating their bioavailability [26]. On the other hand, ECM components can directly bind receptors on satellite cell surface, mediating their anchorage and regulating several signaling pathways[27]. Satellite cells are anchored to sarcolemma and basal lamina through α7/β integrins, and during the muscle damage, the destroy of basal lamina mobilizes the satellite cells contributing to their activation. Several components of ECM can affect satellite cells differentiation. Among them, it was shown that collagene VI is required for satellite cell self-renewal and for the maintenance of satellite cell number following multiple muscle injuries [28].

Local cell populations
Several cell types are involved in the regulation of the satellite cell niche during the muscle regeneration. Secreted factors, vesicles, microsomes and cell-cell signaling may all contribute to satellite cell behavior.

Endothelial cells. Skeletal muscle has dense microvasculature that represented an important component of niche. Indeed, in uninjured muscle, most satellite cells are closely associated with capillaries [29]. The vascular endothelial cells release vascular endothelial growth factor (VEGF) that contributes to satellite cell activation, proliferation and myofiber regeneration [30]. Blood vessels represent also a source of progenitor cells. Indeed, pericytes are mesenchymal cells residing within microvasculature and are multipotent progenitor cells. Pericytes can contribute to muscle regeneration differentiating into myofibers [31].

Immune cells. In uninjured muscle, only a small number of immune cells are present. Instead, upon injury, the immune cells are recruited in the damaged area and rapidly infiltrate the muscle constituting a transient local environment for satellite cells. Inflammatory cells remove the necrotic tissue and secrete growth factors, chemokines and cytokines, such as IL-1, IL-6, TNF-α, that can promote satellite cell activation [32].

Interstitial cells. In addition to satellite cells, there are several muscle interstitial resident stem/progenitor cell populations able to give rise the myofibers. It has been reported that a cell population described as PW1+/Sca1+/CD34+/Pax7- (PICs) can spontaneously enter myogenic differentiation and contribute to new myofiber formation during muscle regeneration [33]. Another interstitial cell population involved in muscle repair process are the fibroadipogenic progenitors (FAPs). FAPs are able to differentiate into fibroblasts and adipocytes [34]. The ability of FAPs to differentiate into myofibers is still outstanding statement. In healthy muscle, FAPs arelocated in the interstitial space between the myofibers and are undifferentiated. After the acute muscle damage, FAPs rapidly enter the cell cycle invading the space between the regenerating myofibers. During the proliferative phases, FAPs release signals inducing the differentiation of primary myoblasts [34, 35]. The number of FAPs increases between 3-4 days after the injury, then declines, returning to pre-injury level. When regeneration fails, such as in muscular dystrophy and chronic damage, FAPs persist in the tissue and differentiate into adipocytes and fibroblasts mediating the fat and extracellular matrix deposition. It was shown that FAPs isolated from dystrophic muscle have a more pronounced ability to form adipocytes in vitro compared with FAPs isolated from healthy muscle [36, 37]. The chronic muscle diseases are frequently characterized by chronic inflammation. Immune cells can regulate FAPs proliferation, differentiation and survival. It has been shown that the type 2 innate immunity stimulates FAPs to facilitate muscle regeneration by the IL4/IL13 signaling. Type 2 cytokine signaling promotes proliferation of FAPs and inhibits their differentiation into

adipocytes [38].Also TNF and TGFβ released by macrophages modulate FAPs expansion. TNF induces apoptosis of FAPs preventing their extended permanence in the tissue and their differentiation into fibroblasts and adipocytes, while TGFβ prevents FAPs apoptosis and triggers their differentiation [39].

1.2 Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a severe form of muscular dystrophy affecting up to one in 3,800–6,000 live male births [40]. DMD is caused by mutations in DMD gene on the X chromosome (Xp21.2) which encodes for dystrophin [41]. Dystrophin is a member of dystrophin-associated glycoproteincomplex (DGC) localized in the sarcolemma. DGC acts as a bridge between the ECM and cytoskeleton and stabilizes the sarcolemma during muscle contraction. Lack of dystrophin in muscle fibers causes membrane instability increases the probability of damage during contraction and leads to fibers degeneration. As a result, dystrophic muscle is characterized by persistence inflammation, triggered by cycles of degeneration and regeneration. Chronic inflammation impairs muscle regeneration and promotes the deposition of fibrotic and adipose tissue, which progressively replace the muscle tissue, reducing the amount of tissue available for therapeutic intervention [29]. DMD patients manifest progressive muscular weakness and wasting of various voluntary muscle of the body and die of respiratory or cardiac muscle failure in early adulthood.

1.2.1 Muscle repair in dystrophic muscle

In DMD patients, the necrotic fibers are initially replaced by new fiber generated by satellite cells. However, the muscle regeneration ability rapidly declines with age. In mdx mice, a DMD animal model, the muscle repair after injury is impaired compared with WT mice (Fig. 4). In DMD patients, muscle regeneration is more rapidly

exhausted compared with mdx mice. Worse recovery of dystrophic muscles could due to the hostile environment and the exhaustion of satellite cell pool. Under normal repair condition, inflammatory cells and ECM components provide the molecular factors and structural scaffold for generation of new tissue. Pathological changes in the muscle environment may impair myogenic function of satellite cells. Both human and mouse dystrophic muscle present an accumulation of CD4+ and CD8+ T cells, macrophages, eosinophils and natural killer T cells infiltrate [42]. Also several cytokines and chemokines are present in dystrophic muscle. Cytokines have pleiotropic effects and may have pro- or anti-inflammatory properties. Several different cytokines have effects on muscle resident cells during the regeneration process. Among up-regulated cytokines in dystrophic muscle, TGFβ play an important role in fibrosis development [43]. After muscle injury, TGFβ is produced by infiltrating inflammatory cells. TGFβ stimulated fibroblast to produce collagen and fibronectin and, moreover, promotes muscle atrophy [44]. The frequent cycles of degeneration-regeneration constantly activate the satellite cells. Continuous activation of satellite cells could exhaust the satellite cell pool over time. It has been shown that cultured satellite cells from mdx muscle display accelerated differentiation compared to non-dystrophic satellite cells, supporting the notion that their proliferative capacity is compromised [45]. Together to the impairment of proliferative activity, also lack of dystrophin may be responsible for reduced muscle repair efficacy in dystrophic muscle. In fact, dystrophin is expressed in differentiated myofibers but also in activated satellite cells, where it appears to be crucial to cell polarity. It has been recently shown that lack of dystrophin in satellite cells perturbs their polarity leading to impaired asymmetric cell division [46]. The impairment of asymmetric cell division leads to reduction of progenitors that are able of contributing to muscle repair in the long term [46]. Moreover, it was shown that, in dystrophic muscle, the decrease in the number of satellite cells during aging is correlated to impairment of Notch signalling. Enforced activation of Notch

pathway rescued the self-renewal of mdx satellite cells [47]. Intriguingly, two Golden Retriever muscular dystrophy (GRMD) dogs, the dog model of DMD, with a milder dystrophic phenotype was identified. These animals display an increase of expression of Jagged1, suggesting that promoting Notch signalling may represent a target for DMD therapy in a dystrophin-independent manner[48]. In fact, as mentioned before, Notch pathway is involved in asymmetric cell division. During cell division, Delta1 and Numb have an asymmetric distribution that leads to the asymmetric inheritance of Delta 1 and numb in committed daughter cell [20]. It remains unknown whether Notch overexpression may restore the asymmetric division in dystrophic satellite cells.
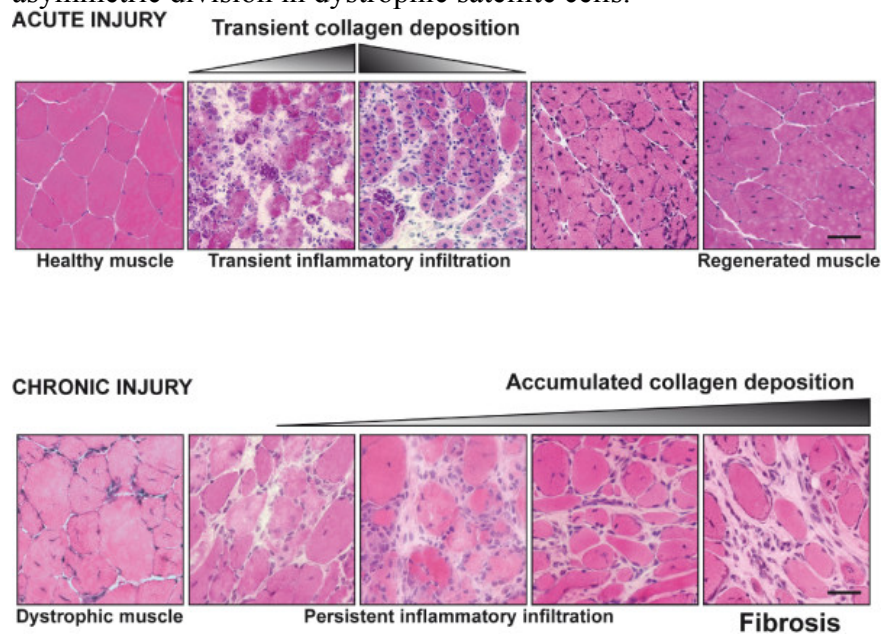

Figure 4. Skeletal muscle regeneration in acute and chronic injury. Dystrophic muscle is characterized by abnormal muscle repair, characterized by extensive infiltration of mononuclear cells and fibrosis [49].

1.2.2 Therapies for DMD

Currently, there is no cure for DMD and treatment is limited to glucocorticoids that prolong ambulation but they have more negative side effects. Multiple treatment strategies are under investigation. Treatment strategies can be divided into two major categories: treatment strategies that aim to restore dystrophin expression and treatment strategies that aim to compensate for lack of dystrophin and counteract the pathology progression by decreasing inflammation and fibrosis and promoting muscle regeneration [46, 50].

Therapies to restore dystrophin expression
- Exon skipping: is a molecular approach that target the specific sequence of pre-mRNA to restore the reading frame within the dystrophin gene. Exon skipping acts through antisense oligonucleotides (ASOs) that hybridize in a complementary manner to pre-mRNA and modulate its splicing process. Among the several ASOs investigated, 2'O-methyl-phosphorothioate oligonucleotide (2'OMetPS) and phosphorodiamidate morpholino oligomer (PMO) are currently under advanced phase in clinical trials [50].
- Read-through therapy: aims at suppression of translation termination at in-frame premature stop codons to restore deficient protein function. Two compounds with read-through function are being investigated in human patients: aminiglicosyde antibiotic gentamicin and atalauren. In the studies using gentamicin and atalauren, patients treated had a modest increase in dystrophin levels and, moreover gentamycin is nephrotoxic. [51, 52]. Novel compounds are also investigated.

- Gene therapy: several virus vectors are studied to deliver functional copies of dystrophin gene. There have been developed the adeno-associated virus (AAV)-based therapies. AAV vectors are the most used vector for their low immunogenicity, the ability to remain stable in nonreplicating cells and tropism for muscle.
- Cell therapy: is a promising clinical option that uses the ability of stem cells to differentiate into a specific lineage and correct the genetic defect of the recipient. Stem cell populations used can be heterologous cells from normal donors or be autologous genetically corrected cells. Several stem cell populations are being investigated in cell therapies for DMD. These populations include myoblasts, CD133+ stem cells, pericytes and mesoangioblasts (Mabs)[50]. Promising results were reported on intramuscular or intra-arterial transplantation of Mabs. Mabs are vessel associated stem/progenitor cells and are able to differentiate into muscle fibers. They possess several receptors for inflammatory cytokines that allow them to migrate to regions where inflammatory process is ongoing. It has been shown that when they are transplanted into the femoral artery of dystrophic animals, they are able to cross the vessel wall and migrate within skeletal muscle, especially in the area where degenerative and regenerative processes are ongoing [53, 54]. Current clinical trials are examining the effectiveness of Mabs transplantation in the treatment of patients with DMD. The phase I/IIa trial of multiple intra-arterial infusions of HLA-matched donor MABs into five DMD patients was reported to be relatively safe but no effects on muscle function was observed [55]. Although this study appeared somehow discouraging, it provided essential

information to modify the plan of future cell therapy protocols in DMD patients. As for example, this study suggested that immunosuppressive treatment is important to preserve Mabs survival but also can reduce the recruitment of Mabs in target tissue and the further trials could target younger patient, where the fibrosis is less.

Therapies that compensate for the lack of dystrophin

- Anti-inflammatory therapies: the inflammatory response to damaged fibers is the main mechanism leading to disease exacerbation. At moment, glucocorticoids, such as prednisone and deflazacort, are the only pharmacologic therapies shown to increase muscle strength and prolong independent ambulation, but present several side effects. Several potential targets are being investigated to inhibit or enhance pro- or anti-inflammatory factors released from DMD skeletal muscles. The new potential target could be more efficient and have less side effects compared with steroidal drugs [56].

- Epigenetic therapies: Epigenetic mechanisms respond to the environment signal through changes in gene expression. Epigenetic modifications are essential for development and differentiation. Aberrant epigenetic modifications occur in several pathologies [57, 58]. It was shown that HDACs are involved in the progression of muscular dystrophy in mdx mice [59]. Histone Deacetylase Inhibitors (HDACi) treatment was reported to improve muscle performance in mdx mice, increasing membrane stability and reducing inflammatory infiltrate [60]. Most of the beneficial effects

of HDACi arise from their ability to inhibit FAPs adipogenic potential and promote their myogenic activity [37]. In fact, HDACi modulate, in FAPs, the SWI/SNF BAF60 variants by up-regulation of myogenic miRs [61]. Phase I/II clinical trial of HDACi treatment on children affected by DMD is currently ongoing [62].

