Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE MORFOLOGICHE UMANE E MOLECOLARI

Ciclo XXV

Settore concorsuale di afferenza: 05/H1
Settore Scientifico disciplinare: BIO/16

TITOLO TESI

Melanocytes: a new potential tool to study and monitoring Duchenne muscular dystrophy

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Esame finale anno 2012

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Abstract

Dystrophin is a subsarcolemmal protein critical for the integrity of muscle fibers by linking the actin cytoskeleton to the extracellular matrix via the dystroglycan complex. It is reported that dystroglycans are also localized in the skin, at dermal-epidermal junction. Here we show that epidermal melanocytes express dystrophin at the interface with the basement membrane. The full-length muscle isoform mDp427 was clearly detectable in epidermis and in melanocyte cultures as assessed by RNA and western blot analysis. Dystrophin was absent in Duchenne Muscular Dystrophy (DMD) patients melanocytes, and the ultrastructural analysis revealed mitochondrial alterations, similar to those occurring in myoblasts from the same patients. Interestingly, mitochondrial dysfunction of DMD melanocytes reflected the alterations identified in dystrophin-deficient muscle cells. In fact, mitochondria of melanocytes from DMD patients accumulated tetramethylrhodamine methyl ester but, on the contrary of control donor, mitochondria of DMD patients readily depolarized upon the addition of oligomycin, suggesting either that they are maintaining the membrane potential at the expense of glycolytic ATP, or that they are affected by a latent dysfunction unmasked by inhibition of the ATP synthase.

Melanocyte cultures can be easily obtained by conventional skin biopsies, less invasive procedure than muscular biopsy, so that they may represent an alternative cellular model to myoblast for studying and monitoring dystrophinopathies also in response to pharmacological treatments.

Abbreviations

ATP adenosine triphosphate

BM basement membrane

BMZ basement membrane zone

CsA Cyclosporin A

CyPD Cyclophilin D

DEJ dermal-epidermal junction

DGP dystrophin–glycoprotein complex

DMD Duchenne muscular dystrophy

ECM extracellular matrix

LM laminin

mDp427 muscular dystrophin protein 427kDa

mdx muscular dystrophy X-linked

mPTP mitochondrial permeability transition pore

n-NOS Neuronal-NOS

NO Nitric Oxide

NOS Nitric Oxide synthase

α-DG alpha-dystroglycan

β-DG beta-dystroglycan

INTRODUCTION

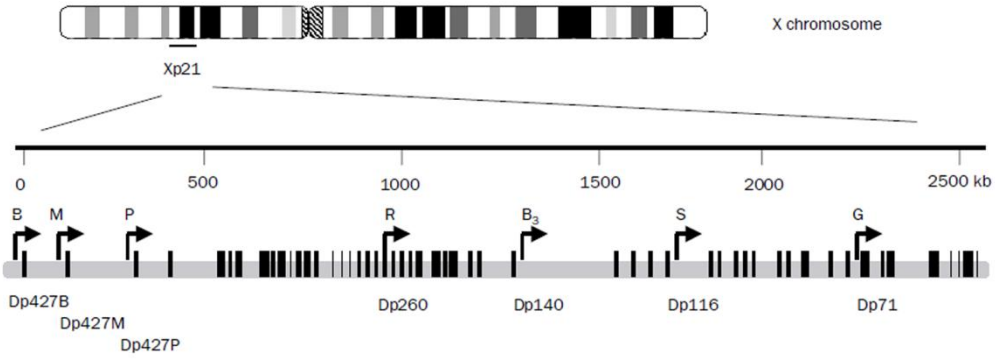
I. DMD gene

The dystrophin gene is the largest gene described in human beings; it spans more than 2.5 million bp of genomic sequence, which correspond to about 0,1% of the total human genome or about 1,5% of the entire X chromosome. Introns represent 99% of the gene whilst the coding sequence includes 86 exons (Muntoni et al., 2003).

Expression of the full-length dystrophin transcript is controlled by three independently regulated promoters, whose names refer to the major but not exclusive site of expression: brain (B), muscle (M) and Purkinje cells (P). The promoters consist of unique first exons spliced to a common set of 78 exons (Blake et al., 2000). The B promoter drives expression primarily in cortical neurons and the hippocampus of the brain, while the P promoter is expressed in the cerebellar Purkinje cells and also in skeletal muscle. The M promoter results in high levels of expression in skeletal muscles and cardiomyocytes and also at low levels in some glial cells in the brain. These three promoters span a large genomic interval of 400kb (Blake et al, 2002) (fig.1).

In addition to these isoforms, the dystrophin gene produces many isoforms generated through alternative splicing events. These splice variants are formed both through the exclusion of some exons from the primary transcript (exon skipping) and by subversion of the reciprocal order of exons (exon scrambling) (Sadoulet-Puccio et al, 1996; Surono et al, 1999). These events, which commonly occur in a tissue-specific

way, generate several dystrophin protein isoforms (promoters R, B_3, S, G)(Muntoni et al., 2003) with different lengths (fig.1).



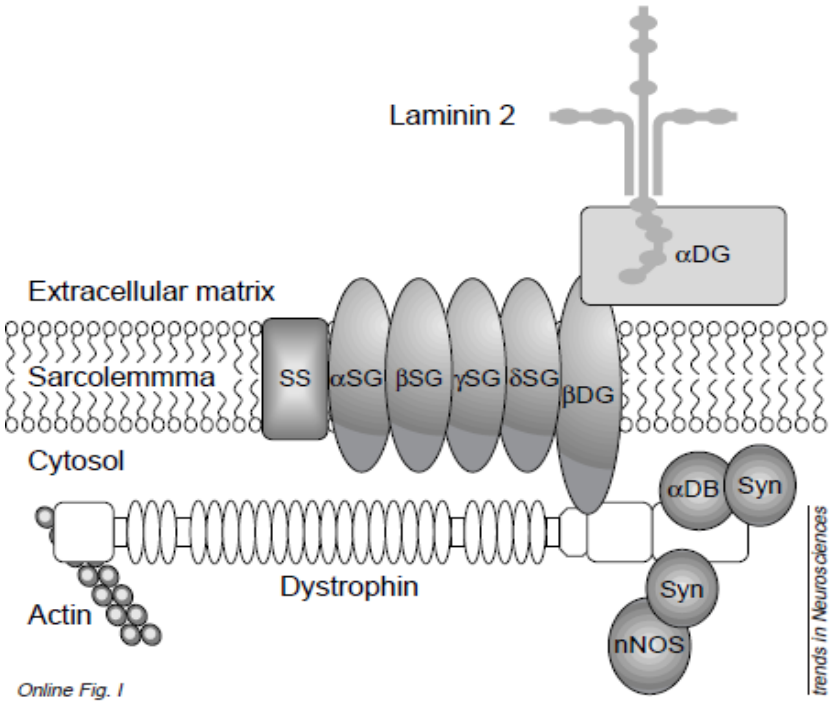
Modified from Muntoni et al.,2003

Figure 1 Genomic organization of the dystrophin gene, located in Xp21. The black vertical lines represent the 79 exons of the dystrophin gene distributed over about 2·5 million bases. The arrows indicate the various promoters: in particular are brain (B), muscle (M), and Purkinje (P) promoters; R, B3, S, and G represent the Dp260 (retinal), Dp140 (brain3), Dp116 (Schwann cells), and Dp71 (general) promoters.

II. Dystrophin protein

Dystrophin is 427kDa cytoskeletal protein belonging the β-spectrin/α-actinin protein family (Muntoni et al., 2003). This family is characterized by an NH2-terminal actin binding domain followed by a variable number of repeating units known as spectrin-like repeats (Blake et al.,2002). Dystrophin can be organized into four separate regions based on sequence homologies and protein-binding capabilities: the amino-terminal domain that has homology with α-actin, the central-rod-domain is a succession of 25 triple-helical repeats similar to spectrin, the cysteine rich domain

and the last-carboxy-terminal-domain (Sadoulet-Puccio et al., 1996). The protein is associated with the plasma membrane (sarcolemma) of cardiac skeletal and smooth muscle (Marbini et al., 1997; Ferlini et al., 2010) and its main role at the sarcolemma is to interact with integral membrane proteins (sarcoglycan, dystroglycans, syntrophin, and dystrobrevin complexes) that are assembled in the dystrophin–glycoprotein complex (DGC) (fig 2).



Modified from Blake et al., 2000

Figure 2, Schematic diagram of the dystrophin complex as found in vertebrate skeletal muscle, showing the currently understood relationships between the better characterized components.

This complex forms a bridge across the sarcolemma and flexibly connects the basal lamina of the extracellular matrix to the inner cytoskeleton binding to F-actin.

One of the main functions of the dystrophin–glycoprotein complex is to stabilize the sarcolemma and to protect muscle fibres from long-term contraction-induced damage and necrosis. in the complex contributes also to cellular communication by acting as a transmembrane signalling complex; in the phosphorylation status it might have a role in signal transduction (Muntoni et al 2003).

III. Dystrophin short isoforms

The dystrophin gene also has at least four internal promoters that give rise to shorter dystrophin proteins lacking the actin-binding terminus but retaining the cysteine-rich and carboxy-terminus domains that contain the binding sites for dystroglycan, dystrobrevin, and syntrophin (Blake et al., 2000). Each of these internal promoters uses a unique first exon that splices into exons 30, 45, 56, and 63 to generate protein products of 260 kDa (Dp260), 140 kDa (Dp140), 116 kDa (Dp116), and 71 kDa (Dp71) (fig. 3). Dp260 is expressed in high concentrations in the retina, where it coexists with the full-length brain and muscle isoforms (Muntoni et al, 2003; Pillers et al, 1993; D'Souza et al, 1995). Dp140 is expressed in brain, retina, and kidney tissues (Lidov et al,1995). Dp116 is only expressed in adult peripheral nerves (Byers et al, 1993). Dp71 is detected in most non-muscle tissues including brain, retina, kidney, liver, and lung and is present in cardiac but not skeletal muscle (Muntoni et al, 2003).

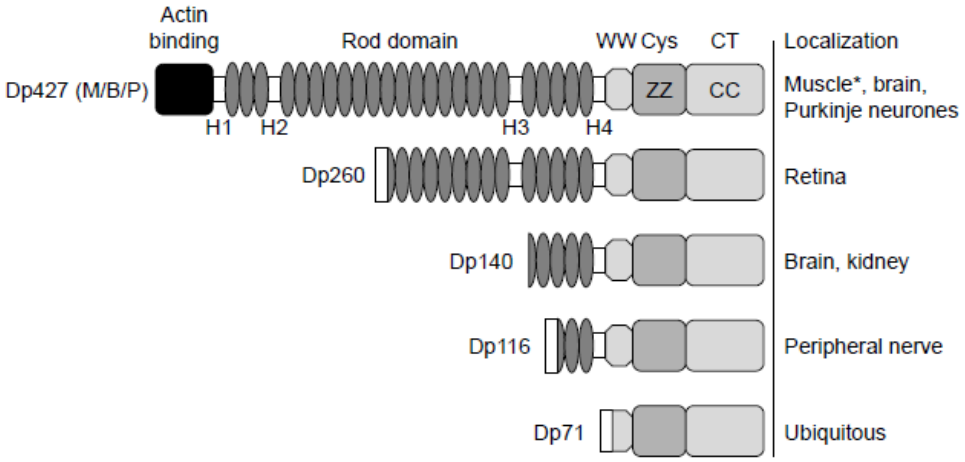


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Figure 3, The domain composition of the different dystrophin isoforms proteins is indicated. The amino terminal domain (Actin binding) is followed by the spectrin like domain (rod domain), the cysteine rich (WW Cys), and the carboxy-terminal domain (CT).

IV. Duchenne muscular dystrophy

Duchenne muscular dystrophy (DMD; Online Mendelian Inheritance in Man [OMIM] reference 310200) is a X-linked disease that affects 1 in 3600–6000 live male births (Drousiotou et al., 1998). DMD occurs as a result of mutations (mainly deletions) in the dystrophin gene (*DMD*; locus Xp21.2). Mutations lead to an absence of or defect in the protein dystrophin, which results in progressive muscle degeneration leading to loss of independent ambulation (Hoffman et al.,1987).

Most patients are diagnosed at approximately 5 years of age, when their physical ability diverges markedly from that of their peers. Individuals with DMD show progressive muscle degeneration (Blake et al., 2000), boys require the use of a

wheelchair before their teens (Bushby et al.,2010) and usually die before the age of 30 from respiratory or cardiac-muscle failure (Blake et al, 2000) (fig 4).

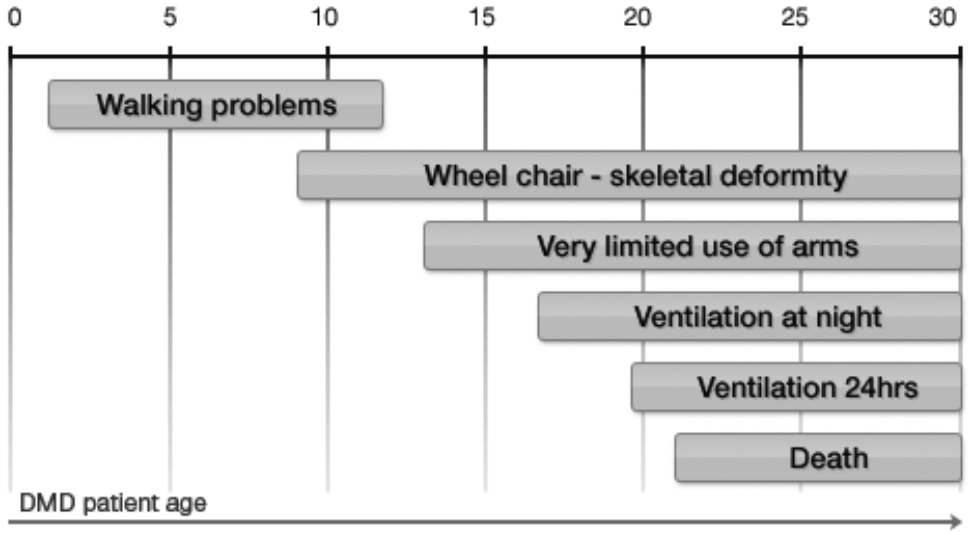

Figure 4, DMD time-line describes the different steps of desease progession.

The reported frequency of different mutations leading to DMD varies widely. According to the Leiden database (www.dmd.nl), duplications of one or several exons correspond to 7% of the mutations, point mutations account for 20%, while deletions are observed in 72% of the patients (Muntoni et al., 2003, Pichavant et al., 2011). Most deletions occur between the exons 44 and 55, corresponding to the dystrophin's rod domain (Nelson et al., 2009). If these mutations alter the reading frame of dystrophin (out of frame-mutation), protein formation is truncated, no dystrophin is produced and the patient develops DMD. If the mutation is "in frame",

the dystrophin is smaller in size but still functional, in which case the patient is diagnosed with Becker muscular dystrophy (Manzur et al., 2008).

Although the molecular origins of DMD have been known since several years, there is still no curative treatment for the disease. To date, the only treatment shown to be effective in slowing the progression of the illness are corticosteroids (Bushby et al., 2010). Many promising therapeutic strategies have since been developed in animal models. Human trials based on these strategies have started, leading to the hope of definitive treatments for this disease (Muntoni et al., 2007).

IV.1. Histological features

Necrotic or degenerating muscle fibers are characteristically seen in all postnatal DMD muscle biopsies even before muscle weakness is clinically observed. Degenerating fibers are often seen in clusters (grouped necrosis) (Gerospe et al., 1997) and necrotic fibers are subject to phagocytosis. Muscle biopsies from DMD patients reveal the presence of inflammatory cells at perimysial and endomysial sites (Arahata et al., 1984 and 1986). A secondary sign of muscle fiber necrosis, at least in the early stages of the dystrophinopathies, is the active regeneration of muscle to replace or repair lost or damaged fibers (Schmalbruch et al., 1984). Progressively, the regenerative capacity of the muscles is lost and muscle fibers are gradually replaced by adipose and fibrous connective tissue, giving rise to the clinical appearance of pseudohypertrophy followed by atrophy (Bell et al., 1968; Bradley et al., 1972; Blake et al., 2002). The combination of progressive fibrosis and muscle

fiber loss results in muscle wasting and ultimately muscle weakness (Blake et al., 2002).

IV. 2. Pathophysiology

Dystrophin is considered a key structural element in the muscle fiber, and the primary function of the dystrophin-associated protein complex is to stabilize plasma membrane, although a role in signaling has been hypothesized (Rybakova et al., 2000).

The gene encoding dystrophin was identified in 1987 (Hoffman et al., 1987), but the mechanisms leading to disease manifestation still remain unclear.

The dystrophic condition is associated with a wide variety of cellular dysfunctions, including membrane instability, deregulation in Ca^{2+} homoeostasis, increased susceptibility to oxidative damage, enhanced proteolytic activity and apoptosis, and impaired energy metabolism (Blake et al., 2002). Altogether, these events lead to muscle fibre death followed by infiltration of activated lymphocytes and macrophages, progressive replacement of muscle tissue by fibrotic and adipose tissue, and culminate in the loss of functional muscle mass (Reutenauer et al., 2008).

Below the currently hypotheses on cellular mechanisms of disease are reported*Membrane permeability*

The discovery of dystrophin and other members of the dystrophin-associated protein complex scaffolding supported the view that the absence of one of these proteins could compromise the muscle membrane integrity of the fibers, particularly after

sustained contractions, as ability to sustain eccentric contraction appears to be dramatically reduced in Duchenne muscular dystrophy (Moens et al., 1993).

Dystrophin-deficient muscle is characterized by increased permeability to macromolecules flowing in and out of the cell following mechanical stress (Blake et al, 2002). Cytoplasmic accumulation of proteins that are normally not present in muscle fibers, such as albumin and immunoglobulins, suggests increased membrane permeability (Deconinck et al., 2007). Membrane fragility in Duchenne muscular dystrophy patients and mdx mice has been reported.

Calcium

Calcium homeostasis is critical to many aspects of muscle function (Berchtold et al., 2000). Calcium accumulation and hypercontracted fibers have been described in DMD patient muscle biopsies (Cullen et al., 1975; Duncan et al., 1978) and it has been demonstrated the increased influx through a dystrophin-deficient membrane (Franco et al., 1990; De Backer et al., 2002).

Calcium mishandling in DMD suggest that dystrophin may regulate calcium-signalling cascade such as calcium entries (Constantin et al., 2006).

Abnormal increase in submembranous calcium concentration was confirmed by the physiologic values of membrane potential (Franco et al, 1990). Nevertheless, if mechanical stress induces microlesions in the fiber membrane, high influx of extracellular calcium inevitably occurs, overriding the capacity to maintain physiologic cytosolic Ca^{2+} concentration. Sustained increase in cytosolic calcium

concentration leads to activation of proteases, particularly calpains, resulting in the destruction of membrane constituents which, in turn, will increase calcium entry (Hopf et al, 1996). Excessive calcium may then lead to cell death (Carafoli et al. 1998) (Fig 5).

Therefore, calcium overload in human DMD myotubes is dependent on their contractile activity suggesting the involvement of channels being active during contraction and/or calcium release (Constantin et al., 2006).