1.3 PKCΘ

PKCΘ is a member of PKC family. PKC family includes 12 isoforms of serine/threonine protein kinases that are subdivided into three groups based on their regulatory domains and their second messenger requirements for activation:

- Classical PKCs: α, βI, βII, and γ require phospholipids and calcium for activation.
- Novel PKCs: δ, ε, θ, and η do not require calcium for their activation
- Atypical PKCs: ζ, and ι/λ can be activated in the absence of diacylglycerol and calcium

PKCΘ is most abundant in hematopoietic cells, especially T-cells, platelets, mast cells and natural killers. PKCΘ plays an important role in t-cell activation, differentiation and survival. Indeed, inhibition of PKC-θ is considered as a potential immunosuppressive strategy for controlling T cell mediated immunity. PKCΘ is highly expressed also in skeletal muscle and regulates the muscle cell development, homeostasis and remodelling [63].

1.3.1 PKCΘ in T-cells

PKCΘ is observed in immunological synapse (IS) [64], a T-cell specialized membrane microdomain where TCR signalling molecules, including the TCR itself, are assembled. Following the antigen stimulation of T cell, the signalling from TCR/CD28 induces the DAG accumulation in plasma membrane, which recruits PKCΘ to IS (Fig. 5). PKC-θ mediates the activation of transcription factors such as NF-κB, AP-1, and nuclear factor of activated T cells (NFAT), that are crucial for activation of IL-2 gene [65-67]. Indeed, PKCΘ activity is involved in T-cell activation and differentiation in Th2 and Th17 cells. It was shown that PKCΘ, after t-cell stimulation, can translocate to the nucleus and form the chromatic-anchored complex localized to the promoter of inducible immune responsive gene [68, 69]. PKCΘ deficient T-cells display defects in activation, survival and differentiation.
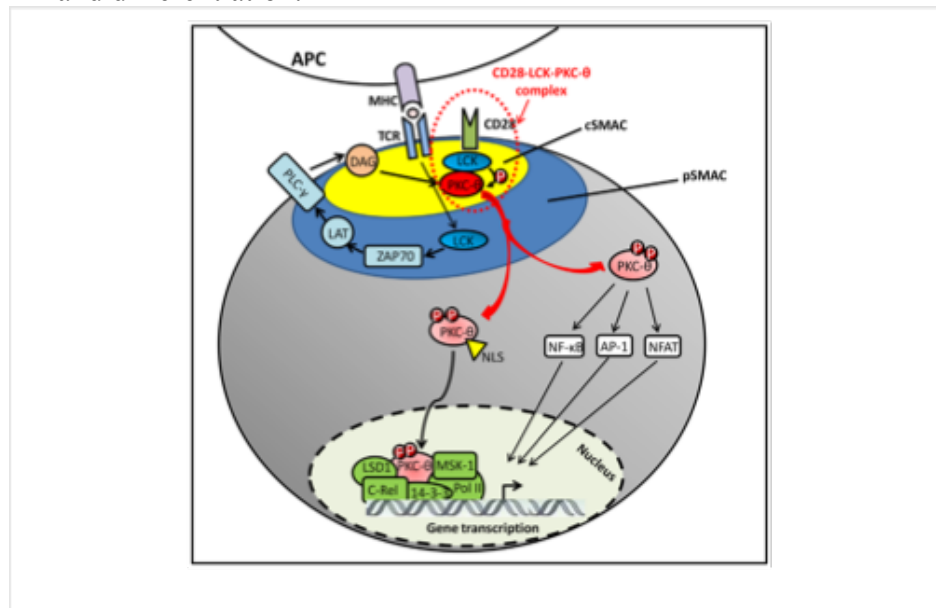

Figure 5. PKCΘ translocation in immune synapse [70].

Given the central role of T-cells in several immune diseases and the crucial role of PKCΘ in T-cell fate, PKCΘ might be considered a selective target for the treatment of immune disorder without causing overt immunosuppression. Indeed, it was shown that PKCΘ deficient mice were resistant to several autoimmune syndrome such as myelin oligodendrocyte glycoprotein –induced encelophalomyelitis and myosin-induced autoimmune myocarditis [71, 72]. Moreover, in cardiac allograft experiment, PKCΘ deficient mice displayed delayed rejection reaction. suggesting that inhibition of PKC-θ can achieving long-term survival of allografts [73, 74].

1.3.2 PKCΘ in skeletal muscle

PKCΘ is the PKC isoform predominantly expressed in skeletal muscle, where it plays multiple roles during development and in the adulthood. During development, PKCΘ expression was detected in fetal myoblast but not in embryonic myoblast, suggesting that it is involved in the differentiation of different muscle precursors [75, 76]. Actually, embryonic and fetal myoblasts display a different sensitivity to TGFβ [77], which contributes to the asynchronous differentiation during limb muscle formation. Embryonic myoblasts are resistant to the inhibitory effect of TGFβ on myotube formation but forced expression of PKCΘ prevented myotube formation in the presence of TGFβ [75]. Moreover, PKCΘ forms a complex with nuclear factor one X (Nifx) which activates fetal and suppresses embryonic gene expression, operating as a transcriptional switch from embryonic to fetal myogenesis [78]. PKCΘ can also regulate gene expression regulating HDAC4/5 nuclear export. In fact, it was previously shown that in C2C12 muscle cells (a myoblast cell line from adult C57 mouse) PKCΘ activity led to phosphorylation dependent HDAC5 nuclear export favouring MEF2 activation [79]. On the other hand, PKCΘ activity is involved in post-natal muscle growth; in fact, it was previously

shown that mice deficient in PKCΘ display a delayed body and muscle-fiber growth during the first weeks of postnatal life. In adult mice, lack of PKCΘ delays also myofiber formation after injury due to reduced up-regulation of genes involved in fiber fusion such as caveolin-3 and β1D- integrin [80]. Although PKCΘ muscle deficient does not show any morphological alterations, it displays altered signalling pathways involved in response to environment stimuli. Indeed, it was also shown that lack of PKCΘ prevents ER-stress-induced autophagy in starved and immobilized muscle [81].

1.3.3. PKCΘ in DMD

As mentioned before, inflammation plays an important role in DMD progression. Indeed, several anti-inflammatory strategies are proposed for the DMD treatment. In DMD patients and mdx mice infiltrating macrophages and T cells are detected in muscle during the early stages of disease [82]. Mdx mice treated with antibody depleting CD8 or CD4 cells and mdx genetically depleted of T and B-cells (scid/mdx mice) present a significant reduction of fibrosis suggesting that the T cells activity is crucial in dystrophic muscle [83]. In our laboratory, we showed that lack of PKCΘ in mdx reduced muscle wasting and infiltration. Bone marrow transplantation experiments demonstrated that the phenotype observed in mdxΘ-/- mice is mainly due to the lack of PKCΘ in hematopoietic cells [84] (Marrocco et al. unpublished observation). Indeed, current investigation in the laboratory is showing that PKCΘ is required for very early T cells infiltration in dystrophic muscle, orchestrating the immune response (Lozanoscha-Ochser et al *ms in preparation*). We also observed significant improvement in muscle morphology and function in mdx mice treated with PKCΘ inhibitors (Marrocco et al. submitted). Together, these observations make PKCΘ an attractive target for DMD treatment. PKCΘ inhibition, in DMD, could not only slow down the progression of diseases but may

also favour the efficacy of therapy aiming to restore the dystrophin expression. Indeed, cell therapy needs to be combined with the appropriate immunomodulation therapy to regulate the adaptive immune response. However, it is still necessary to better characterize the effect of lack or inhibition of PKCΘ in immune cell infiltrate in dystrophic muscle.

2. AIMS

2.1 AIM 1: To establish whether lack of PKCΘ in mdx mice improves the efficiency of the resident cell populations to repair damaged muscle

Previous results obtained in the laboratory showed that lack of PKCΘ in 8 week old mdx mice reduced muscle necrosis and immune cell infiltrate improving muscle regeneration. It was thus proposed that lack of PKCθ makes a more favourable environment for muscle precursor cells to differentiate. To verify this hypothesis, as first, I wanted to understand whether muscle regeneration was boosted in dystrophic muscle lacking PKCθ, assessing the kinetics of muscle regeneration and muscle degeneration in mdx and mdxΘ-/- mice, during the progression of the disease Moreover, I wanted to compare the ability to repair damaged muscle following an acute muscle injury in both genotypes. In addition, I evaluated the activity of cell populations mainly involved in skeletal muscle regeneration: satellite cells and FAPs. In fact, satellite cells behaviour changes during the progression of DMD and the preservation of their self-renewal and regenerative ability is an important therapeutic strategy. On the other hand, FAPs are the interstitial muscle cells that may participate in muscle regeneration supporting the satellite cell myogenic activity, but also in muscle degeneration differentiating promoting fibrosis and fat deposition. Modulation of FAPs activity is thus proposed to improve muscle repair.

2.2 AIM 2: To establish whether lack of PKCΘ in mdx mice improves the engraftment of transplanted stem cells

Since PKCΘ has been shown to play a key role in the development of autoimmune disease and in allograft rejection, I aimed to verify whether its inhibition may contribute to the

effectiveness of cell therapy in dystrophic muscle, promoting the survival of transplanted cells. To this aim, I wanted to verify engraftment and survival of transplanted beta-gal expressing mesoangioblasts in mdx muscle lacking PKCΘ, compared to mdx

3. RESULTS

3.1 Lack of PKCΘ improves muscle repair and promotes satellite cell pool maintenance in *mdx* mice

3.1.1 Lack of PKCΘ reduces the muscle degeneration in mdx mice and boosts the muscle regeneration

It is well known that in mdx mice, the progression of the disease follows distinctive phases of muscle degeneration and regeneration. Up to two weeks of age, the muscle morphology is similar in mdx and healthy mice. The process of muscle degeneration starts at 3 weeks of age and peaks at 4 weeks. The high level of muscle degeneration is followed by high levels of muscle regeneration. From 3 months of age, both the cycles of degeneration and regeneration are attenuated and the skeletal muscle enters a stable phase [82, 85].

Previously we showed that lack of PKCΘ in mdx mice at 2 month of age prevented muscle wasting and improved muscle regeneration, maintenance and performance. I here analyzed muscle degeneration and regeneration at different ages in mdx and mdxΘ-/- mice to characterize the kinetics of disease in both genotypes. Muscle degeneration was evaluated in tibilis anterior (TA) as the extent of damage area in H&E stained cryosection, while muscle regeneration as the extent of area positive for embryonic myosin heavy chain (eMHC) immunostaining.

As shown in Figure 6, muscle damage was very high in 4week old mdx mice, as expected, to suddenly drop down by 6 weeks of age; after this age, muscle damage continued to slowly decrease, up to 12 months of age. By contrast, muscle damage was much lower in 4 week old mdx mice lacking PKCΘ, and this low level was maintained throughout the ages, besides a slight increase at 6 months of age. On the other hand, in mdx muscle, the high level of muscle regeneration observed at 4 weeks of age, dropped drastically by 6 weeks and continued at very low level until 12 months of age. In mdxΘ-/- muscle the level of muscle regeneration was low but

persistent throughout the ages. Expressing these results as the ratio of regenerating area over damage area, in mdxϴ-/- muscle the ratio is higher at all the ages examined, suggesting that the regeneration process is boosted compared to mdx during the progression of the disease.
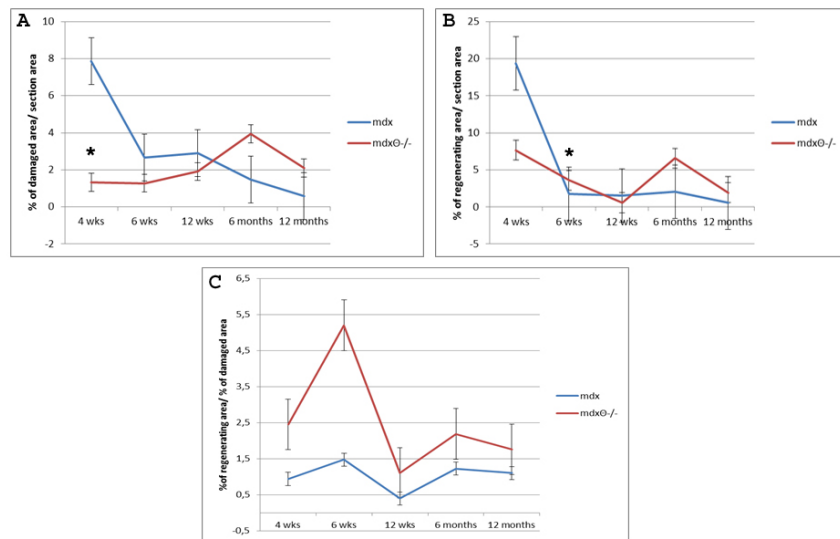
Figure 6. Muscle degeneration and regeneration kinetics in mdx and mdxϴ-/- mice. A Muscle degeneration was quantified as the percentage of damaged area over the total area in H&E stained TA cryosections. **B.** Muscle regeneration was quantified as the percentage of eMHC positive area over total area of TA cryosections. **C.** Ratio of regenerating area on damaged area. The results are mean±SEM (n= 4-5 for each age; *P<0.05 two-tailed Student's t-test).

During the progression of disease, the chronic damage and inflammation is known to lead to an increase in fibroblast ECM production that gives rise to the fibrotic tissue. Fibrosis is one of most deleterious aspects of DMD since the aberrant deposition of ECM limits the amount of contractile muscle tissue [86]. I thus analyzed the collagen deposition in TA muscle derived from mdx and mdxϴ-/- mice at different ages. The Masson's trichrome

staining of TA sections (Fig. 7) showed a significant increase of collagen deposition in mdx mice compared to WT mice at 6 weeks, as expected. The difference in collagen deposition between mdx and WT increased further at 6 and 12 months. By contrast, the lack of PKCΘ partly prevented the collagen deposition in mdx muscle, at the examined ages. The decreased deposition of ECM components in dystrophic muscle should therefore preserve the architecture and the functionality of muscle tissue, and assure the correct and complete repair process after the injury.
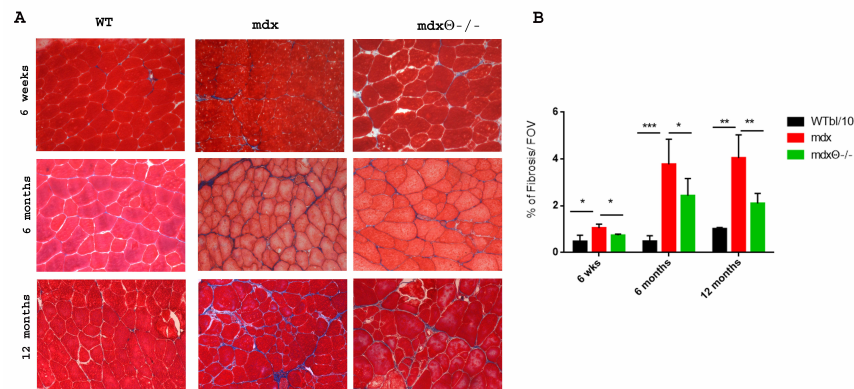
Figure 7. Fibrosis accumulation is reduced in mdxΘ-/- muscle compared with mdx. A Representative images of Masson's trichrome staining of cryosections derived from WT/, mdx and mdxΘ-/- TA muscle at 6 weeks, 6 and 12 months**. B.** Fibrosis was quantified as percentage of Masson's trichrome positive area per field of view in TA section.(*P<0.05, **P<0.01, ***P<0.001 two-tailed Student's *t*-test).