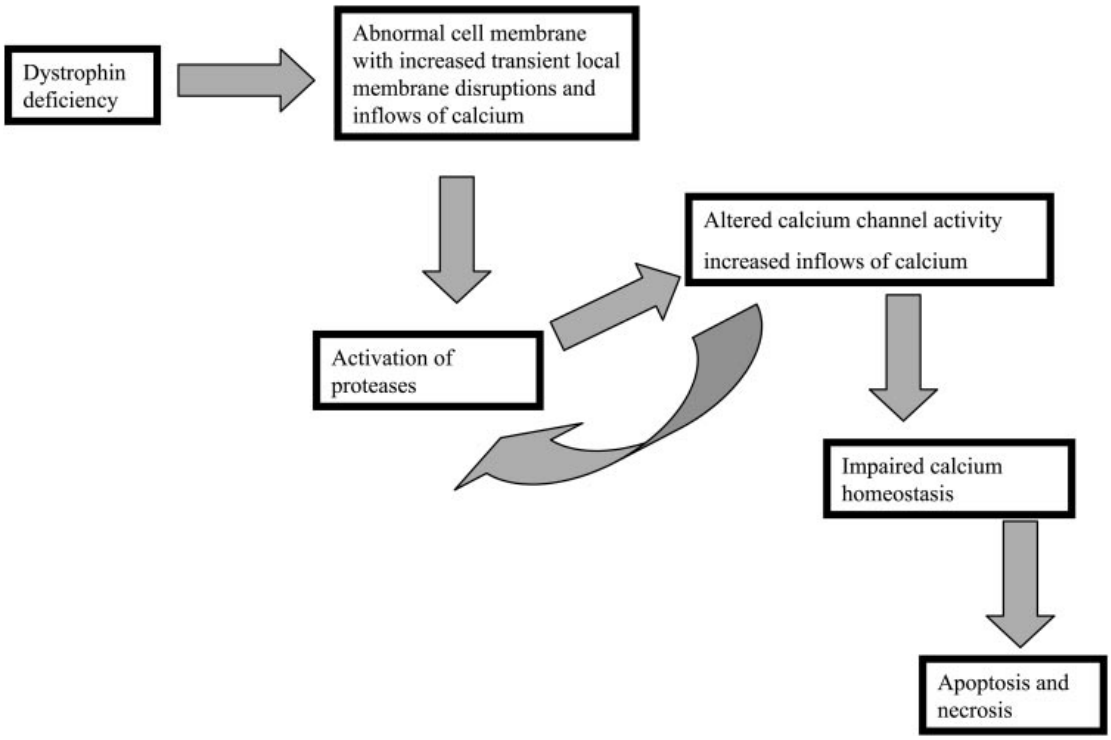

Figure 5, The pathophysiology of dystrophin deficiency. This diagram illustrates the scheme described by Steinhardt and others.

Mitochondria and mitochondrial permeability transition pore (mPTP)

A specific decrease in all mitochondrial inner membrane enzymes, most probably as a result of $Ca2^{+}$ overload, has been postulated to be the cause for the bioenergetic deficits in dystrophin-deficient skeletal muscle (Kuznetsov et al., 1998). In addition to the established role of mitochondria in energy metabolism, regulation of cell death has emerged as a major function of these organelles (Bouchier-Hayes et al., 2005; Orrenius et al., 2007). Taking into account the key role played by mitochondrial Ca^{2+} handling in the control of cell death, these organelles are potential targets of impaired Ca^{2+} homoeostasis in muscular dystrophy (Robert et al., 2001). Calcium deregulation induces mitochondrial depolarization and apoptosis (Hajnoczky et al., 2003). Indeed, depolarization of the inner mitochondrial membrane results in the opening of mitochondrial permeability transition pore (mPTP) and Ca^{2+} efflux from mitochondrial matrix through both the mPTP and the Na^{+}/Ca^{2+} exchanger (Bernardi, 1999).

The mPTP, the structure of which is still not established (Forte and Bernardi, 2005), behaves as a voltage-dependent, high-conductance and nonspecific megachannel of the inner mitochondrial membrane. In the fully open state, its apparent diameter is about 3 nm and the open–closed transitions of the pore are highly regulated by multiple effectors. Indeed, pore opening can be promoted by a high concentration of matrix Ca^{2+} levels, oxidative stress, pyridine nucleotide oxidation, thiol oxidation, alkalinization or low transmembrane potential (Bernardi, 1999; Crompton, 1999). Persistent mPTP opening is followed by equilibration of ionic gradients and of

species with molecular weight lower than 1.5 kDa, which may cause swelling, cristae unfolding and outer membrane rupture associated with the release of cytochrome c and pro-apoptotic factors (Fig 6) (Petronilli et al., 2001). Opening of the mPTP is inhibited by Cyclosporin A (CsA) after its binding to cyclophilin D (CyPD), a member of the cyclophilin family of cis–trans isomerases (Halestrap and Davidson, 1990). CyPD is involved in modulation of the mPTP affinity for $Ca2^{+}$, and conversely $Ca2^{+}$ modulates the efficacy of mPTP inhibition by CsA (McGuinness et al., 1990; Bernardi, 1992). The mPTP is activated early in apoptosis and its inhibition has been shown to protect against apoptosis (Bernardi et al., 2001).

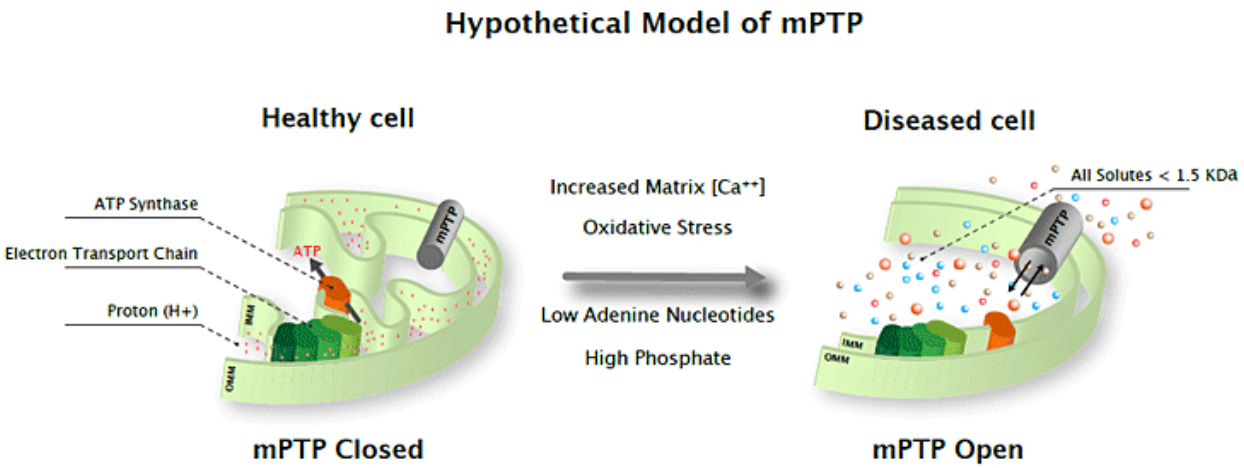

Figure 6, Hypothetical model of mPTP. Various factors, including calcium overload and oxidative stress, open, in the inner mitochondrial membrane, the mitochondrial permeability transition pore (mPTP). This uncouples oxidative phosphorylation and compromises intracellulare ATP levels eventually leading to necrotic cell death.

Free Radicals

Many studies have illustrated the fact that the majority of ROS production seen in cells occurs via the mitochondria as a toxic by-product of oxidative phosphorylation, which is the central means of ATP and heat production for our bodies (Whitehead et al., 2006). Initially, ROS are encased within the mitochondria and are unable to harm other intracellular structures. However, if the amount of ROS within the mitochondria becomes too high, they may begin to damage proteins, lipids, and acids inside the mitochondria, decrease oxidative phosphorylation output, and increase ROS production even further (Wallace et al., 2005). In this event, the cell becomes significantly impaired and must be removed by activation of the mPTP. The expanding role of increased levels of ROS in DMD has caused this mechanism to gain traction as another source of excessive ROS through a cycle of amplified ROS formation causing increased mtPTP opening and vice versa (Menazza et al., 2010).

Aside from ROS production due to oxidative phosphorylation, monoamine oxidases A and B (MAO-A; MAO-B) have displayed the ability to cause oxidative deamination of certain neurotransmitters and dietary amines in the outer mitochondrial membrane. This process creates aldehydes, ammonia, and hydrogen peroxide, which are all considered ROS. Various studies involving the mitochondria have exhibited reduced ROS levels in muscle cells in *mdx* mice animal models using MAO-inhibition (Menazza et al., 2010) or PTP inhibition through ablation of cyclophilin D, a mitochondrial protein which promotes PTP activity (Millay et al., 2008; Palma et al., 2009).

Free radicals may also interact with inflammatory cells to cause muscle damage in the disease process of DMD. Reactive oxygen species have been found to trigger the activation of the NF-κB pathway. NF-κB, a transcription factor, controls the presence of pro-inflammatory factors including TNFα and IL-1β (Kumar, 2003). Increased levels of these pro-inflammatory factors have been found in *mdx* mice before muscle death even occurs. Besides promoting inflammation, another role of TNFα is the production of mitochondrial free radicals. This system, therefore, is self-perpetuating in that increased ROS activates the expression of NF-κB, which in turn activates TNFα, causing a production of more mitochondrial ROS (Kumar, 2003). Furthermore, other inflammatory cells such as neutrophils and macrophages have the ability to produce ROS. In fact, macrophage-driven free radicals have been hypothesized to cause a significant amount of the muscle damage seen in *mdx* mice (Tidball et al., 2007) The presence of NO can inhibit these free radicals. However, a reduction in nNOS present in dystrophin-deficient muscle does not produce sufficient amounts of NO to neutralize these free radicals, and therefore, cell damage by ROS occurs (Wehling et al., 2001).

Reduction in nNOS/NO

Necrotic fibers are often observed in clusters in affected Duchenne muscular dystrophy; therefore, early pathophysiologic hypotheses postulated a pathogenicrole of the muscle vasculature (Koehler et al., 1977). More recent insights into the local vasodilatator role of nitric oxide (NO) in skeletal muscle may be relevant to Duchenne muscular dystrophy pathophysiology. NO is produced in muscle cells by

the neuronal isoform of NO synthase (n-NOS) that is normally bound to dystrobrevin and syntrophin. In dystrophin-deficient muscles, n-NOS is delocalized from its subsarcolemmal anchorage, floating freely in cytoplasm, and its content is reduced. During exercise, when need in oxygen is increased, muscle ischemia may occur in Duchenne muscular dystrophy (Sander et al., 2000).

Even it n-NOS does not play a direct role in Duchenne muscular dystrophy, it could contribute to the extent of damage, as suggested by intracellular pH dysregulation found *in vivo* in repetitively stimulated *mdx* mouse muscles (Wheling et al.,2001).

Gene Regulation

Dystrophin-associated protein complex is involved also in the signal transduction (mechanotransduction) of muscle activity-related gene expression, so absence of dystrophin results in selective regulation of various genes (Deconinck et al., 2007). Several genes involved in intracellular signaling molecules, such as calcineurin, p38 mitogen-activated protein kinase, c-Jun N-terminal kinases, and other protein kinases, have been demonstrated to be upregulated by mechanical stress specifically in the hearts of dystrophin deficient mice (Nakamura et al.,2001) . Moreover, recent studies have demonstrated similar patterns of muscle gene expression in Duchenne muscular dystrophy and in healthy subjects undergoing endurance exercise training (Timmons et al., 2007). This suggests that the Duchenne muscular dystrophy gene upregulation profile might reflect a compensatory mechanism mainly involving the integrin signaling pathway.

Inflammation and fibrosis

DMD patients show chronic inflammation and impaired muscle regeneration. Once necrosis starts, DMD muscle contains an increased number of a variety of inflammatory cells (CD4 and CD8 T lymphocytes) (McDouall et al., 1990; Blake et al., 2002) and genome profiling studies provide evidence for coordinated activity of numerous components of a chronic inflammatory response, including cytokine and chemokine signaling, leukocyte adhesion and diapedesis, invasive cell type-specific markers, and complement system activation (Haslett et al., 2002).

Conjugate immune response signals and local overexpression of extracellular matrix genes are evident (Spencer et al., 2001) and cause the extensive fibrosis and sequent adipocyte tissue substitution. In fact, following the inflammatory cascade, it is hypothesized that profibrotic cytokine transforming growth factor (TGF-β1) is induced, leading to muscle wasting due to failed regeneration (Chen et al., 2005; Tidball et al., 2005). In patients with DMD, the rennin-angiotensin system increases the fibrotic cytokine TGF-β and upregulates TNF-alpha, which further perpetuates the inflammatory process. Fibrosis occurs as matrix metalloproteinase (MMP) inhibitors such as TIMP1 and 2 are upregulated (Ichim et al., 2010).

Moreover, the ability for muscle cells to regenerate is lost in DMD, as a consequence of exhaustion of satellite cells during ongoing degeneration and regeneration cycles (Ichim et al., 2010)

Apoptosis

Apoptosis is a specific form of cell death in which apoptotic cells shrink and are then rapidly phagocytized by neighboring cells (Sandri et al., 2001). This genetically controlled cell suicide takes place through one of two possible mechanistic pathways (Sandri et al., 2001). One potential pathway is via an extrinsic system Fas and FasL involving transmembrane receptors of the death receptor family (Abdel-Salam et al., 2009). FasL ligand induces apoptosis through cognate interactions with its receptor Fas family (Abdel-Salam et al., 2009). Fas is not typically expressed in normal muscle tissue, but has been found in diseased muscle fibers (Danielsson et al., 2009). The second pathway is via an intrinsic, endogenous system such as the mitochondrial Bax/Bcl-2 family (Abdel-Salam et al., 2009). Bcl-2 is a major and well-characterized anti-apoptotic protein that prevents the release of pro-apoptotic proteins through the outer mitochondrial membrane (Danielsson et al., 2009). Bax, on the other hand, is a protein that promotes cell death in response to apoptotic stimuli family (Abdel-Salam et al., 2009). A common end-point for both of these pathways is the activation of a series of cysteine proteases known as caspases (Sandri et al., 2001). These caspases can be broken down into two families: effectors and initiators. Effectors, such as caspase 3, are responsible for proteolytic cleavage that leads to cell disassembly (Sandri et al., 2001).

Apoptosis, along with other mechanisms, has been proposed to be involved in the pathogenesis of several neuromuscular diseases (Miller and Girgenrath, 2006). Dystrophin deficiency associated with DMD results in chronic inflammation and

severe skeletal muscle degeneration, where the extent of muscle fibrosis contributes to disease severity (Abdel-Salam et al., 2009). Normally dystrophin interacts with several members of the dystrophin glycoprotein complex, which forms a mechanical as well as signaling link from the extracellular matrix to the cytoskeleton (Abdel-Salam et al., 2009). Mutations in dystrophin result in membrane damage, allowing massive infiltration of immune cells, chronic inflammation, apoptosis, necrosis, and severe muscle degeneration. Normal muscles have the capacity to regenerate in response to injury. This ability is absent in DMD, resulting in severe skeletal muscle degeneration and triggering of apoptosis (Abdel-Salam et al., 2009). It is now accepted that the onset of death in dystrophin deficient muscles is an apoptotic process and that later stages of the disease involve both apoptosis and necrosis (Tidball et al., 2005).

Apoptotic events were reported to precede muscle fiber necrosis in mdx mice (Sandri et al., 1997). These findings supported the results already found in patients with DMD displaying apoptosis-associated features such as DNA fragmentation and the upregulation of Bcl-2, Bax, and caspases (Tews et al., 2002). Apoptotic nuclei, which are very rare in normal muscles (less than 0.1%), were detected in the dystrophic muscles of DMD patients as well as mosaic patterns of Bcl-2 and Bax which typically characterize dystrophic muscles (Sandri et al., 1998). A correlation has been found between apoptosis and caspase 3, being caspase 3 activity only found in dystrophin-deficient muscle fibers (Sandri et al., 2001). In patients with DMD, all muscles were found to have some fibers expressing Fas, Bax expression was

significantly higher, and Bcl-2 expression was significantly lower (Danielsson et al., 2009).

V. Skin: cellular and molecular components

The skin is organised in three layers including, from the top to bottom:

- the epidermis, with its associated appendages, pilosebaceus follicles and sweat glands;
- the dermis, separated from epidermis by the dermal-epidermal junction (DEJ);
- the hypodermis.

Embryologically, the epidermis and its appendages are of ectodermal origin, whereas the dermis and hypodermis are of mesodermal origin (Kanitakis J et al, 2002) (Fig.6).

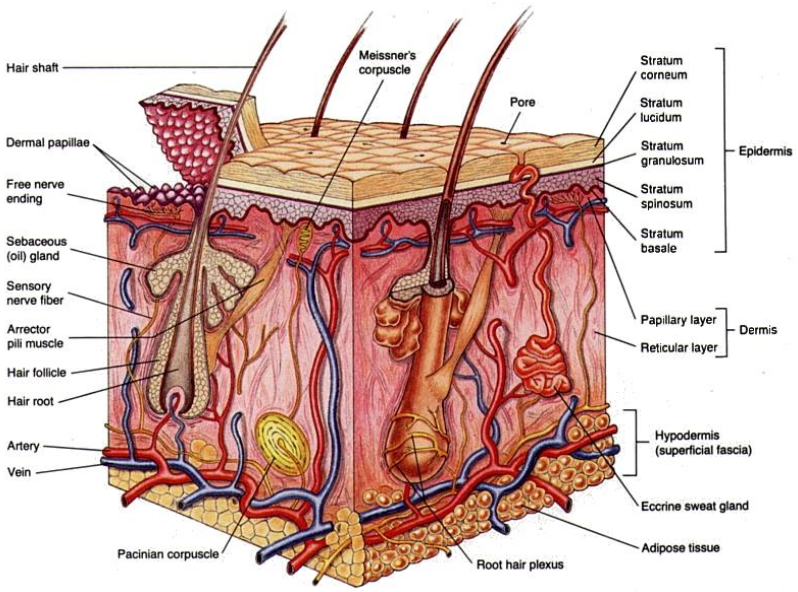

Figure 7, Skin representation.

The epidermis is a stratified epithelium that renews itself continuously. It is composed of different cell types, the majority of which (90-95%) are keratinocytes, 5-10% are instead non-keratinocytes, including melanocytes, Langerhans cells and Merkel cells (Fig.2). Epidermal cells are arranged in continuous layers (from bottom to top): the basal layer (single layer), the stratum spinosum (5-15 layers), the stratum granulosum (1-3 layers), the stratum lucidum and, the most superficial layer, the stratum corneum (5-10 layers)(Fig.8 and Fig.9).

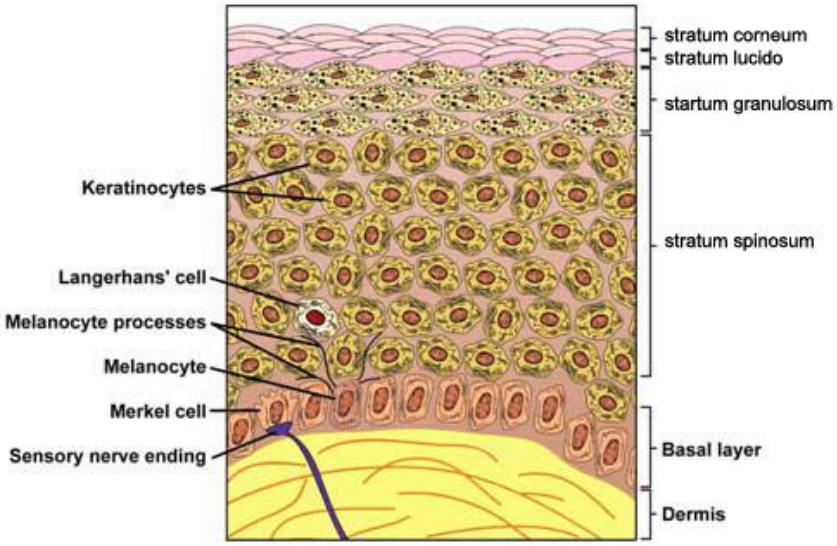

Figure 8, Schematic representation of epidermis layers.

The dermis is a supportive, compressible and elastic connective tissue that includes several cells (fibroblasts, dermal dendrocytes and mast cells) , fibrous molecules (collagens I,III, IV, VII) and ground substance (glycoproteins and proteoglycans). Its

structure varies depending on depth (superficial, papillary dermis and deep, reticular dermis).

Between dermis and epidermis there is a complex basement membrane zone (BMZ), called DEJ, synthesised by basal keratinocytes and dermal fibroblasts. It plays a fundamental role as a mechanical support for the adhesion of epidermis to dermis and regulates the exchanges of metabolic products between these two compartments. The DEJ is a highly dynamic and complex structure that is important in the regulation of cell adhesion, differentiation, and motility; in the transmission of extracellular signals and growth factor; and in the formation of permeability barriers (Hashmi et al, 2011). By electron microscopy 50-nm electron-dense zone called lamina densa and a 40-nm electron-lucent zone referred to as lamina lucida canbe detected (Timpl et al., 1986; Leblond et al., 1989) . Collectively, the lamina densa and the lamina lucida form the basal lamina.