3.1.2 The muscle repair after injury is improved in mdxΘ-/- compared with mdx mice.

The continuous cycles of degeneration and regeneration and the hostile dystrophic environment are believed to exhaust the regenerative capacity. Indeed, after acute injury, the muscle repair is impaired in mdx mice compared with WT mice [87] and gets even

worse with the ages [47]. We thus wondered whether lack of PKCΘ may improve the regenerative ability of dystrophic muscle upon muscle injury. TA muscle of 6 month old WT, mdx and mdxΘ-/- mice were injured by cardiotoxin (CTX) injection. It has been previously shown that CTX-stimulated increase of satellite cells is prevented in 6 month old mdx mice [47]. The mice were sacrificed 7 days after CTX injection and the number of regenerating myofibers, identified as the centrally nucleated fibers, was counted in H&E stained section of TA muscles. As expected, the number of regenerating fibers in the damaged area was lower in dystrophic muscle compared with WT muscle (Fig. 8). By contrast, a significant higher number of regenerating fibers was found in TA muscle derived from mdxΘ-/- mice compared with mdx. Skeletal muscle repair following injury requires also the deposition, remodelling and reorganization of ECM. ECM deposition occurs within a week post-injury and it can go on for several weeks, to ensure complete repair. However, in cases of chronic injuries, such as in dystrophic muscle, the newly generated ECM can transform into scar tissue [88]. As shown in Figure 8, at 7 days after CTX injury, the amount of ECM deposition in the injured area was greater in the mdx mice than WT mice, as expected. However, lack of PKCΘ partly prevented ECM deposition in mdx mice. Together these results suggest that the lack of PKCΘ in dystrophic muscle preserves the ability of skeletal muscle to repair the damaged area.
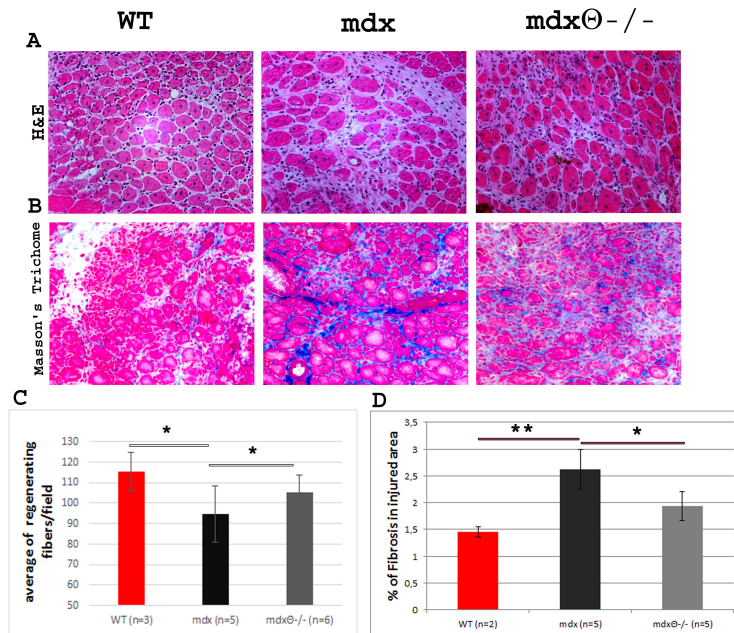
Figure 8 Muscle regeneration upon injured is improved in mdxΘ-/- mice compared with mdx. Representative images of TA stained with H&E **(A)** and Masson's trichrome staining of 6 month old WT/bl10, mdx and mdxΘ-/- mice at 7 days after CTX injury **(B)**. Average of number of regenerating fibers **(C)** and quantification of collagene deposition **(D)** per field of view. (*p<0.05, **p<0.01, two-tailed Student's *t*-test).

3.1.3 Lack of PKCΘ in mdx mice preserves the self-renewal ability of satellite cells.

Given the reduction of muscle damage during the early stages of disease in mdxΘ-/-, we wondered whether the improvement of repair ability was due to more efficient maintenance of satellite cell pool compared with mdx mice. In fact, it is believed that in muscular dystrophy the continuous rounds of damage can exhaust the self-renewal ability of satellite cells [87]. In the early stage of muscle repair process, quiescent MuSCs migrate to the site of injury and

proliferate. Following the proliferation phase, a subset of MuSCs undergoes myogenic differentiation, downregulating Pax7 expression and upregulating MyoD expression. Another subset of proliferating MuSCs mantains Pax7 expression, instead, and returns to a quiescent state ensuring the long-term self-renewal [89-91].

Since MuSC self-renewal may be improved in dystrophic muscle lacking PKCΘ, it is conceivable that the observed improved regeneration was due to maintenance of satellite cells number within mdxΘ-/- muscle. The number of total Pax7 positive cells in TA section from mdx and mdxΘ-/- mice at different ages, was then counted. As shown in Figure 9, the number of Pax7 positive cells was significantly higher in TA muscle from mdxΘ-/- than mdx mice at any of the examined age (Fig.9).
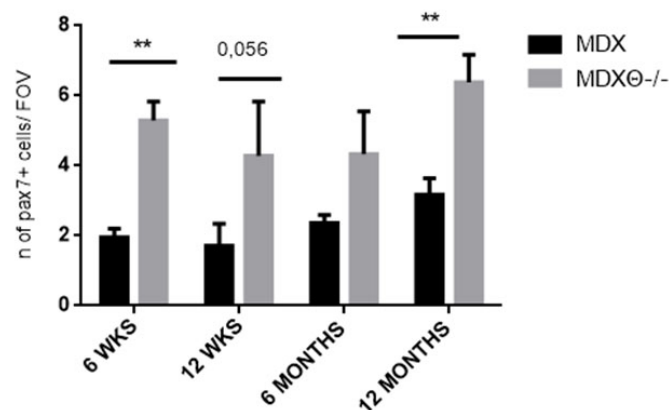
Figure 9. Lack of PKCΘ in mdx mice increases the number of Pax7 positive cells in vivo. Number of Pax7+ cells in TA section of mdx and mdxΘ-/- mice at 6weeks, 12 weeks, 6 and 12 months of age. The number of Pax7+ cells was calculated by counting the number of PAX7+ cells per field of view in immune-stained sections. The results are the means±STDEV for three different mice in each genotype at different ages. (*p<0.05, **p<0.01, two-tailed Student's *t*-test).

Further, we found, by qRT-PCR analysis, that the level of expression of Myogenin, was higher in mdxϴ-/- mice compared with mdx mice at 12 months of age (Fig.10). Myogenin expression is up-regulated in differentiating satellite cells, and it is considered as a marker of muscle regeneration. This increase was not observed in muscle derived from PKCϴ-/- as compared with WT, suggesting that it is a consequence of lack of PKCϴ specifically in dystrophic muscle. Together, these results demonstrate that lack of PKCϴ in mdx mice increases the number and the regenerative ability of satellite cells, compared with mdx
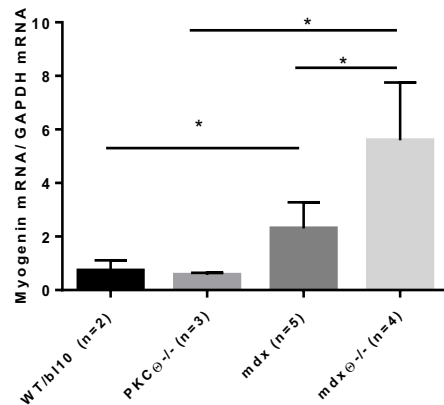
Figure 10. Increased Myogenin level in mdxϴ-/- mice at 12 months of age. Myogenin mRNA levels in PKCϴ-/-, mdx and mdxϴ-/- TA muscle was normalized to those in WT/bl10 TA muscle. (*$p<0.05$, two-tailed Student's *t*-test).

Together, these results suggest that lack of PKCθ in dystrophic muscle improves MuSCs self-renewal, thus maintaining muscle regenerative ability. Several factors pathways are involved in MuSCs self-renewal. Many studies showed that Notch signalling is essential for maintenance of satellite cell pool and is perturbed in ageing and muscle diseases [23, 47, 92].
 To study the effect of lack of PKCϴ in dystrophic muscle on satellite cell function, I isolated the MuSCs from limb muscle of 6 week old mdx and mdxϴ-/- mice by FACS to analyse the gene

expression involved in satellite cell self-renewal. We choose 6 weeks of age because this is the age when muscle regeneration starts to decline in mdx muscle. MuSCs were isolated as Ter119$^-$/ CD45$^-$/ CD31$^-$/ CD34$^+$/ α7-integrin $^+$/ Sca-1$^-$ cells . The expression level of Pax7, MyoD, Notch1-2-3, Delta1 and Numb was then analysed by qRt-PCR analysis. As shown in Figure 11A, Pax7 and Notch1 level of expression was significantly higher in MuSCs freshly isolated from mdxΘ-/- mice compared with those isolated from mdx mice. An aliquot of the sorted cells was cultured for 62hrs, instead, and analysed for the expression of PAX7 and MYOD by immunofluorescence. Based on the expression of PAX7 and MYOD, cultured satellite cells are defined as self-renewing (Pax7$^+$/MyoD$^-$), activated (Pax7$^+$/MyoD$^+$) or differentiating (Pax7$^-$/MyoD$^+$) [93, 94]. As shown in Figure 11B-C, the percentage of Pax7$^+$/MyoD$^-$ "self-renewing" cells over the total number of cells, was higher in MuSCs derived from in mdxΘ-/- muscle than mdx. The higher level of expression of Pax7 and Notch1 in MuSCs isolated from mdxΘ-/- muscle compared with mdx, together with the higher number of Pax7$^+$/MyoD$^-$ when MuSCs isolated from mdxΘ-/- muscle are cultured, suggest that in lack of PKCΘ in mdx preserves the self-renewal of MuSCs.
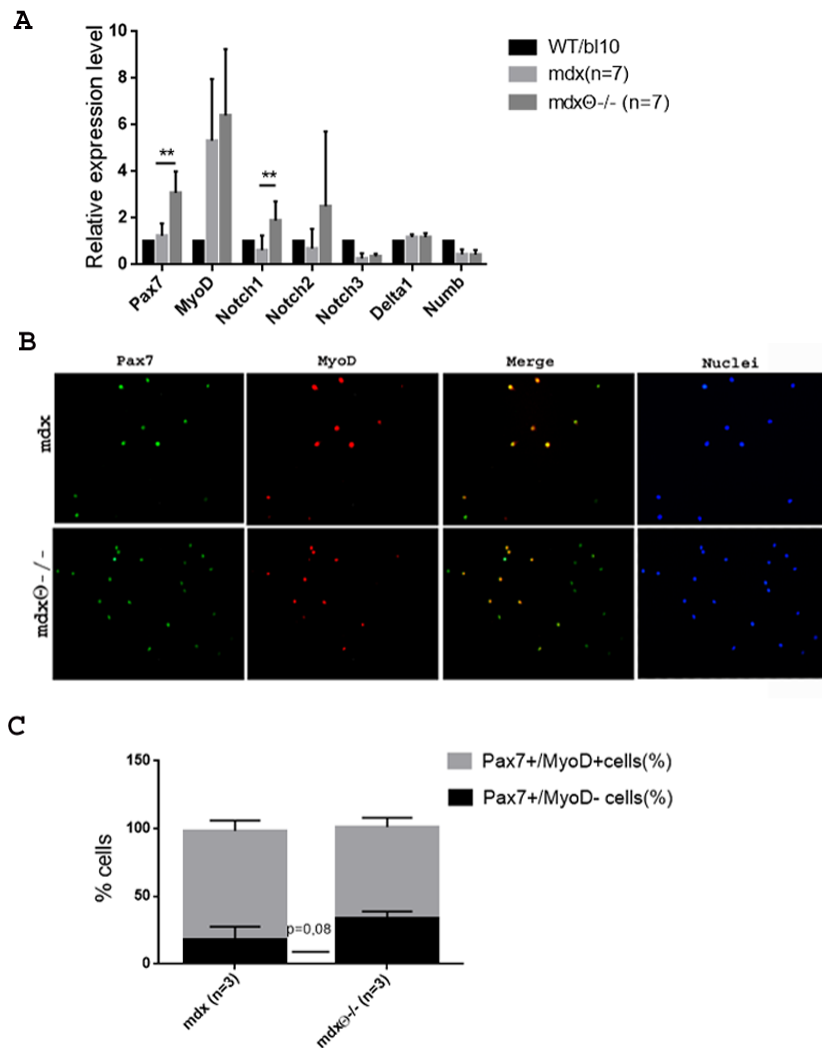
Figure 11. Lack of PKCΘ in dystrophic muscle improves the self-renewal capacity of MuSCs. A Sorted MuSCs were analysed for the level of expression of Pax7, MyoD, Notch receptors, Delta1 and Numb by qRT-PCR. RNA expression level in mdx and mdxΘ-/- MuSCs was normalized to those in WT/bl10 MuSCs. Samples represent duplicate experiment of pooled RNA from MuSCs sorted from three mice for each sample. (*p<0.05, **p<0.01, two-tailed Student's *t*-test). **B** Representative images of Pax7 (green) and MyoD (red)

immunofluorescence of MuSCs, isolated from mdx and mdxΘ-/- muscle and cultured for 62h. Nuclei were counterstained with Hoechst (blue). **C** Percentages of Pax7+/MyoD- and Pax7+/MyoD+ cells over the total number of nuclei. The results are mean±STDEV (n=3/genotype).