The DEJ represents a complex and precise assembly of both intracellular and extracellular proteins that function in a collective manner to preserve tissue integrity; the main components, depicted in the BMZ representation (Fig 8), are: perlecan, laminin-511, laminin-332, laminin-311, nidogen, type IV, VII, XVII(BP180) collagens, plectin, integrin $\alpha6\beta4$, $\alpha6\beta1$ (specific for melanocytes), and $\alpha3\beta1$ (Hashmi et al, 2011). Moreover, α and β-dystroglycan (Durbeej et al., 1996) and δ-sarcoglycan and dysferlin (Vainzof et al., 2000), are expressed in skin.

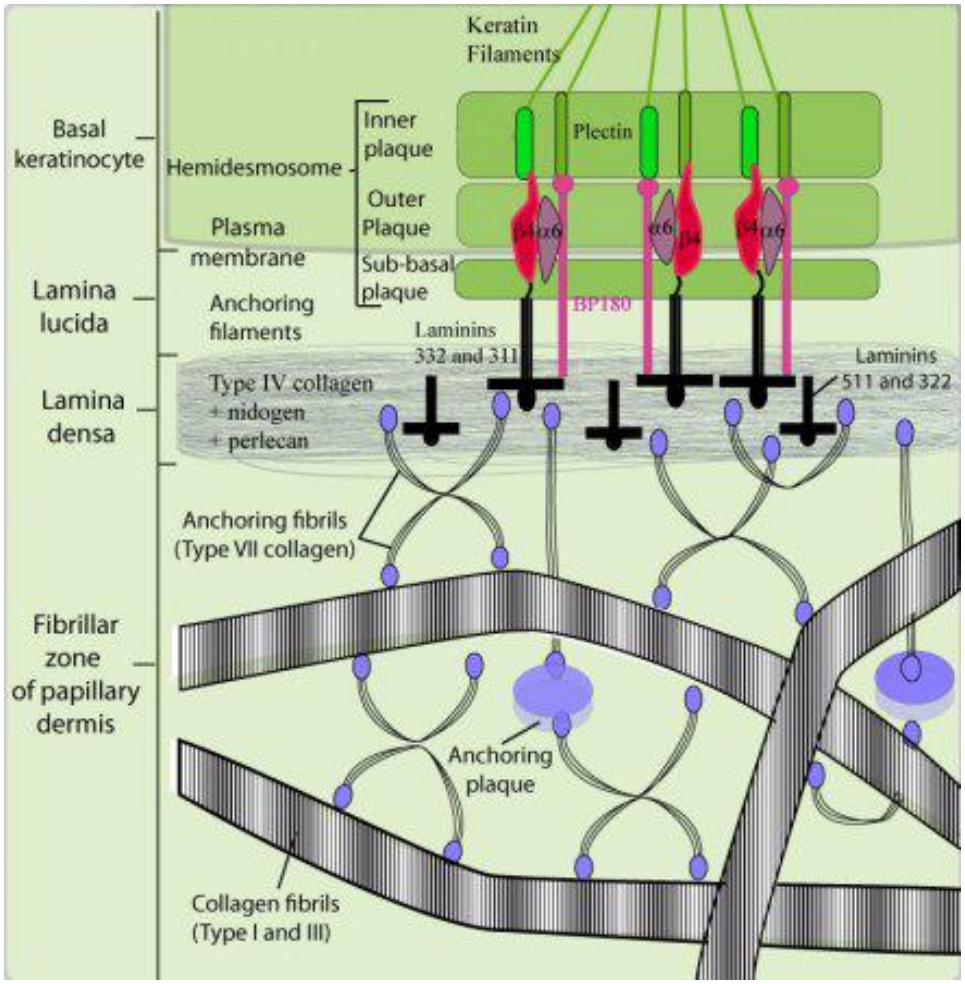

Figure 9, BMZ molecular composition. The most superior aspects of the dermal-epidermal BMZ contain the specialized intracellular keratin linker proteins plectin and BPAG1 (BP230). These in turn are connected to specialized transmembrane proteins, which include integrins α6β4 and α3β1 and type XVII collagen (BP180/BPAG2). Collectively, these intracellular and transmembrane proteins form the hemidesmosome. The integrins and type XVII collagen also connect to specialized extracellular proteins laminin-332 and laminin-311, together forming anchoring filaments. The interaction between these specialized extracellular proteins and ubiquitous proteins, which include laminin-511, type IV collagen, and nidogen, form the lamina densa. Finally, extending out of the lamina densa is another specialized extracellular structure known as the anchoring fibril. Anchoring fibrils are thick-banded structures, extending as perpendicular extensions from the lamina densa, looping through interstitial collagen fibrils in the dermis, and reinserting back onto the lamina densa. The primary constituent of anchoring fibrils is type VII collagen in the papillary dermis.

V.1. Melanocytes

Melanocytes are neural crest-derived cells that synthesize and store the melanin. The major known function of melanin is to provide protection against UV-induced DNA damage by absorbing and scattering UV radiation (Park et al., 2009). Melanocytes of the skin and hair transfer their melanin to surrounding keratinocytes. With the help of its dendritic projections, each epidermal melanocyte provides melanin to approximately 36 keratinocytes (Fitzpatrick, 1983; Van Den Bossche et al, 2006). Melanin is packaged and delivered to keratinocytes by melanosomes that are lysosome-like structures. Once in keratinocytes, melanosomes are distributed and, in response to UV radiation, positioned over the nuclei to form cap-like structures (Haass et al., 2005).

In the epidermis, melanocytes are interspersed among every 5–10 basal keratinocytes, forming the epidermal melanin unit (Haass and Herlyn, 2005). Despite the dynamic nature of epidermal turnover, the proliferation and function of melanocytes are strictly controlled under physiologic conditions. This balance is maintained through regulated induction of melanocyte division and is only disturbed during transformation into a nevus or a melanoma (De Luca et al., 1993). During childhood, so during expansion of the total skin surface, there is a continuous need for melanocyte proliferation to maintain a stable ratio with the basal keratinocytes (Haass et al., 2005). In order to proliferate, melanocytes need to decouple from the basement membrane and from the keratinocytes, retract their dendrites, divide, and migrate along the basement membrane before they finally recouple to the matrix and

to keratinocytes to form another epidermal melanin unit (Haass et al., 2004). The half-life of melanocytes is not well understood. In any case melanocytes during adulthood, continue to proliferate albeit at a low rate and only upon specific stimulation such as sunlight exposure or wounding (Bissell and Radisky, 2001).

During human development, the density of melanocytes reaches a peak of about 2300 cells/mm^3 at the end of the third month and reaches a final value of about 800 cells/mm^3, that represents a significant proportion of the cells of the epidermis in the adult (Ernfors, 2010).

The homeostasis of melanocytes is mainly due to the influence of soluble growth factors and on their direct molecular contact to the cellular (i.e. keratinocytes) and acellular microenvironment (i.e. extracellular matrix (ECM), basement membrane (BM)) (Valyi-Nagy et al., 1993; Krengel et al., 2005).

Melanocyte is a bipolar cells, so they have functions and adhesion protein complex different in the upper side, where interacts with keratinocytes, and lower side, where anchors the BM.

The molecular interaction between melanocytes and the BM is not fully understood. Early electron microscopic investigations showed the presence of dense plates on the basal side of melanocytes, which are similar but not identical to hemidesmosomes on keratinocytes (Tarnowski et al., 1970). By immunoelectron microscopy, it was demonstrated the presence of α3–, α6–, αV- and b1-integrins on the melanocyte plasma membrane (Zambruno et al., 1993). The epidermal BM contains laminin (LM) type-1, -5 and -6, reflecting the contribution of epithelial (keratinocytes) and

mesenchymal cells (fibroblasts) (Fleischmajer et al., 1998). The attachment of melanocytes to LM molecules in the lamina lucida is mediated by integrins α3β1 and α6β1. In vitro, melanocytes bind to collagen type IV through integrin α2β1, leading to increased melanocyte migration (Morelli et al., 1993). Binding to fibronectin, a small glycoprotein of the dermal ECM, is mediated by integrins α5β1 and αVβ3 (Scott et al., 1992). In vivo, integrin αVβ3 is only expressed in the vertical growth phase of malignant melanoma, suggesting that fibronectin-binding integrins might be of importance in melanocytic tumor progression. Additionally, in certain situations, e.g. wound healing, adhesion molecules that promote the migration of melanocytes (α2β1) or their binding to dermal ECM molecules (α5β1, αVβ3) may play a significant role (Krengel et al., 2005).

Moreover, skin melanocytes express α and β-dystropglycan (Korner et al., 2007). α-DG is an extracellular laminin-2-binding protein anchored to β-DG, which directly links mDp427 in the sarcolemma.

At the apical and lateral sides melanocytes adhere to keratinocytes via E-cadherin and desmoglein which enable them to communicate with each other through gap junctions. In melanoma cells E-cadherin is down-regulated (Haass et al., 2005).

Keratinocytes stimulate melanocyte growth, regulate the melanocyte-keratinocyte ratio, melanocyte morphology and melanin synthesis, and specifically direct the proper spatial and physiological organization of melanocytes within the basal layer (De Luca et al., 1993).

AIMS OF THE STUDY

The main aim of the study is to characterize the skin melanocytes as a cellular dystrophin-expressing model mirroring muscle cells.

In particular, the study was focused on:

- the evaluation and localization of dystrophin-protein in human normal skin
- the characterization of dystrophin isoforms expressed by the skin melanocytes
- the study of the expression of the full length muscle dystrophin (mDp427) in cultured melanocytes
- the assessment of mDp427 expression and the mitochondrial morphology in DMD patients, both *in vivo* and *in vitro*
- the evaluation of mitochondrial functionality in cultured myoblasts and melanocytes from the same DMD patients

MATERIAL AND METHODS

Patients. Skin biopsies from three healthy subjects and three DMD patients were collected; samples were frozen in isopentane pre-chilled and stored in liquid nitrogen. All patients were previously diagnosed by genetic, histochemical and biochemical analysis. DMD1 patient carried deletion of exon 51, while DMD2 and DMD3 patients deletion of exon 45 in *DMD* gene. All participants provided written informed consent, and approval was obtained from the Ethics Committee of the University of Ferrara.

Immunofluorescence analysis. Unfixed frozen sections (7 μm-thick) of biopsies from healthy donors and DMD patients were incubated with rabbit polyclonal antibody which recognizes an internal region of dystrophin (amino acids 801-1100) of dystrophin (Santa Cruz) and detected with anti-rabbit TRITC-conjugated IgG (DAKO); mouse monoclonal DYS1, DYS2, DYS3, utrophin, β-dystroglycan (Novocastra) and α-dystroglycan (Upstate Technologies, UBI) antibodies were revealed with secondary anti-mouse TRITC or FITC-conjugated antibodies (DAKO). Samples, when indicated, were double labeled with anti-pMEL-17 (Monosan), cytokeratin (Sigma) or laminin α1 antibodies (Chemicon). Samples were mounted with an anti-fading reagent (Molecular Probes) and observed with a Nikon epifluorescence microscope.

***Melanocyte and muscle cell cultures*.** Primary cultures of normal melanocytes were obtained from the leg skin of four healthy donors and two DMD patients. Skin fragments were cut into small pieces and the epidermis was separated from the dermis after overnight incubation in 0.5% dispase II (Roche) at 4° C. Melanocytes were maintained in M254 culture medium (GIBCO) supplemented with phorbol-12-myristate 13-acetate, transferrin, hydrocortisone, insulin, bovine pituitary extract, basic fibroblast growth factor and fetal calf serum (HMGS supplement, GIBCO). Muscle cell cultures from tibialis anterior of one healthy subject and from DMD patients were established as previously reported (Angelin et al., 2007).

***Western blot analysis*.** Human melanocyte cultures in 100 mm-diameter dishes and epidermal samples, obtained as previously described, were washed three times in PBS and it was added 100 µl of protein extraction buffer (RIPA lysis buffer). Total proteins were quantitated with a Bio-Rad DC Protein Assay Kit. One hundred micrograms of protein from each samples were boiled in Laemmli sample buffer and loaded onto a 6% polyacrylamide gel. The fractionated proteins were electroblotted onto a nitrocellulose membrane at 35 V ON at 4°C and unreacted binding sites on the membrane were blocked with 5% (wt/vol) dry milk solution for 60 min at room temperature. Membranes were probed with DYS1 (Novocastra), pMEL 17 (Monosan) and GAPDH (Millipore) and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000; Santacruz) for 20 min. Chemiluminescent

detection of proteins was carried out with ECL detection reagent Kit (GeHealthcare Amersham) according to the supplier's instructions.

Electron microscopy. Skin fragments were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for two hours, post-fixed with 1% osmium tetroxide and embedded in Epon 812 epoxy resin. Melanocytes and muscle cell cultures were grown onto uncoated well plates, fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for two hours, post-fixed with 1% osmium tetroxide. After dehydration, cells were detached with propylene oxide, and embedded in Epon812 epoxy resin (Ognibene et al., 1999). Ultrathin sections were observed with a Philips EM400 electron microscope operated at 100 kV. The quantity of altered mitochondria was evaluated on three independent experiments, and expressed as mean percentage. At least 700 mitochondria for each sample were studied for statistical evaluation; data were analyzed according to Mann-Whitney test, and the criterion for statistical analysis was $P<0.05$.

RT-PCR analysis of dystrophin transcripts. Total RNA was isolated from frozen skin biopsies and melanocyte cultures using the Rneasy Kit (Qiagen) following the manufacturer's instructions. Before cDNA synthesis, RNA was treated with DNAse I (Roche). Reverse Trascription (RT) was performed using random hexanucleotide primers and Superscript III enzyme (Invitrogen) according to the protocol supplied. All PCRs reactions were carried out in a volume of 25µl containing the cDNA

template and oligonucleotide primers designed to amplify the full-lenght and short dystrophin isoforms and utrophin A and B (sequence available upon request). PCR reactions were analyzed on agarose gels containing ethidium bromide prior to photography.

Mitochondrial membrane potential assay. Mitochondrial membrane potential was measured based on the accumulation of tetramethylrhodamine methyl ester (TMRM) (Molecular Probes) (Angelin et al., 2007). Primary cultures of melanocytes obtained as described above from a healthy donor and two DMD patients were seeded onto 24-mm-diameter round glass coverslips and grown for 2 days in M254 culture medium with HMGS supplement (GIBCO). The medium was then replaced with serum-free M254 medium supplemented with 10 nM TMRM for 30 min, and cellular fluorescence images were acquired with an Olympus IX71/IX51 inverted microscope, equipped with a xenon light source (75 W) for epifluorescence illumination and with a 12-bit digital cooled CCD camera (Micromax, Princeton Instruments). Data were acquired and analyzed using Cell R Software (Olympus). For detection of fluorescence, 568 ± 25 nm band-pass excitation and 585 nm long-pass emission filter settings were used. Images were collected with exposure time of 100 msec using a 40x, 1.3 NA oil immersion objective (Nikon). The extent of cell and hence mitochondrial loading with potentiometric probes is affected by the activity of the plasma membrane multidrug resistance pump. In order to normalize the loading conditions, in all experiments with TMRM the medium was

supplemented with 1.6 μM CsH, which inhibits the multidrug resistance pump but not the PTP (Bernardi et al., 1999). At the end of each experiment, mitochondria were fully depolarized by the addition of 4 μM of the protonophore carbonyl cyanide-*p*-trifluoromethoxy-phenyl hydrazone (FCCP). Clusters of several mitochondria were identified as regions of interest, and fields not containing cells were taken as the background. Sequential digital images were acquired every 2 min and the average fluorescence intensity of all relevant regions was recorded and stored for subsequent analysis.

RESULTS

Melanocytes express dystrophin at the interface with the dermal-epidermal junction

Perpendicular sections of skin from normal subjects were immunolabeled with a polyclonal anti-dystrophin antibody, raised against the internal domain of the protein. In the epidermis, dystrophin labeling was detected in the basal layer, while it was absent in the upper layer of epidermis (Fig. 1A, upper panel). Double labeling with anti-laminin γ1 chain, a marker of BM, showed that dystrophin expression was restricted to the cell plasma membrane facing the DEJ. Interestingly, only a fraction of the cells of the basal layer expressed dystrophin (Fig. 1A, lower panel). By double labeling with specific markers of keratinocytes (cytokeratin) and melanocytes (p-MEL-17) dystrophin was selectively detected in melanocytes, while it was absent in basal keratinocytes (Fig. 1B, upper panel). On the other hand, β-dystroglycan and α-dystroglycan, two dystrophin associated components, were homogeneously expressed along the epidermal basement membrane (Fig. 1B, lower panel).

Dystrophin isoforms in melanocytes

The immunohistochemical analysis of dystrophin with DYS1, DYS2 and DYS3 antibodies, specific for the dystrophin rod, C and N terminal domain, respectively, showed a strong labeling at the membrane of melanocytes (Fig. 2A). Western blot analysis with DYS1 antibody, which specifically recognizes the full length isoform,

was performed on a sample of normal epidermis: a band at 427kD was detected in the epidermis lysate, albeit at lower levels with respect to a normal skeletal muscle sample, due to the relative low number of melanocytes present in the epidermis (Fig. 2B).

RT-PCR analysis of dystrophin isoforms in skin epidermis obtained by enzymatic digestion with dispase II confirmed the presence of muscle full length mDp427. In addition, the Dp260, Dp116 and Dp71 shorter isoforms were also detected (Fig. 2C).

Dystrophin expression in cultured melanocytes

Dystrophin expression was also evaluated in primary melanocyte cultures from normal skin, both at the protein and transcript level. Previous studies described different patterns of dystrophin localization, depending on the time of culture and cell proliferation, in normal muscle cell cultures (Trimarchi et al., 2006). Thus, melanocytes were examined at 12, 48 and 72 hous after plating. The rate of proliferation was determined by immunofluorescence analysis of Ki-67, a nuclear factor express exclusively in proliferating cells (Fig. 3B). After 12 hours of plating, when the cells were quiescent (Ki-67-negative), dystrophin labeling with DYS1 antibody was detected at the plasma membrane, while it was absent along the dendritic processes. A change of the dystrophin pattern was noted in proliferating cells (Ki-67-positive); in fact, dystrophin labeling was restricted to the perinuclear area, with a cytoplasmic pattern (Fig. 3A). The expression of dystrophin, evaluated by western blotting with DYS1 antibody, indicated that the amount of the protein

present in melanocytes at passage 3 is similar to that in differentiated myoblasts (Fig. 3C). The reduction observed at passage 6 could be associated with *in vitro* dedifferentiation (Kormos et al., 2011). RNA analysis in cultures showed a clearly detectable full-length muscle isoform (Fig. 3D).

On the contrary, α-dystroglycan and β-dystroglycan membrane labeling was maintained in proliferating melanocytes, both at the cell body and dendritic processes (Fig. 3E).

Dystrophin is absent in DMD skin melanocytes

Skin biopsies obtained from three genetically characterized DMD patients were analyzed for dystrophin expression. Dystrophin was absent at DEJ despite the high number of epidermal melanocytes detectable at the basal layer (p-MEL positive cells) (Fig. 4A). Dystrophin was also absent in the dermis, in particular, in the arrector pili smooth muscle cells and myoepithelial cells (not shown). Dystrophin absence in DMD skin correlated with the expression pattern in muscle biopsies of the same patients, with the exception of rare (<1%) dystrophin-positive revertant fibers (Fig. 4B), which could result from alternative skipping leading to the restoration of a functional protein (Klein et al., 1992).

DMD melanocytes display defective adhesion

To evaluate whether dystrophin absence may affect melanocyte attachment to the DEJ, we performed double labeling with pMEL-17 and perlecan, BM protein component, in skin biopsies of two DMD patients. Dystrophin-deficient melanocytes localized in the basal layer of the epidermis, similarly to normal melanocytes. However, they displayed an altered shape and a reduced surface of attachment to the DEJ (Fig. 5A) as we could seen also in the skin semithin sections Toluidine blue stained (Fig. 5B). The ultrastructural analysis revealed the presence of duplication of the basement membrane exclusively underlying melanocytes; aspects of detachment of melanocyte plasmamembrane from the BM were commonly found (Fig. 5C). By contrast, DMD keratinocytes appeared attached to the DEJ basement membrane (not shown).