Notch signalling in satellite cells is activated by the binding of Notch receptor with Jagged and delta1, known ligand of Notch, expressed mainly on the myofiber surface. It is known that Delta1 decrease in aged and dystrophic muscle [23, 47]. I thus analysed the expression level of Delta1 in TA muscle derived from mdx and mdxΘ-/- by qRT-PCR analysis. As shown in Figure 7, Delta1level of expression is significantly higher in TA muscle derived from mdxΘ-/- than that from mdx muscle at 6 and 12 weeks and at 12 months (Fig. 12). The high Delta1 level of expression in mdxΘ-/- may suggest an active role of myofibers to boost the self-renewal process by Notch signalling. Interestingly, no increase of Delta1 level of expression was observed in muscle derived from 6mo old mdx/theta- compared to age-matching mdx. As shown before, in mdxΘ-/- mice, muscle degeneration peaks at 6 months of age, which may temporarily compromise myofiber quality.
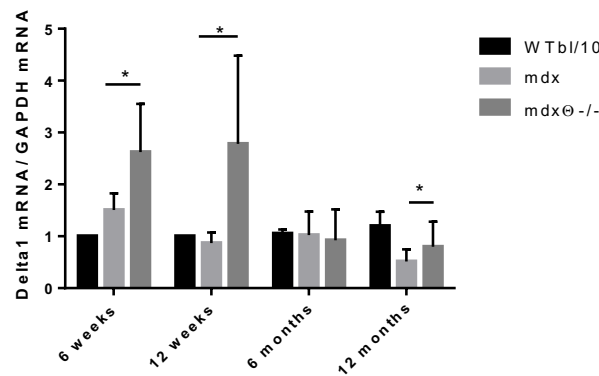
Figura 12. Increased Delta1 level in mdxΘ-/- mice at different ages. Delta1 mRNA level in mdx and mdxΘ-/- TA muscle was normalized to those in WT/bl10 TA muscle. (*p<0.05, two-tailed Student's *t*-test).

To verify whether PKCϴ is directly regulating Delta1 expression we analysed Delta1 expression in TA muscle derived from WT/bl6 and PKCϴ-/- mice. In healthy non-dystrophic muscle, Delta1 expression was similar in WT and PKCϴ-/- (Fig. 13A). However, 7 days after CTX-induced muscle injury, the level of expression of Delta1 was higher in PKCϴ-/- muscle compared with WT (Fig.13b). These results suggest that PKCϴ modulates Delta1 expression in regenerating myofibers, and its lack results in increased Delta1 expression, which, in turn, may favour MuSCs *self-renewal*.
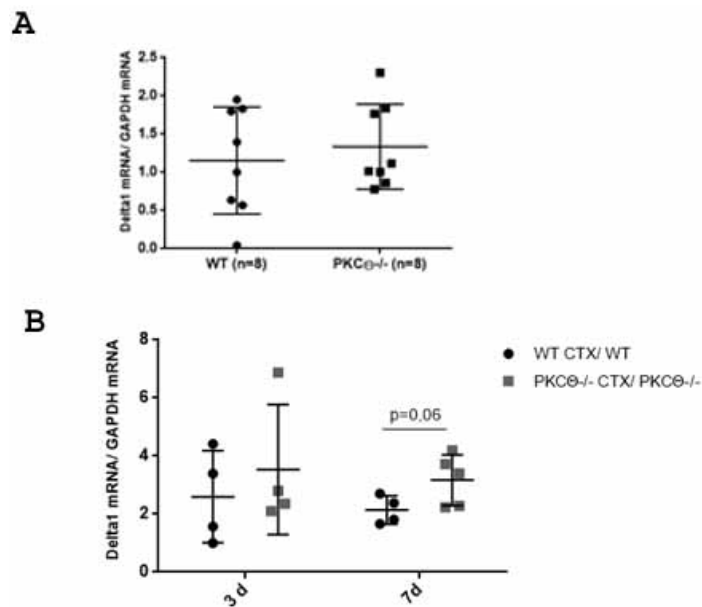
Figura 13. Lack of PKCϴ increases the levels of Delta1 expression after injury. A qRT-analysis of Delta1 expression in WT and PKCϴ TA muscle . **B** qRT-analysis of Delta1 expression in WT/bl6 and PKCϴ TA muscle at 3and 7 days after CTX injury

3.1.4 Lack of PKCΘ in dystrophic muscle changes the differentiation fate of FAPs and improves their pro-myogenic activity

Since lack of PKCΘ in mdx improve muscle regeneration, I wondered whether it may affect the activity of FAPs. FAPs are interstitial cells involved in both muscle regeneration and muscle degeneration. Indeed, during muscle chronic diseases can mediate fat and ECM deposition.

FAPs were isolated by FACS from limb muscles of 6 week old mice as Ter119−/CD45−/CD31−/CD34+/α7-integrin−/Sca-1+ cells. As first the expression level of PKCΘ was analysed, by Western blot analysis, in FAPs isolated from mdx mice and cultured in either growth or adipogenic differentiation medium. As shown in Figure 14, PKCΘ was expressed in both culture conditions.
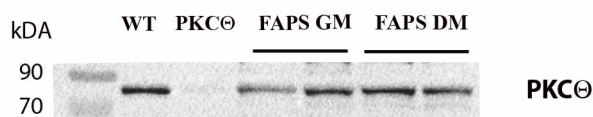
Figure 14. PKCΘ expression in FAPs. Western blot analysis of FAPs isolated from mdx muscle and cultured in growth medium (GM) or adipogenic differentiation medium (DM). Whole muscle protein lysates from WT and PKCΘ-/- mice were used as positive and negative control.

To verify whether lack of PKCΘ may alter the expression level of other PKCs, whose activity may affect FAPs behaviour, the expression level of PKCα, PKCδ and PKCε was determined in FAPs isolated from mdx and mdxΘ-/- by qRT-PCR analysis.-. As shown in Figure 15, the absence of PKCΘ in the FAPs did not appear to change the level of expression of the other PKCs.
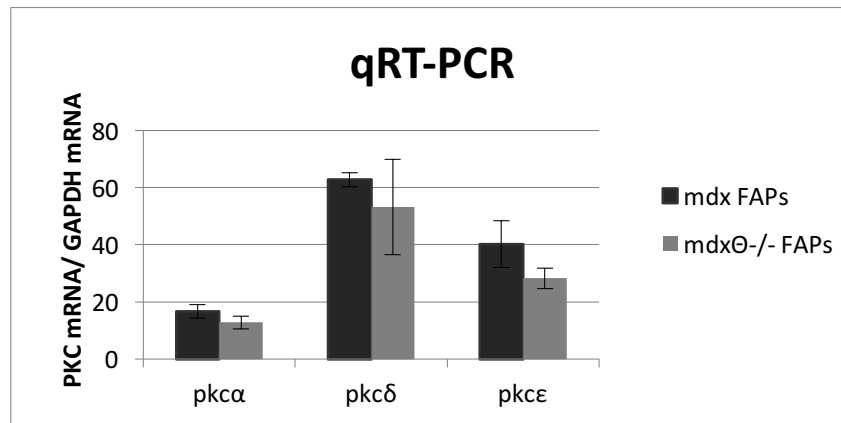
Figure 15. PKC expression in FAPs. FAPs were analysed for levels of expression of PKCα, PKCδ, PKCε by qRT-PCR. RNA expression levels for each gene was normalized to those of PKC in whole WT muscle.

Since FAPs are known to functionally interact with MuSCs and to regulate each other behaviour, we wonder whether lack of PKCΘ in mdx muscle, may affect functional interactions between FAPs and MuSCs. We used ex vivo trans-well co-culture system, since it is known that FAPs enhance the differentiation rate of MuSCs in co-culture [34]. FAPs and MuSCs were isolated from muscles of mdxΘ/- or mdx. MuSCs from mdx or from mdxΘ-/- were plated in the bottom chamber, while FAPs from the same genotype or from the other were plated on the upper chamber. The effect of FAPs on intrinsic myogenic potential of MuSCs was determined by MyHC staining after 7 days of co-culture in growth medium and quantified by fusion index. As shown in Figure 16, MuSCs fusion index, derived both from mdx and mdxΘ-/- muscle, was further increased when co-cultured with FAPs isolated from muscles of mdxΘ-/- compared with FAPs isolated from mdx (Fig. 16). These results demonstrate that lack of PKCΘ in dystrophic muscle enhances FAPs myogenic potential.
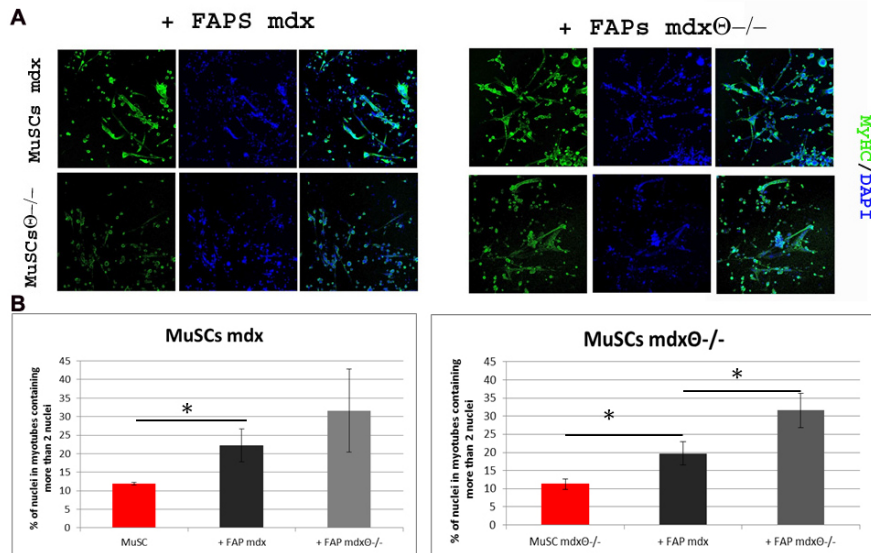
Figure 16. Functional interactions between FAPs from mdx and mdxϴ-/- muscle and MuSCs from mdx and mdxϴ-/muscle. A Representative immunofluorescence images of co-culture stainred for MyHC and DAPI**. B** Fusion rate was determined as the percentage of nuclei contained in myotubes (containing ≥ 3 nuclei) with respect to the total number of nuclei. The results are mean±STDEV for three different experiments. (*p<0.05, two-tailed Student's *t*-test).

To evaluate the eventual "global" changes in gene expression induced by lack of PKCϴ in FAPs, the transcriptome of FAPs freshly isolated from muscle of mdx and mdxϴ-/- mice, was analysed by RNAseq. FAPs sorted from 4 mice/genotype were pooled to generate one sample and two samples for genotype were used for RNAseq analysis. As shown in the heat map (Fig.17a), there were few differentially expressed genes comparing FAPs from mdx and mdxϴ-/-. A total of 138 genes were significantly differentially expressed between the samples. Among them, 117 were upregulated while 21 were downregulated in FAPs from mdxϴ-/- muscle compared with mdx. Among the identified

transcripts, transcripts of genes involved in muscle determination and differentiation, such as Pax7, MyoD and Myf5, were up-regulated in FAPs isolated from mdxΘ-/- mice, compared to those isolated from mdx mice. By contrast, no changes in the expression level of genes involved in adipogenic differentiation, such as C-Ebpβ, Pparγ, Adiponectin and Perilipin) were observed. Moreover, we found that Kdm6b (also called JMJD3) and Smyd1 are up-regulated in FAPs from mdxΘ-/- muscle (Fig.17C). Kdm6b and SmyD1 are involved in histone modification. Kdm6b mediates the removal of repressive trimethylation of histone H3 at lysine 27 (H3K27me3) while SmyD1 mediates the activate methylation histone H3 at Lys-4 (H3K4me). Although these results need to be validated, they suggest that lack of PKCΘ in dystrophic muscle modifies FAPs fate and plasticity, up-regulating muscle specific genes.