Characteristic mitochondrial alterations in DMD skin and cultured melanocytes

The ultrastructural analysis of skin biopsies from patients DMD2 and DMD3 showed mitochondrial changes in epidermal melanocytes, including increased size, reduced matrix density and disrupted cristae, consistent with swelling. On the other hand, mitochondrial alterations were not detected in DMD keratinocytes (Fig. 6A) and in melanocytes of normal skin (not shown). The presence of mitochondrial alterations was confirmed in melanocyte cultures of the same DMD patients. DMD mitochondria appeared generally enlarged; in longitudinal sections, multiple focal swelling areas were detected along their major axis (Fig. 6B). The percentage of

altered mitochondria was 32% in DMD2 (SD ± 8.5, $p<0.005$) and 38% (SD ± 9.7, $p<0.005$) in DMD3 patient's melanocytes. On the other hand, mitochondrial abnormalities were only occasionally detected in melanocytes of healthy subjects. We compared melanocytes with myoblasts cultures from the same DMD patients by ultrastructural analysis. Interestingly, muscle cell mitochondria displayed focal swelling, similarly to melanocytes (Fig. 6C). The percentage of swollen mitochondria was 3% (SD ± 0.2), 12% (SD ± 3.4) and 15.7% (SD ± 2.1) in normal, DMD2 and DMD3 muscle cells, respectively.

Latent mitochondrial dysfunction in melanocytes from DMD patients

To test whether a functional defect of mitochondria could be underlie the ultrastructural alterations, we studied mitochondrial function in primary cultures of melanocytes from one normal donor and two DMD patients. Mitochondria readily accumulated TMRM, indicating that they are energized irrespective of the presence of dystrophin. On the other hand, and at variance from mitochondria of normal donor (Fig. 7A- panel a), upon the addition of the F_0F_1 ATPase inhibitor oligomycin mitochondria of DMD patients readily depolarized after the expected hyperpolarization (Fig. 7A – Panel b and c), suggesting a latent dysfunction (e.g. Ca^{2+} deregulation) (Angelin et al., 2008) that can be unmasked by inhibition of the ATP synthase. Mitochondrial functionality was also evaluated in myoblast cultures of the same DMD patiens (DMD 2 and DMD 3), to compare the data obtained in the cultured melanocytes. TMRM analysis proved the muscle cells of DMD patients

exhibit a similar response profile seen in the melanocytes i.e. a depolarization upon the addiction of olygomycin (Fig. 7B).

CONCLUSION & DISCUSSION

Our investigation revealed several new findings. First, melanocytes, but not adjacent keratinocytes, express the dystrophin full length isoform both in vivo and in vitro, while the protein is absent in melanocytes from DMD patients. Second, DMD melanocytes display morphological alterations of mitochondria similar to those detected in dystrophin-deficient muscle cells and a latent mitochondrial dysfunction unmasked by inhibition of the ATP synthase.

Interestingly, we found that melanocytes express dystrophin with a restricted localization to the plasma membrane facing the dermal epidermal junction. Epidermal melanocytes are polarized cells, with basal and apical different functions, which are recapitulated by differential composition of the membrane at different sides (Pinon and Wehrle-Haller, 2011). DEJ consists of a sheet-like structure, composed by extracellular matrix proteins, which forms an adhesion interface between epidermal basal cells (keratinocytes and melanocytes) and the underlying extracellular matrix. The DEJ acts as a permeability barrier; it controls cell organization and differentiation by mutual interactions between cell-surface receptors and molecules in the extracellular matrix (Santiago-Walker et al., 2009). DEJ exhibits features common to muscle cell sarcolemma. In particular, laminin alpha2 chain (Sewry et al., 1996), and dystroglycans (DGs) alpha and beta subunits have been detected at the DEJ (Herzog et al., 2004). In epithelia, DGs are essential for basement membrane formation (Barresi and Campbell, 2006) and are involved in maintenance of epithelial cell polarity (Durbeej et al., 1995; Masuda-Hirata et al., 2009; Michele et al., 2002), providing a link with the extracellular matrix through the

α-dystroglycan glycosylated epitope. The dystrophin localization we have found in melanocytes may indicate a specific role of the protein at the dermal-epidermal junction.

It has been recently reported that high levels of the Dp427m protein are expressed in primary human melanocytes, whilst very reduced amounts of the protein are detectable in melanoma cell lines (Korner et al., 2007). The muscle-specific full-length isoform (*Dp427m*) is highly expressed in skeletal and cardiac muscles, and at a reduced level, in Purkinje cerebellar neurons (Muntoni et al., 2003).There are several other tissue specific isoforms of dystrophin, some exclusively or predominantly expressed in the brain or the retina (Waite et al., 2012). Here we show that, in addition to the Dp427m, melanocytes express the Dp116, Dp260 and Dp71 short isoforms.

The dystrophin expressed by human melanocyte cultures, analyzed at both the mRNA and protein level, corresponds to the muscle-specific full length mDp427 isoform, as previously reported (Korner et al., 2007). Interestingly, dystrophin continues to be expressed when melanocytes are maintained in culture; however, whilst the protein is retained at the plasma membrane in resting adherent melanocytes, its localization at the peri-nuclear cytoplasm characterizes proliferating melanocytes.

Whilst dystrophin is expressed by normal epidermal melanocytes, it is absent in DMD patient epidermis as well as in the muscle biopsies of the same patients. The absence of dystrophin, both in muscle and skin melanocytes, is in agreement with the

type of mutations and with the frame-shift hypothesis (Koenig et al., 1989). The spontaneous restoration of reading-frame of dystrophin occurring in some muscle fibers (revertant) correlates with degeneration/regeneration cycles (Yokota et al., 2006) indicating that this mechanism could be muscle specific, as a consequence of the dystrophic process.

When DMD skin biopsies are analyzed at the ultrastructural level, melanocytes appear detached with respect to the basement membrane of the DEJ. This data suggests that dystrophin may act in stabilizing melanocyte adhesion to the basement membrane and that this function is impaired in DMD patients. A previous study showed DMD gene deletions in melanoma cell lines; the occurrence of DMD mutations correlated with increased migration, whereas re-expression of DMD attenuated the phenotype (Körner et al., 2007). Our data support the hypothesis that dystrophin may critically change the adhesion and migratory capacity of melanocytes.

The second finding of the present investigation is related to the observation that DMD melanocytes show characteristic alterations of the mitochondrial morphology, consistent with swelling, which are not present in the neighbouring keratinocytes. These mitochondrial alterations persist in melanocytes in culture conditions, arising the question of whether they are mechanistically linked to disease pathogenesis, as is the case for collagen VI-related muscular dystrophies that involve inappropriate PTP opening (Irwin et al., 2003).

We evaluated mitochondrial function in primary melanocyte and myoblast cultures. In cells from healthy donor the addition of the F0F1 ATPase inhibitor oligomycin caused hyperpolarization, as expected of phosphorylating cells. Indeed, in respiring cells the mitochondrial membrane potential is maintained by proton pumping through the respiratory chain, and ATP synthesis draws a fraction of the proton gradient, which therefore increases when the ATP synthase is blocked. Strikingly, the initial hyperpolarization induced by oligomycin was instead followed by fast depolarization in melanocytes and myoblasts from the two DMD patients. This behaviour is reminiscent of the anomalous response we detected in myoblasts from UCMD patients (Angelin et al., 2007) and suggests a latent mitochondrial dysfunction in DMD cells like the one found in mdx mouse model (Pauly et al., 2012). Indeed, the initial hyperpolarization indicates that mitochondria are respiring and making ATP normally, yet oligomycin initiates a set of events ending in fast depolarization.

For the first time here we show that a similar mechanism may be responsible for mitochondrial alterations in both DMD melanocytes and myoblasts.

The idea that Ca^{2+} dependent mitochondrial dysfunction is a causative event in onset of DMD has been put forward as early as in 1976 (Wrogemann and Pena, 1976), and recently reinforced by the partial rescue from muscle pathology observed after inhibition of mitochondrial cyclophilin D, a positive effector of the PTP, with Debio025 in *mdx* mice (Millay et al., 2008; Wissing et al., 2010).

Skin biopsy as a diagnostic tool in dystrophinopathies has been already reported, as dystrophin is expressed at the plasma membrane of arrector pili smooth muscle cells (Ferlini et al., 2010; Tanveer et al., 2009). However, the use of this method remains uncommon since the uneven distribution of arrector pili and the localization of myoepithelial cells in deep dermis requires large and deep biopsies. Our finding of dystrophin expression by epidermal melanocytes make skin biopsy an appealing source for dystrophin detection, with diagnostic utility, and may represent an extremely useful tool to monitor the effect of therapeutic treatments. In addition, considering that melanocyte cultures can be easily obtained by conventional skin biopsy and that pure melanocyte cultures can be obtained and maintained stable for several passages by using selective culture medium (Kormos et al., 2011), they may represent a feasible and reliable tool alternative to muscle biopsy for dystrophinopathies diagnosis, disease progression monitoring and mitochondrial functional studies.

FIGURES AND LEGENDS

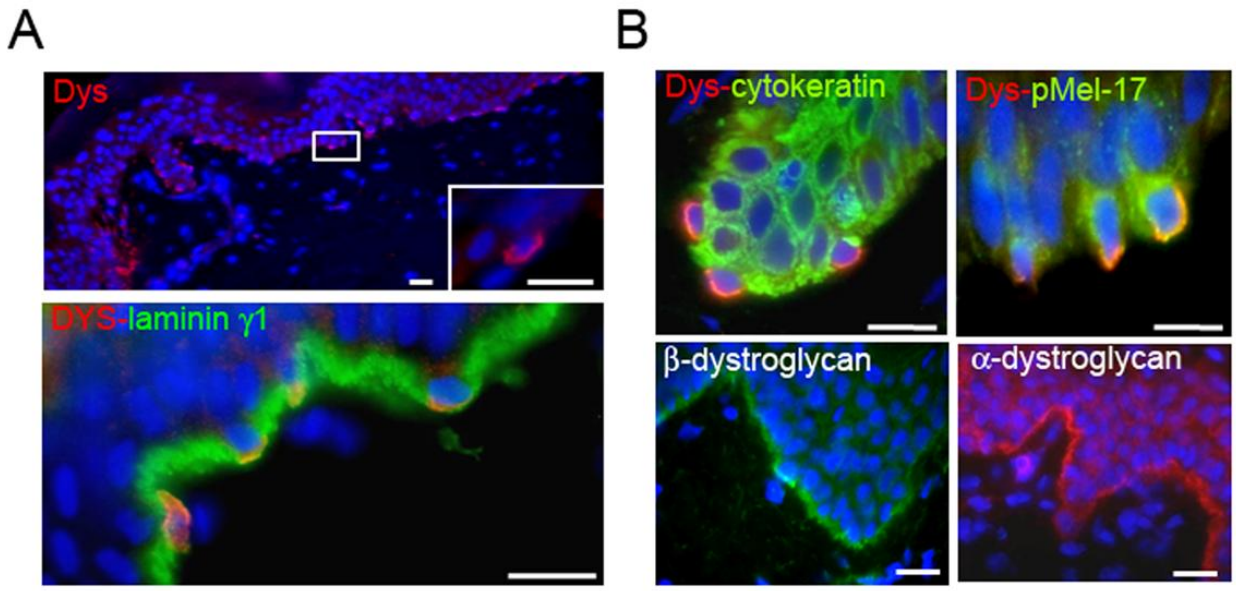

Fig 1. Dystrophin expression in the epidermis.