A

COLOR KEY

0   3

mdx1   mdx2   mdxΘ-/-1   mdxΘ-/-2

B

0.0 4.3 8.6
absolute(Log10(corrected P-value))

tissue development
system development
striated muscle tissue development
striated muscle contraction
striated muscle cell differentiation
striated muscle cell development
skeletal myofibril assembly
skeletal muscle tissue development
skeletal muscle thin filament assembly
skeletal muscle organ development
skeletal muscle fiber development
skeletal muscle contraction
single-organism process
single-multicellular organism process
sarcoplasmic reticulum calcium ion transport
sarcomere organization
response to chemical stimulus
release of sequestered calcium ion into cytosol...
regulation of muscle system process
regulation of muscle contraction
regulation of multicellular organismal process
regulation of leukocyte apoptotic process
regulation of homeostatic process
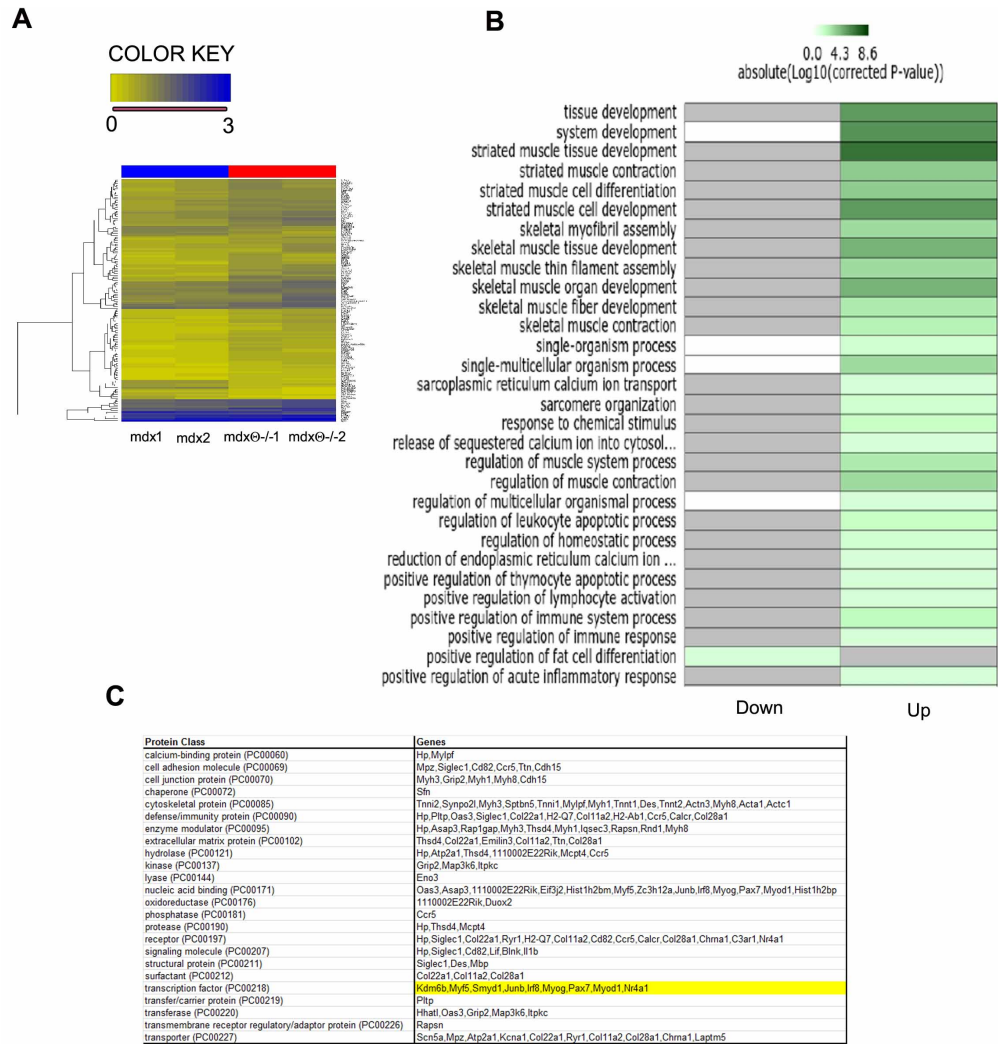reduction of endoplasmic reticulum calcium ion ...
positive regulation of thymocyte apoptotic process
positive regulation of lymphocyte activation
positive regulation of immune system process
positive regulation of immune response
positive regulation of fat cell differentiation
positive regulation of acute inflammatory response

Down   Up

C

Protein Class	Genes
calcium-binding protein (PC00060)	Hp,Mylpf
cell adhesion molecule (PC00069)	Mpz,Siglec1,Cd82,Ccr5,Ttn,Cdh15
cell junction protein (PC00070)	Myh3,Grip2,Myh1,Myh8,Cdh15
chaperone (PC00072)	Sfn
cytoskeletal protein (PC00085)	Tnni2,Synpo2l,Myh3,Sptbn5,Tnni1,Mylpf,Myh1,Tnnt1,Des,Tnnt2,Actn3,Myh8,Acta1,Actc1
defense/immunity protein (PC00090)	Hp,Pltp,Oas3,Siglec1,Col22a1,H2-Q7,Col11a2,H2-Ab1,Ccr5,Calcr,Col28a1
enzyme modulator (PC00095)	Hp,Asap3,Rap1gap,Myh3,Thsd4,Myh1,Iqsec3,Rapsn,Rnd1,Myh8
extracellular matrix protein (PC00102)	Thsd4,Col22a1,Emilin3,Col11a2,Ttn,Col28a1
hydrolase (PC00121)	Hp,Atp2a1,Thsd4,1110002E22Rik,Mcpt4,Ccr5
kinase (PC00137)	Grip2,Map3k6,Itpkc
lyase (PC00144)	Eno3
nucleic acid binding (PC00171)	Oas3,Asap3,1110002E22Rik,Eif3j2,Hist1h2bm,Myf5,Zc3h12a,Junb,Irf8,Myog,Pax7,Myod1,Hist1h2bp
oxidoreductase (PC00176)	1110002E22Rik,Duox2
phosphatase (PC00181)	Ccr5
protease (PC00190)	Hp,Thsd4,Mcpt4
receptor (PC00197)	Hp,Siglec1,Col22a1,Ryr1,H2-Q7,Col11a2,Cd82,Ccr5,Calcr,Col28a1,Chrna1,C3ar1,Nr4a1
signaling molecule (PC00207)	Hp,Siglec1,Cd82,Lif,Blnk,Il1b
structural protein (PC00211)	Siglec1,Des,Mbp
surfactant (PC00212)	Col22a1,Col11a2,Col28a1
transcription factor (PC00218)	Kdm6b,Myf5,Smyd1,Junb,Irf8,Myog,Pax7,Myod1,Nr4a1
transfer/carrier protein (PC00219)	Pltp
transferase (PC00220)	Hhatl,Oas3,Grip2,Map3k6,Itpkc
transmembrane receptor regulatory/adaptor protein (PC00226)	Rapsn
transporter (PC00227)	Scn5a,Mpz,Atp2a1,Kcna1,Col22a1,Ryr1,Col11a2,Col28a1,Chrna1,Laptm5

Figura 17. The transcripts of genes involved in muscle determination and differentiation are up-regulated in FAPs isolated from mdxΘ-/- mice. A Hierarchical clustering analysis of gene expression profiles in FAPs isolated from mdx and mdxΘ-/- muscle using log2 ratios. Each column presents one sample and each horizontal line refers to a gene. Color legend is on the top of the heatmap. The intensity represents the magnitude of the difference. The yellow tiles represent downregulated genes and blue tiles indicate upregulated genes. **B** The

enrichment of up and down-regulated genes for each biological category. **C** The enrichment of up regulate genes for each protein class.

To characterize the differentiation potential of FAPs isolated from muscles of mdx and mdxΘ-/- mice, the FACS-purified FAPs were cultured in adipogenic differentiation medium or in myogenic differentiation medium. The adipogenic potential was measured by Oil red O staining, while the myogenic potential was measured by MyHC immunostaining. As shown in Figure18, FAPs isolated from mdxΘ-/- mice showed reduced ability to differentiate into Oil red-positive adipocytes compared with mdx mice; surprisingly, when cultured in myogenic differentiation medium, FAPs derived from mdxΘ-/-, but not to FAPs derived from mdx, are able to differentiate into myotubes. We cannot exclude that the up-regulation of the expression of myogenic genes in freshly isolated FAPs and the formation of myotubes in the culture of FAPs from mdxΘ-/- could be due to contamination with MuSCs during FACS sorting. However, it is unlikely that the FAPs isolated from mdxΘ-/- are consistently more contaminated in all the experiments.
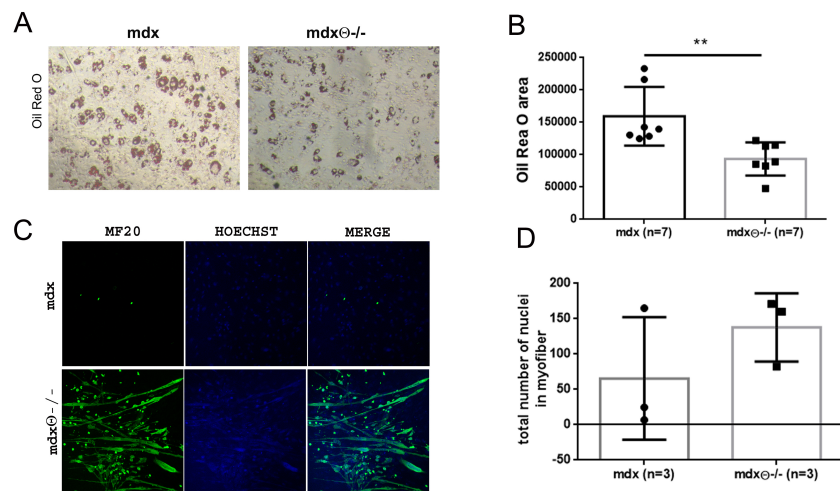

Figura 18. Differentiation potential of FAPs from mdx and mdxΘ-/- muscle.
A Representative images of Oil Red O staining in FAPs cultures. **B** Quantification

of red oil area, reported as pixel^2/field. **C** Representative images of MyHC (green) immunofluorescence in FAPs cultures. Nuclei were counterstained with DAPI (blue). **D** Number of MHC positive nuclei per well.

To further analyse these aspects, FAPs isolated from 6 week old mdx mice were cultured in presence or not of the PKCΘ inhibitor ,C20 (Marrocco et al 2016), prior the exposure to differentiation media. Isolated FAPs were treated with increasing concentrations of C20 (0,5μM, 1μM and 5μM) 15 hours before induction of either adipogenic or myogenic differentiation. As shown in Figure19, C20 treated FAPs did not show statistically significant differences in the ability to differentiate into adipocytes compared with untreated FAPs. By contrast, FAPs treated with 1μM C20 showed a significant increase of ability to differentiate in myotubes compared with untreated FAPs. This results suggest that the inhibition of PKCΘ could directly affect the differential fate of FAPs and promotes their myogenic differentiation.
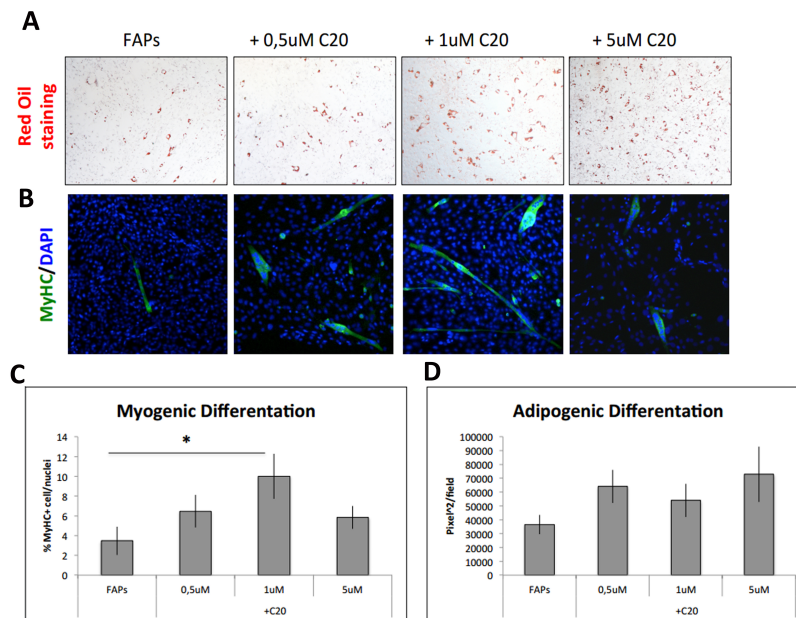
figure 19. Differentiation potential of C20 treated FAPs. A Representative images of Oil Red O staining in FAPs cultures. **B** Representative images of MyHC (green) immunofluorescence in FAPs cultures. Nuclei were counterstained with DAPI (blue).**C** Quantification of red oil area, reported as pixel^2/field. **D** Fusion rate was determined as the percentage of nuclei contained in myotubes (containing ≥ 3 nuclei) with respect to the total number of nuclei. The results are mean±STDEV for three different experiments. (*p<0.05, two-tailed Student's *t*-test

3.2. Lack of PKCΘ in mdx mice modifies the muscle environment to improve the engraftment of transplanted stem cells.

Given the improvement of regenerative capacity of resident stem cells in mdxΘ-/- mice and the role of PKCΘ in T cells and allograft rejection [73, 95], we wondered whether lack of PKCΘ could improve also the regenerative activity of exogenous stem cells improving the efficacy the stem cell engraftment. To this aim, we used MABs as exogenous stem cells.

3.2.1 MABs recruitment in mdx muscle is not affected by lack of PKCΘ

To verify whether lack of PKCΘ in the dystrophic muscle environment can be beneficial to graft transplant, $5x10^5$ nuclear β-Galactosidase expressing Mabs (nLacZ-Mabs) were intrarterialy transplanted into 6 week old mdx and mdxΘ-/-. Three days after injection, mice were sacrificed and TA muscles were collected to analyse the presence of β-gal expressing cells in cryosections and the level of expression of LacZ mRNAby qRT-PCR analysis. Surprisingly, we found that the number of nLacZ-Mabs and of nLacZ expression were higher in mdx than in mdxΘ-/- (Fig. 20) . However, given that trafficking, adhesion and infiltration of Mabs is regulated by the signals released by the damaged inflamed muscle, this result may depend on the different level of inflammation and

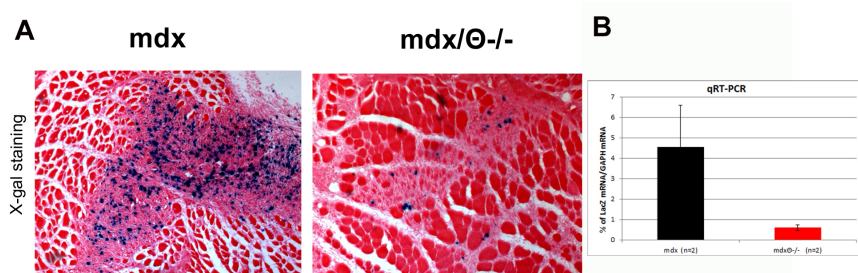damage within the dystrophic muscle expressing or not expressing PKCΘ.

Figure 20. Mabs recruitment in injured TA muscle derived from mdx and mdx Θ-/- TA 72h after Mabs injection into femoral artery. **A** Representative staining for X-gal activity in TA cryosections derived from mdx and mdx Θ-/- . **B** qRT-PCR analysis of LacZ expression in the same samples.

To verify whether the lower recruitment was due to the lower levels of degeneration and inflammation or to lack of PKCΘ in the environment, nLacZ-Mabs were transplanted in injured WT an PKCΘ-/- mice. TA muscles of WT an PKCΘ-/- were damaged by cardiotoxin or by freeze injury. After 24 hrs $5x10^5$ nLacZ-Mabs were injected into the femoral artery. The mice were sacrificed 1 and 3 days after transplantation. As shown in Figure 21, 24 hrs after transplantation, the number of nLacZ-Mabs and nLacZ mRNA within the injured muscle appeared to be similar in WT an PKCΘ-/- mice. By contrast, when mice were sacrificed 3 days after intra-arterial Mabs transplantation, nLac-Mabs were detectable only in TA muscle derived from PKCΘ-/- mice, but not in that from WT, as revealed by both histochemistry and qRT-PCR for nLacZ expression. These results support the idea that lack of PKCΘ does not affect Mabs recruitment in damaged area, but it may favour the survival of transplanted cells.
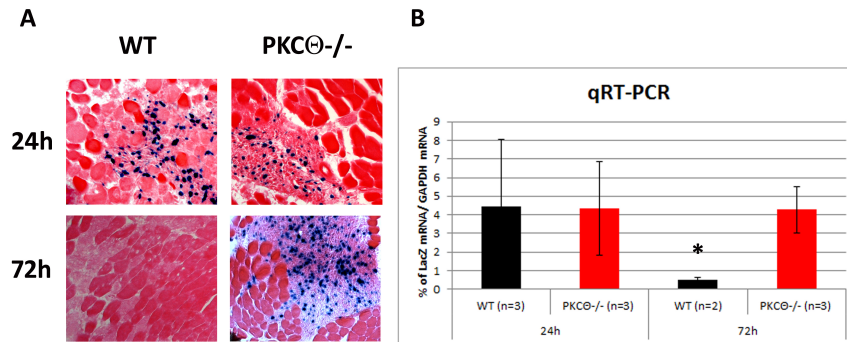
Figure 21. Mabs recruitment in injured TA muscle derived from WT and PKCϴ-/- 24 and 72h after Mabs injection into femoral artery. A Representative staining for X-gal activity in TA cryosections derived from WT and PKCϴ-/-. **B** qRT-PCR analysis of LacZ expression in the same samples

3.2.2 Lack of PKCϴ improves Mabs survival and differentiation in myofibers in injured and dystrophic muscle.

To analyse the survival of transplanted Mabs, Mabs were transplanted via intra-muscular injection to prevent any differences due to recruitment. In particular, TA muscle of WT and PKCϴ-/- mice was first damaged by freeze-injury. 24 hours after muscle injury, 5×10^5 nLacZ-Mabs were transplanted via intramuscular injection. The mice were sacrified 1, 3, 6 and 14 days after transplantation. As shown in Figure 22, the number of nLacZMabs within the injured muscle appears to be similar in WT and PKCϴ-/- mice up to 6 days after transplantation. 14 days after transplantation, nLacZ-Mabs were detectable in TA muscle derived from PKCϴ-/- mice, but not in that from WT. Moreover, LacZ positive nuclei were localized within muscle fibers, showing that transplanted Mabs contributed to the formation of regenerating myofibers.
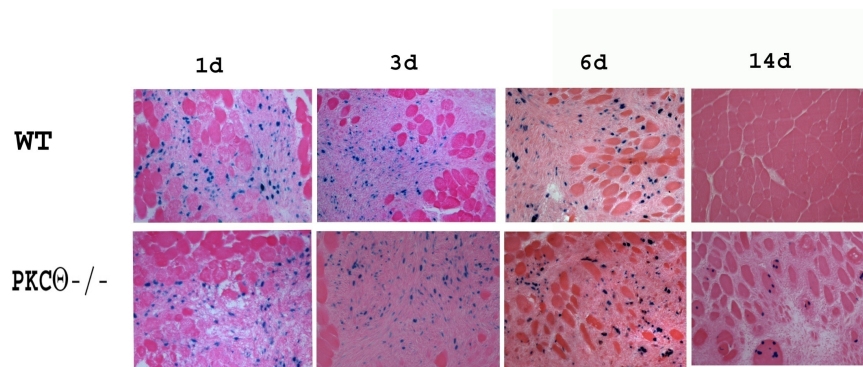
Figure 22. Time course of Mabs survival in injured TA muscle derived from WT and PKCϴ-/- following intra-muscular injection. Representative cryosections of TA muscle derived from WT and PKCϴ-/- sacrificed 1, 3, 6 or 14 days after transplantation, as indicated and assayed for X-gal activity

We then analysed the survival of transplanted Mabs in dystrophic muscle. Mabs were transplanted via intra-muscular injection in TA muscle of 6 week old mdx and mdxϴ-/- mice. The mice were sacrificed 3, 7, 14 and 30 days after transplantation. As in non-dystrophic background, the number of nLacZ/Mabs within the muscle appeared to be similar in mdx and mdxϴ-/- mice 3 and 7 days after transplantation; however, we LacZ positive nuclei in mdx mice were mostly localized in the interstium, while were mostly found within muscle myofibers in mdxϴ-/- mice (Fig.23). This results may suggest that the muscle environment in mdxϴ-/- mice improves Mabs ability to fuse in muscle fibers compared with mdx mice. Importantly, 14 days after transplantation, nLacZ/Mabs were detected in muscle derived from mdxϴ-/- mice, but not from mdx mice. 30 days after transplantation no lacZ positive nuclei were found in both mdx and mdxϴ-/- mice. Together these results demonstrate that lack of PKCϴ prolongs survival and sustains differentiation of transplanted MABs, probably because of the reduced inflammatory environment. However, the MABs we used expressed the bacterial beta-galactosidase. It is thus possible that,

since lack of PKCΘ affects T cells activity modulating the adaptive immune response but not the acute immune response, it may not prevent rejection to antigens of bacterial origin.
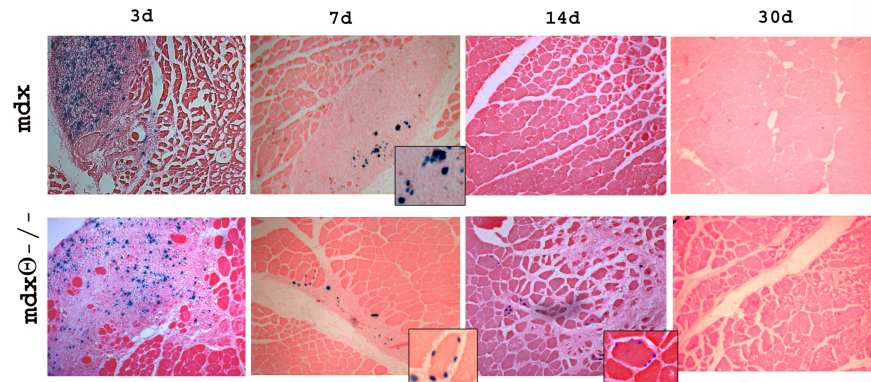

Figure 23. Time course of Mabs survival in injured TA muscle derived from mdx and mdxΘ-/- following intra-muscular injection. Representative cryosections of TA muscle derived from mdx and mdxΘ-/- sacrificed 3,7, 14 or 30 days after transplantation, as indicated and assayed for X-gal activity

4. DISCUSSION

The present study shows that the lack of PKCΘ in mdx mice, improves muscle repair promoting satellite cell pool maintenance, myogenic and pro-myogenic activity of FAPs and favours the engraftment of transplanted stem cells. In our laboratory it has been previously shown that lack of PKCΘ reduced muscle loss and inflammatory infiltrate and increased muscle performance in 8 week old mdx mice. Analysing the degeneration and regeneration level at different stages of the disease in mdx and mdxΘ-/- muscle, I found that the high level of muscle damage observed in 4week old mdx mice, is almost blunted in mdxΘ-/- mice and collagen deposition is partly prevented during the progression of the disease, when compared with mdx mice. By contrast, the area of regenerating myofibers, compared to degenerating area, is increased at different ages in mdxΘ-/- mice compared to mdx muscle. Increased regeneration was also observed, after acute injury, supporting the hypothesis that myogenic ability of endogenous cell populations is maintained and promoted in the absence of PKCΘ. These results might appear in contrast to our previous observation that lack of PKCΘ delayed muscle repair after freeze-injury in non-dystrophic background. However, we also showed that the observed delayed muscle repair was not associated to reduced activity of satellite cells, rather to the delay in the addition of fusing cells to regenerating myofibers, a process known as "secondary fusion" [80]. In the dystrophic background, impaired muscle repair is rather due to impaired satellite cells activation and maintenance. Thus, the different muscle regenerative ability of mdx and mdxΘ-/- could be due to the different behaviour of satellite cells due to different quality of environment. I show here that lack of PKCΘ blunts the early peak of muscle degeneration in mdx, which, in turn, may prevent the exhaustion of satellite cell pool and preserve their self-renewal ability. Indeed, I show that: 1. the number of Pax7 positive cells in muscle is higher in mdxΘ-/-mice compared with mdx mice, and 2. the level of Pax7 and Notch1expression in satellite cells

isolated from muscle derived from mdxΘ-/- mice is higher compared with those from mdx. Notch1 signalling is known to be required for maintaining regenerative ability and self-renewal of satellite cells. It is well known that Notch signalling declines in aged mice and in muscular dystrophy [23, 47]. Notch signalling is activated by the interaction of Delta or Jagged ligands to Notch receptors at the level of cell membrane. After injury, Notch ligands are upregulated on the myofiber surface, suggesting that the activation of Notch signalling is crucial during the muscle repair. Satellite cells are directly attached to myofibers and Notch ligand on myofibers should activate Notch signalling in satellite cells. Interestingly, I found that in mdxΘ-/- muscle Delta1 expression is higher than mdx muscle suggesting that the myofibers could support the Notch signalling in satellite cells. In basal condition, PKCΘ doesn't affect Delta1 expression level; in fact, its expression is similar in WT and PKCΘ-/- muscle. In contrast, after the acute injury, the level of Delta1 expression is higher in PKCΘ-/- muscle compared with WT muscle, suggesting that the PKCΘ could directly affect Delta1 expression level after the injury. This observation suggests that the up-regulation of Delta1 expression is due to lack of PKCΘ in myofibers and not to the different muscle environment. In fact, lack of PKCΘ does not alter the immune response in acute muscle injury, where the activity of T cells is not involved, but preserves the acute immune response that is important during the regenerating process (Lozanoska-Ochser et al ms in prep).

Together to improving satellite cells maintenance, self-renewal and differentiation ability, lack of PKCθ in mdx also improved FAPs ability to contribute to muscle repair. In fact, when co-cultured with MuSCs FAPs derived from mdxΘ-/- muscle exert higher pro-myogenic activity compared to FAPs derived from mdx muscle. Surprisingly, I found that FAPs express high level of PKCΘ, when cultured both in growing and in pro-adipogenic medium. Moreover, I show that FAPs isolated from mdxΘ muscle in culture exert ability to differentiate into muscle cells and reduced to differentiate into adipocytes, in culture, compared to FAPs derived from mdx muscle.

Similar ability was observed also when PKCθ was pharmacologically inhibited in FAPs isolated from mdx. Accordingly, RNA seq analysis showed that FAPs freshly isolated from mdxΘ-/- muscle expressed high level of genes involved in muscle differentiation. The ability of FAPs to differentiate in myoblasts is very much controversial. Indeed, it is believed that FAPs do not contribute directly to regenerated muscle fibers, but support MuSCs behaviour. However, previous studies showed that treatment with TSA, an HDAC inhibitor, is able to de-repress a ''latent'' myogenic program in FAPs. Thus, the epigenetic changes can promote the myogenic potential of FAPs [61]. Interestingly, RNAseq showed that the expression of JMJD and SMYD were upregulates in FAPs from mdxΘ-/-, suggesting that lack of PKCΘ in dystrophic muscle may induce epigenetic modifications.

However, further studies will be necessary to discriminate whether the observed changes in FAPs phenotype and behavior depends primarily to the modified environment or to the lack of PKCΘ in FAPs.

Interestingly, lack of PKCΘ in mdx, promotes not only the regenerative activity of endogenous myogenic cell populations, but also the regenerative activity of exogenous transplanted stem cells. In fact, lack of PKCΘ in mdx muscle improved MABs survival and differentiation in myofibers. I show here that 14 days after transplantation, MABs are still detectable in mdxΘ-/- muscle and are incorporated into myofibers; by contrast, in mdx muscle MABs are detectable up to 7 day, mostly in the interstitial spaces. It is conceivable that lack of PKCΘ modifying the inflammatory environment, favors MABs survival and differentiation ability. On top, as being PKCΘ involved in allograft response, its lack may partly prevent rejection, since the mice were not immune-suppressed; although the used MABs derived from the same genetic background, they express beta-gal which is anyway highly immunogenic. Moreover, improved transplanted MABs myogenic differentiation within mdxΘ-/- muscle, may also depend, at least in part, to the observed increase in Delta1 expression on myofibers. In

fact, it was recently shown that the Dll1-Notch1 axis regulates the myogenic potential of MABs and ameliorates in vivo MAB-driven regeneration [96].

Together, these results suggest that lack of PKCΘ in dystrophic muscle makes a more favourable environment for both endogenous and exogenous cell populations to contribute to muscle maintenance and repair. This phenotype is probably dependent on modifications in the quality of the immune response. However, the obtained results may also suggest that PKCΘ activity might also directly regulate muscle cell populations phenotype, contributing to worsen the disease. The underlying mechanisms are under investigation and may contribute to the identification of additional molecular / cellular targets for intervention aiming to shift the balance between muscle regeneration and fibroadipogenic degeneration in DMD and to improve cell mediated therapeutic strategies.

5. MATERIAL AND METHODS

5.1 Animal models

PKCθ-/- mice (C57BL/6J background) were provided by Dan Littman (New York University, New York). In these mice, the gene encoding PKCθ was inactivated in all cells of the body, as previously described [98].

Mdx mice (C57BL/10ScSn-Dmdmdx/J) were purchased from Jackson laboratory and mdx PKCθ-/- transgenic mice were generated in our laboratory (C57BL/6j-C57BL/10ScSn background). C57BL/10ScSn control mice were purchased from Jackson laboratory. Only males were used.

The animals were housed in the Histology Department–accredited animal facility. All the procedures were approved by the Italian Ministry for Health and were conducted accordingto the U.S. National Institutes of Health (NIH) guidelines.

5.2 PCR genotyping

For genomic DNA PCR, DNA was extract from the Bone Marrow, by an ON incubation, at 55°C with agitation, in extracting buffer for DNA composed of:1 ml of KCl 2,5M; 0,5 ml of TrisHCl 1M pH 8,4; 0,1 mg MgCl2 1M; o,225 ml NP40; 0,225 ml Tween 20 ; Proteinase K 0,06 mg/ml; and 46,5 ml H_2O autoclaved. The day after, incubated for 10' at 100°C and centrifuged at 14000 rpm for 10'. After centrifugation, the pellet was discarded and the supernatant containing DNA was used for PCR analysis. For the PCR, the EmeraldAmp®GT PCR Master Mix was used, following the manufacturing protocol and for the amplification the PTC-100™ thermocycler was used.

For PKCθ$^{-/-}$ mutation, a 700bp PCR fragment is expected for the mutated PKCθ gene, while a 400bp PCR fragment for the WT one.
Primers: PKCθ-REV: TAAGAGTAATCTTCCAGAGC
FOR: TTGGTTCTCTTGAACTCTGC
NEO: ACTGCATCTGCGTGTTCGAA

5.3 Muscle injury procedure

To induce muscle cardiotoxin injury, 10 μl cardiotoxin (10 μmol in H2O, Sigma-Aldrrich) was injected in two different area of the TA using a 30 Gauge microsyringe. To induced freeze injury, after skin incision and muscle exposition, Tibialis anterior (TA) muscles were frozen with three consecutive cycles of freeze-thawing by applying for 15 seconds a liquid nitrogen cooled metallic rod. The skin was then sutured with 4.0 suture string.

5.4 Mesoangioblast transplantation

Animals were anesthetized with Avertina. Bilateral intra-femoral artery injection was performed with 5×10^5 cells/50 μl saline solution, using 32-gauge needles under STEMI SV11 stereomicroscope (Zeiss, Oberkochen, Germany). Intramuscular injection was performed in TA with 5×10^5 cells/50 μl saline solution, using 30-gauge needles.

5.5 Cell cultures

nLacZMabs: nLacZ-Mabs derived from C57/bl6 mice, were gently provided by Dr. Cesare Gargioli, Department of Biology, Tor Vergata Rome University, Rome, Italy. Mabs were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS (both from Gibco; Invitrogen, Carlsbad, CA, USA) in a humidified 5% CO2 atmosphere at 37°C.

FAPs and MuSCs: Faps and MuSCs were prepared from limb skeletal muscles. Dissected muscles were hashed and then digested with 2 μg/ml Collagenase A (Roche), 2.4 U/ml Dispase I (Roche), 10 ng/ml DNase I (Roche), 0.4 mM CaCl2 and 5 mM MgCl2 for 90 min at 37°C. After digestion, the obtained cells were washed with Dulbecco's phosphate-buffered saline (DPBS) containing 0,2% BSA and and then filtered with cell strainer 70 and 40 μm. Cells were incubated with primary antibodies (10 ng/ml) CD31-PacificBlue (Invitrogen), CD45-eFluor450 (eBioscience), Ter119-eFluor450 (eBioscience), CD11b-Pacific blue, Sca-1-FITC (BD Pharmingen), CD34-Biotin (eBioscience) and α7integrin-APC

(kindly provided by Dr. Fabio Rossi) for 30 min on ice. A subsequent incubation, 30 min on ice, with Streptavidin-PE-Cy7 (1/500; BD Pharmingen) was performed. Cells were finally washed and resuspended in HBSS containing 0.2% w/v BSA and 1% v/v Penicillin–Streptomycin. Flow cytometry analysis and cell sorting were performed on a DAKO-Cytomation MoFlo High Speed Sorter. Muscle satellite cells (MuSCs) were isolated as Ter119−/CD45−/CD31−/CD34+/α7-integrin+/Sca-1− cells; FAPs cells were isolated as Ter119−/CD45−/CD31−/CD34+/α7-integrin−/Sca-1+ cells. FAPs were grown in BIOAMF-2 complete medium (ATGC) as a growth medium (GM). After 7 days in GM, cells were exposed for 3 days to adipogenic induction medium (DMEM containing 10% FBS, 0.5 mM IBMX, 0.25 μM dexamethasone and 10 μg/ml insulin), followed by further 3 days in adipogenic maintenance medium, (DMEM containing 10% FBS and 10 μg/ml insulin). Cell culture inserts with 1.0-μm pore and 12-well culture plates (BD bioscience) were used for transwell co-culture. Plates were coated with 0.1% gelatin. 4×10^4 freshly sorted MuSCs were plated in the bottom of the plate, while 8×10^4 FAPs cells were plated on the upper insert. Transwell co-cultures were maintained in GM for 7 days and then harvested for analyses.

FAPs were treated with an PKCϴ-specific inhibitor compound 20 (C20) which was gently provided by Dr. Maryanne Brown (BoehringerIngelheim Pharmaceuticals Inc., Ridgefield, CT, USA). Sorted FAPs from 6 week old mdx were plated in BIOAMF-2 complete medium and treated with C20 (0,5μM-1μM-5μM). After 15 h, BIOAMF-2 complete medium was changed with adipogenic induction medium.

5.6 RNA isolation and RT

For RNA isolation from cells, the cells were collected in TRIsure™ (Sigma-Aldrich). For RNA preparation from muscle, the muscles were homogenized in Trissure with the ULTRA-TURRAX T25 (Janke&kunkel IKA Labortechnik) and syringed 4 times with a 21G

needle syringe. Then the samples were incubated in TRIsure^{TM} at RT for 10 minutes, centrifuged at 12.000 g for 10 minutes at 4°C and the pellet was discarded. For every ml of TRIsure^{TM} used, 200ul of Chloroform were added. After centrifugation for 15 minutes at 12000 g, an aqueous phase containing the RNA separates. For every ml of TRIsure^{TM} used, 500ul of isopropanol were added to the aqueous phase. After 10 minutes of incubation at RT and centrifugation for 10 minutes at 12000 g, the RNA precipitates. The RNA was aqueous phase as washed with a solution of cold 75 % Ethanol in water and then resuspended in RNAse and DNAse free water. The RNA was converted in cDNA by a RT PCR. For the RT PCR the High-Capacity cDNA RT kit from Applied Biosystem.

5.7 Real time pcr analysis and primer list
For RT-qPCR analysis the Bioline SensiMixTM SYBR Low-ROX Kit was used, following the manufacturer's protocol. For data analysis, the 75000 Software v2.0.5, provided by Applied Biosystem,was used. Primer list:

Notch1 for rev	GGT CGC AAC TGT GAG AGT GA TTG CTG GCA CAT TCA TTG AT
Notch2 for rev	GCA GGA GCA GGA GGT GAT AG GCG TTT CTT GGA CTC TCC AG
Notch3 for rev	GTC CAG AGG CCA AGA GAC TG CAG AAG GAG GCC AGC ATA AG
Delta1 for rev	CCG GCT GAA GCT ACA GAA AC GAA AGT CCG CCT TCT TGT TG
Pax7 for	GTCCCAGTCTTACTGCCCAC

rev	TGTGGACAGGCTCACGTTTT
MyoD	
for	CGACACCGCCTACTACAGTG
rev	GGTGGTGCATCTGCCAAAAG
Numb	
for	CCGGCATGCTCCAATTG
rev	TCTGGCTAAGAGCAGGAAAACC
MyoG	
for	GCATGGAGTTCGGTCCCAA
rev	TATCCTCCACCGTGATGCTG
GAPDH	
for	ACC CAG AAG ACT GTG GAT GG
rev	CAC ATT GGG GGT AGG AAC AC
LacZ	
for	ATCTCTATCGTGCGGTGGTT
rev	GAGCTGACCATGCAGAGGAT)

5.8 Western blot

Cells were homogenized in ice-cold buffer containing 20 mMTris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 5 mM DTT, 200 mg/ml leupeptin, 10 mg/ml Trasylol, 1 mM PMSF, and 0.1% Triton X-100 and then disrupted by sonication. The homogenate was incubated for 30 min on ice accompanied by repeated vortexing, then centrifuged at 12,000g for 10 min at 4°C. The pellet was discarded. An aliquot of the supernatants was used for protein determination using the Comassie Plus protein assay reagent (Pierce, Rockford, IL), according to the manufacturer's instruction, while the remainder was used for Western blot analysis. An equal amount of protein from each sample was loaded onto 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membranes were incubated with PKCΘ primary antibodies (Cell signalling).

10. RNA sequencing

Duplicates of mRNA samples isolated from FAPs were sent to the IGA technologies services company, which provided us with the sequencing results aligned with the genome. The obtained data were analysed by Dr. Loredana Le Pera, from ITT, as previously described [97].

5.9 Statistical analysis

Quantitative data are presented as means ± SD of at least three experiments. Statistical analysis to determine significance was performedusing paired Student's t tests and with ANOVA test. Differences were considered to be statistically significant at the $p < 0.05$ level.

6. REFERENCE LIST

1. Mauro, A., *Satellite cell of skeletal muscle fibers.* J Biophys Biochem Cytol, 1961. **9**: p. 493-5.
2. Ciciliot, S. and S. Schiaffino, *Regeneration of mammalian skeletal muscle. Basic mechanisms and clinical implications.* Curr Pharm Des, 2010. **16**(8): p. 906-14.
3. Fielding, R.A., et al., *Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle.* Am J Physiol, 1993. **265**(1 Pt 2): p. R166-72.
4. Arnold, L., et al., *Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis.* J Exp Med, 2007. **204**(5): p. 1057-69.
5. Chazaud, B., et al., *Satellite cells attract monocytes and use macrophages as a support to escape apoptosis and enhance muscle growth.* J Cell Biol, 2003. **163**(5): p. 1133-43.
6. Cantini, M., et al., *Macrophage-secreted myogenic factors: a promising tool for greatly enhancing the proliferative capacity of myoblasts in vitro and in vivo.* Neurol Sci, 2002. **23**(4): p. 189-94.
7. Gayraud-Morel, B., F. Chretien, and S. Tajbakhsh, *Skeletal muscle as a paradigm for regenerative biology and medicine.* Regen Med, 2009. **4**(2): p. 293-319.
8. Olguin, H.C. and B.B. Olwin, *Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal.* Dev Biol, 2004. **275**(2): p. 375-88.
9. Seale, P., et al., *Pax7 is required for the specification of myogenic satellite cells.* Cell, 2000. **102**(6): p. 777-86.
10. Oustanina, S., G. Hause, and T. Braun, *Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification.* EMBO J, 2004. **23**(16): p. 3430-9.

11. Olguin, H.C., et al., *Reciprocal inhibition between Pax7 and muscle regulatory factors modulates myogenic cell fate determination.* J Cell Biol, 2007. **177**(5): p. 769-79.

12. Davis, R.L., H. Weintraub, and A.B. Lassar, *Expression of a single transfected cDNA converts fibroblasts to myoblasts.* Cell, 1987. **51**(6): p. 987-1000.

13. Almada, A.E. and A.J. Wagers, *Molecular circuitry of stem cell fate in skeletal muscle regeneration, ageing and disease.* Nat Rev Mol Cell Biol, 2016. **17**(5): p. 267-79.

14. Ehm, O., et al., *RBPJkappa-dependent signaling is essential for long-term maintenance of neural stem cells in the adult hippocampus.* J Neurosci, 2010. **30**(41): p. 13794-807.

15. Pellegrinet, L., et al., *Dll1- and dll4-mediated notch signaling are required for homeostasis of intestinal stem cells.* Gastroenterology, 2011. **140**(4): p. 1230-1240 e1-7.

16. Pannerec, A., G. Marazzi, and D. Sassoon, *Stem cells in the hood: the skeletal muscle niche.* Trends Mol Med, 2012. **18**(10): p. 599-606.

17. Jarriault, S., et al., *Signalling downstream of activated mammalian Notch.* Nature, 1995. **377**(6547): p. 355-8.

18. Mourikis, P. and S. Tajbakhsh, *Distinct contextual roles for Notch signalling in skeletal muscle stem cells.* BMC Dev Biol, 2014. **14**: p. 2.

19. Wen, Y., et al., *Constitutive Notch activation upregulates Pax7 and promotes the self-renewal of skeletal muscle satellite cells.* Mol Cell Biol, 2012. **32**(12): p. 2300-11.

20. Conboy, I.M. and T.A. Rando, *The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis.* Dev Cell, 2002. **3**(3): p. 397-409.

21. Bjornson, C.R., et al., *Notch signaling is necessary to maintain quiescence in adult muscle stem cells.* Stem Cells, 2012. **30**(2): p. 232-42.

22. Lin, S., et al., *Brief report: Blockade of Notch signaling in muscle stem cells causes muscular dystrophic phenotype and*

impaired muscle regeneration. Stem Cells, 2013. **31**(4): p. 823-8.

23. Conboy, I.M., et al., *Notch-mediated restoration of regenerative potential to aged muscle.* Science, 2003. **302**(5650): p. 1575-7.

24. Bentzinger, C.F., et al., *Cellular dynamics in the muscle satellite cell niche.* EMBO Rep, 2013. **14**(12): p. 1062-72.

25. Sanes, J.R., *The basement membrane/basal lamina of skeletal muscle.* J Biol Chem, 2003. **278**(15): p. 12601-4.

26. Hynes, R.O., *The extracellular matrix: not just pretty fibrils.* Science, 2009. **326**(5957): p. 1216-9.

27. Gattazzo, F., A. Urciuolo, and P. Bonaldo, *Extracellular matrix: a dynamic microenvironment for stem cell niche.* Biochim Biophys Acta, 2014. **1840**(8): p. 2506-19.

28. Urciuolo, A., et al., *Collagen VI regulates satellite cell self-renewal and muscle regeneration.* Nat Commun, 2013. **4**: p. 1964.

29. Christov, C., et al., *Muscle satellite cells and endothelial cells: close neighbors and privileged partners.* Mol Biol Cell, 2007. **18**(4): p. 1397-409.

30. Arsic, N., et al., *Vascular endothelial growth factor stimulates skeletal muscle regeneration in vivo.* Mol Ther, 2004. **10**(5): p. 844-54.

31. Dellavalle, A., et al., *Pericytes resident in postnatal skeletal muscle differentiate into muscle fibres and generate satellite cells.* Nat Commun, 2011. **2**: p. 499.

32. Tidball, J.G. and S.A. Villalta, *Regulatory interactions between muscle and the immune system during muscle regeneration.* Am J Physiol Regul Integr Comp Physiol, 2010. **298**(5): p. R1173-87.

33. Mitchell, K.J., et al., *Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development.* Nat Cell Biol, 2010. **12**(3): p. 257-66.

34.   Joe, A.W., et al., *Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis.* Nat Cell Biol, 2010. **12**(2): p. 153-63.

35.   Uezumi, A., et al., *Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle.* Nat Cell Biol, 2010. **12**(2): p. 143-52.

36.   Uezumi, A., et al., *Fibrosis and adipogenesis originate from a common mesenchymal progenitor in skeletal muscle.* J Cell Sci, 2011. **124**(Pt 21): p. 3654-64.

37.   Mozzetta, C., et al., *Fibroadipogenic progenitors mediate the ability of HDAC inhibitors to promote regeneration in dystrophic muscles of young, but not old Mdx mice.* EMBO Mol Med, 2013. **5**(4): p. 626-39.

38.   Heredia, J.E., et al., *Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration.* Cell, 2013. **153**(2): p. 376-88.

39.   Lemos, D.R., et al., *Nilotinib reduces muscle fibrosis in chronic muscle injury by promoting TNF-mediated apoptosis of fibro/adipogenic progenitors.* Nat Med, 2015. **21**(7): p. 786-94.

40.   Mendell, J.R., et al., *Evidence-based path to newborn screening for Duchenne muscular dystrophy.* Ann Neurol, 2012. **71**(3): p. 304-13.

41.   Hoffman, E.P., R.H. Brown, Jr., and L.M. Kunkel, *Dystrophin: the protein product of the Duchenne muscular dystrophy locus.* Cell, 1987. **51**(6): p. 919-28.

42.   Villalta, S.A., A.S. Rosenberg, and J.A. Bluestone, *The immune system in Duchenne muscular dystrophy: Friend or foe.* Rare Dis, 2015. **3**(1): p. e1010966.

43.   Chen, Y.W., et al., *Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy.* Neurology, 2005. **65**(6): p. 826-34.

44.   Mendias, C.L., et al., *Transforming growth factor-beta induces skeletal muscle atrophy and fibrosis through the*

induction of atrogin-1 and scleraxis. Muscle Nerve, 2012. **45**(1): p. 55-9.

45. Yablonka-Reuveni, Z. and J.E. Anderson, *Satellite cells from dystrophic (mdx) mice display accelerated differentiation in primary cultures and in isolated myofibers.* Dev Dyn, 2006. **235**(1): p. 203-12.

46. Dumont, N.A., et al., *Dystrophin expression in muscle stem cells regulates their polarity and asymmetric division.* Nat Med, 2015. **21**(12): p. 1455-63.

47. Jiang, C., et al., *Notch signaling deficiency underlies age-dependent depletion of satellite cells in muscular dystrophy.* Dis Model Mech, 2014. **7**(8): p. 997-1004.

48. Vieira, N.M., et al., *Jagged 1 Rescues the Duchenne Muscular Dystrophy Phenotype.* Cell, 2015. **163**(5): p. 1204-13.

49. Mann, C.J., et al., *Aberrant repair and fibrosis development in skeletal muscle.* Skelet Muscle, 2011. **1**(1): p. 21.

50. Shimizu-Motohashi, Y., et al., *Recent advances in innovative therapeutic approaches for Duchenne muscular dystrophy: from discovery to clinical trials.* Am J Transl Res, 2016. **8**(6): p. 2471-89.

51. Malik, V., et al., *Gentamicin-induced readthrough of stop codons in Duchenne muscular dystrophy.* Ann Neurol, 2010. **67**(6): p. 771-80.

52. Finkel, R.S., et al., *Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy.* PLoS One, 2013. **8**(12): p. e81302.

53. Sampaolesi, M., et al., *Cell therapy of alpha-sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblasts.* Science, 2003. **301**(5632): p. 487-92.

54. Sampaolesi, M., et al., *Mesoangioblast stem cells ameliorate muscle function in dystrophic dogs.* Nature, 2006. **444**(7119): p. 574-9.

55. Cossu, G., et al., *Intra-arterial transplantation of HLA-matched donor mesoangioblasts in Duchenne muscular dystrophy.* EMBO Mol Med, 2015. **7**(12): p. 1513-28.

56. Miyatake, S., et al., *Anti-inflammatory drugs for Duchenne muscular dystrophy: focus on skeletal muscle-releasing factors.* Drug Des Devel Ther, 2016. **10**: p. 2745-58.

57. Brookes, E. and Y. Shi, *Diverse epigenetic mechanisms of human disease.* Annu Rev Genet, 2014. **48**: p. 237-68.

58. Jaenisch, R. and A. Bird, *Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals.* Nat Genet, 2003. **33 Suppl**: p. 245-54.

59. Colussi, C., et al., *HDAC2 blockade by nitric oxide and histone deacetylase inhibitors reveals a common target in Duchenne muscular dystrophy treatment.* Proc Natl Acad Sci U S A, 2008. **105**(49): p. 19183-7.

60. Consalvi, S., et al., *Preclinical studies in the mdx mouse model of duchenne muscular dystrophy with the histone deacetylase inhibitor givinostat.* Mol Med, 2013. **19**: p. 79-87.

61. Saccone, V., et al., *HDAC-regulated myomiRs control BAF60 variant exchange and direct the functional phenotype of fibro-adipogenic progenitors in dystrophic muscles.* Genes Dev, 2014. **28**(8): p. 841-57.

62. Bettica, P., et al., *Histological effects of givinostat in boys with Duchenne muscular dystrophy.* Neuromuscul Disord, 2016. **26**(10): p. 643-649.

63. Marrocco, V., et al., *Targeting PKCtheta in skeletal muscle and muscle diseases: good or bad?* Biochem Soc Trans, 2014. **42**(6): p. 1550-5.

64. Arendt, C.W., et al., *Protein kinase C-theta;: signaling from the center of the T-cell synapse.* Curr Opin Immunol, 2002. **14**(3): p. 323-30.

65. Lin, X., et al., *Protein kinase C-theta participates in NF-kappaB activation induced by CD3-CD28 costimulation*

through selective activation of IkappaB kinase beta. Mol Cell Biol, 2000. **20**(8): p. 2933-40.

66. Baier-Bitterlich, G., et al., *Protein kinase C-theta isoenzyme selective stimulation of the transcription factor complex AP-1 in T lymphocytes.* Mol Cell Biol, 1996. **16**(4): p. 1842-50.

67. Pfeifhofer, C., et al., *Protein kinase C theta affects Ca2+ mobilization and NFAT cell activation in primary mouse T cells.* J Exp Med, 2003. **197**(11): p. 1525-35.

68. Sutcliffe, E.L., et al., *Chromatin-associated protein kinase C-theta regulates an inducible gene expression program and microRNAs in human T lymphocytes.* Mol Cell, 2011. **41**(6): p. 704-19.

69. Li, J., et al., *Nuclear PKC-theta facilitates rapid transcriptional responses in human memory CD4+ T cells through p65 and H2B phosphorylation.* J Cell Sci, 2016. **129**(12): p. 2448-61.

70. Brezar, V., W.J. Tu, and N. Seddiki, *PKC-Theta in Regulatory and Effector T-cell Functions.* Front Immunol, 2015. **6**: p. 530.

71. Tan, S.L., et al., *Resistance to experimental autoimmune encephalomyelitis and impaired IL-17 production in protein kinase C theta-deficient mice.* J Immunol, 2006. **176**(5): p. 2872-9.

72. Marsland, B.J., et al., *TLR ligands act directly upon T cells to restore proliferation in the absence of protein kinase C-theta signaling and promote autoimmune myocarditis.* J Immunol, 2007. **178**(6): p. 3466-73.

73. Manicassamy, S., et al., *A critical role for protein kinase C-theta-mediated T cell survival in cardiac allograft rejection.* J Immunol, 2008. **181**(1): p. 513-20.

74. Wang, L., et al., *Deficiency of protein kinase C-theta facilitates tolerance induction.* Transplantation, 2009. **87**(4): p. 507-16.

75. Zappelli, F., et al., *The inhibition of differentiation caused by TGFbeta in fetal myoblasts is dependent upon selective*

expression of PKCtheta: a possible molecular basis for myoblast diversification during limb histogenesis. Dev Biol, 1996. **180**(1): p. 156-64.

76. Biressi, S., et al., *Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells.* Dev Biol, 2007. **304**(2): p. 633-51.

77. Cusella-De Angelis, M.G., et al., *Differential response of embryonic and fetal myoblasts to TGF beta: a possible regulatory mechanism of skeletal muscle histogenesis.* Development, 1994. **120**(4): p. 925-33.

78. Messina, G., et al., *Nfix regulates fetal-specific transcription in developing skeletal muscle.* Cell, 2010. **140**(4): p. 554-66.

79. D'Andrea, M., et al., *Protein kinase C theta co-operates with calcineurin in the activation of slow muscle genes in cultured myogenic cells.* J Cell Physiol, 2006. **207**(2): p. 379-88.

80. Madaro, L., et al., *PKCtheta signaling is required for myoblast fusion by regulating the expression of caveolin-3 and beta1D integrin upstream focal adhesion kinase.* Mol Biol Cell, 2011. **22**(8): p. 1409-19.

81. Madaro, L., et al., *Intracellular signaling in ER stress-induced autophagy in skeletal muscle cells.* FASEB J, 2013. **27**(5): p. 1990-2000.

82. Evans, N.P., et al., *Dysregulated intracellular signaling and inflammatory gene expression during initial disease onset in Duchenne muscular dystrophy.* Am J Phys Med Rehabil, 2009. **88**(6): p. 502-22.

83. Farini, A., et al., *T and B lymphocyte depletion has a marked effect on the fibrosis of dystrophic skeletal muscles in the scid/mdx mouse.* J Pathol, 2007. **213**(2): p. 229-38.

84. Madaro, L., et al., *PKC theta ablation improves healing in a mouse model of muscular dystrophy.* PLoS One, 2012. **7**(2): p. e31515.

85. McGreevy, J.W., et al., *Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy.* Dis Model Mech, 2015. **8**(3): p. 195-213.

86. Kharraz, Y., et al., *Understanding the process of fibrosis in Duchenne muscular dystrophy.* Biomed Res Int, 2014. **2014**: p. 965631.

87. Irintchev, A., M. Zweyer, and A. Wernig, *Impaired functional and structural recovery after muscle injury in dystrophic mdx mice.* Neuromuscul Disord, 1997. **7**(2): p. 117-25.

88. Garg, K., B.T. Corona, and T.J. Walters, *Therapeutic strategies for preventing skeletal muscle fibrosis after injury.* Front Pharmacol, 2015. **6**: p. 87.

89. Zammit, P.S., et al., *Kinetics of myoblast proliferation show that resident satellite cells are competent to fully regenerate skeletal muscle fibers.* Exp Cell Res, 2002. **281**(1): p. 39-49.

90. Day, K., et al., *Nestin-GFP reporter expression defines the quiescent state of skeletal muscle satellite cells.* Dev Biol, 2007. **304**(1): p. 246-59.

91. Halevy, O., et al., *Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal.* Dev Dyn, 2004. **231**(3): p. 489-502.

92. Carey, K.A., et al., *Impaired expression of Notch signaling genes in aged human skeletal muscle.* J Gerontol A Biol Sci Med Sci, 2007. **62**(1): p. 9-17.

93. Zammit, P.S., et al., *Muscle satellite cells adopt divergent fates: a mechanism for self-renewal?* J Cell Biol, 2004. **166**(3): p. 347-57.

94. Smeriglio, P., et al., *Phosphotyrosine phosphatase inhibitor bisperoxovanadium endows myogenic cells with enhanced muscle stem cell functions via epigenetic modulation of Sca-1 and Pw1 promoters.* FASEB J, 2016. **30**(4): p. 1404-15.

95. Hayashi, K. and A. Altman, *Protein kinase C theta (PKCtheta): a key player in T cell life and death.* Pharmacol Res, 2007. **55**(6): p. 537-44.

96. Quattrocelli, M., et al., *Notch signaling regulates myogenic regenerative capacity of murine and human mesoangioblasts.* Cell Death Dis, 2014. **5**: p. e1448.

97. Cialfi, S., et al., *The loss of ATP2C1 impairs the DNA damage response and induces altered skin homeostasis: Consequences for epidermal biology in Hailey-Hailey disease.* Sci Rep, 2016. **6**: p. 31567.

7. LIST OF PUBBLICATION

"Pharmacological inhibition of PKCθ counteracts muscle disease in a mouse model of Duchenne Muscular Dystrophy". Marrocco V., **Fiore P**., Pisu S., Benedetti A., Rizzuto E., Musarò A., Madaro L., Lozanoska-Ochser B., and Bouché M. Ebios, Under Review

"PKCθ signaling is required for myoblast fusion by regulating the expression of caveolin-3 and β1D integrin upstream focal adhesion kinase" Madaro L, Marrocco V, **Fiore P**, Aulino P, Smeriglio P, Adamo S, Molinaro M, Bouché M. Mol Biol Cell. 2011 Apr 15;22(8):1409-19.

"Targeting PKCθ in skeletal muscle and muscle diseases: good or bad?" Marrocco V, **Fiore P**, Madaro L, Crupi A, Lozanoska-Ochser B, Bouché M. Biochem Soc Trans. 2014 Dec ;42(6):1550-5 . Review.