(A) Immunofluorescence analysis of dystrophin on perpendicular frozen sections of normal skin. Dystrophin is detected at the basal layer of the epidermis (upper panel). The high magnification (inset) evidences the discontinuous labeling pattern at the DEJ. Double labeling with anti-laminin γ1 chain reveals that dystrophin localizes at interface with the DEJ, where it co-localizes with laminin γ1 chain (orange staining). Nuclear staining, DAPI. Bar, 50 μm.

(B) Immunohistochemical characterization of dystrophin-expressing cells. Double labeling with anti-dystrophin and specific markers of keratinocytes (cytokeratin) and melanocytes (p-MEL-17) showing that dystrophin labeling is restricted to melanocytes, while it is absent in keratinocytes. β–dystroglycan and α-dystroglycan localize at DEJ with a continuous pattern involving all the different cells at this side. Nuclear staining, DAPI. Bar, 50 μm.

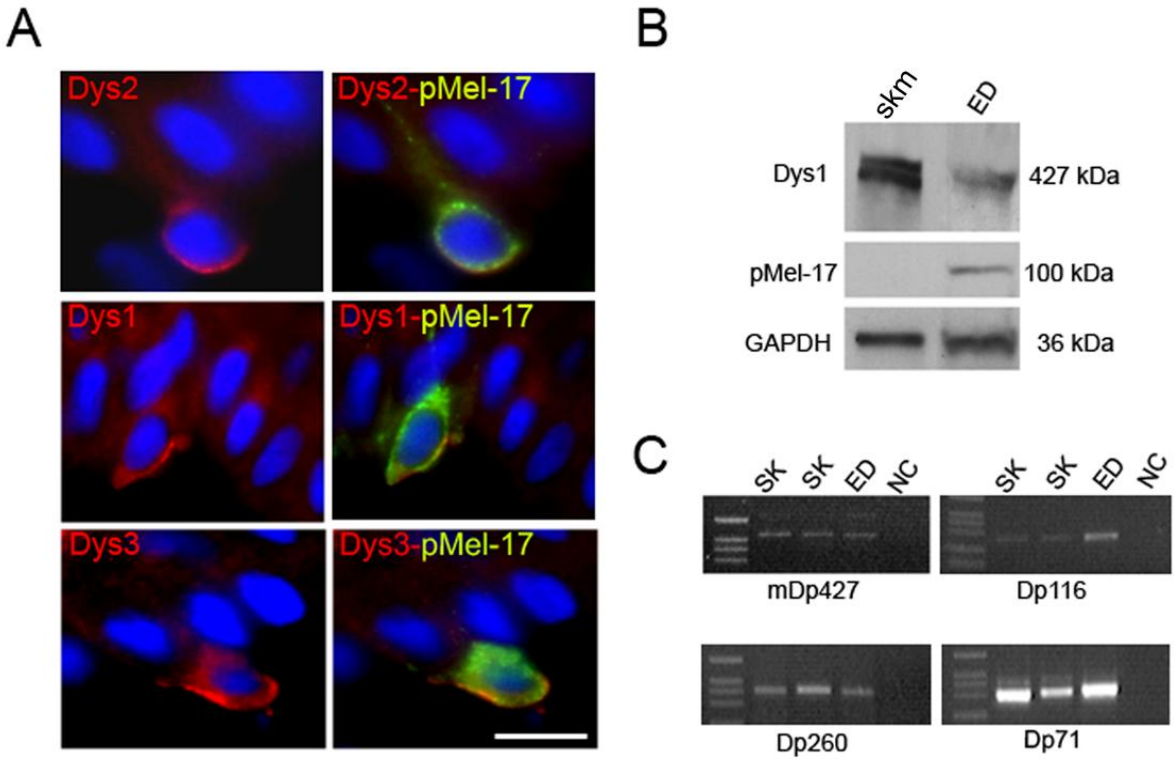

Fig 2, Dystrophin isoforms

(A) Immunofluorescence analysis of dystrophin with DYS2, DYS1 and DYS3 antibodies in perpendicular sections of normal skin. Melanocytes, identified by pMEL-17 antibody (green fluorescence) are double labeled with anti-dystrophin antibodies raised against carboxy-terminal (DYS2), internal (DYS1) and amino-terminal (DYS3) protein domain. Dystrophin staining is clearly detectable with all the antibodies, supporting the presence of the full length isoform. Nuclear staining, DAPI. Bar, 20 μm.

(B) Western blot analysis of dystrophin in the epidermis (ED) and skeletal muscle (skm). DYS1 antibody reveals the presence of a band at 427kD in both samples; the amount of dystrophin in the epidermis is lower with respect to the normal skeletal muscle, possibly due to the relative low number of melanocytes present in the epidermis, as indicated by the low amount of melanocyte marker (pMEL-17). GAPDH has been used as loading control.

(C) RT-PCR analysis of dystrophin transcripts. RNA analysis of dystrophin isoforms in two skin samples (SK) and epidermis (ED) reveals the presence of muscle full length mDp427 in all the samples examined. In addition, Dp260, Dp116 e Dp71 isoforms were also detected. NC, negative control.

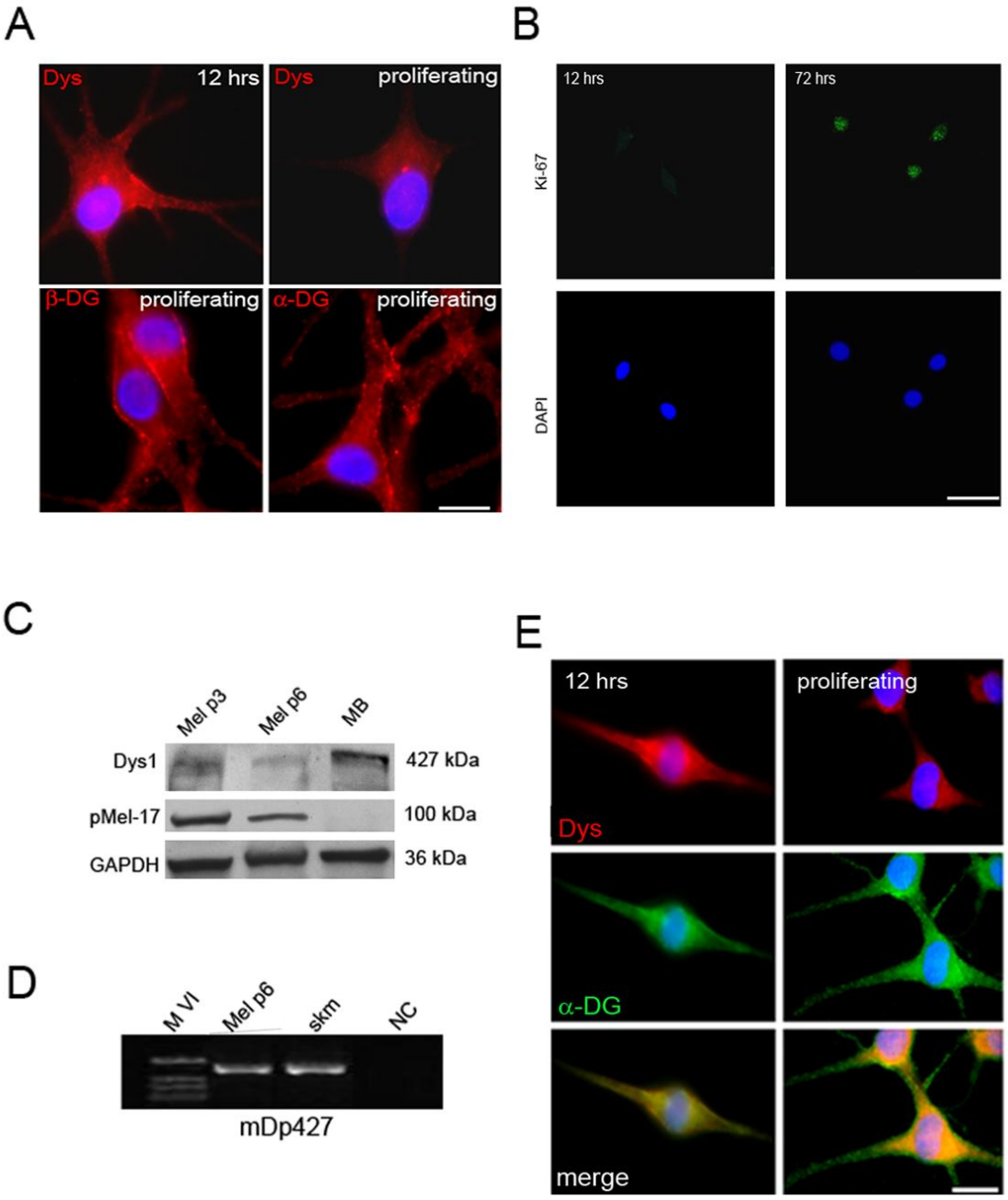

Fig 3, Dystrophin expression in cultured melanocytes.

(A) and (B) Melanocytes were extracted from skin epidermis by enzymatic digestion and grown onto coverslips for 12 hours (quiescent- Ki-67 negative cells) or 3 days (proliferating – Ki-67 positive cells). After 12 hours dystrophin staining with DYS1 antibody (Dys) is detected at the membrane, while in proliferating cells, dystrophin localization is restricted to the perinuclear area. α-dystroglycan (α-DG) and β-dystroglycan (β-DG) show a membrane pattern in proliferating cells. Nuclear staining, DAPI. Bar scale, 50 μm.

(C) Western blot analysis of dystrophin in cultured melanocytes at passage 3 (Mel p3) and 6 (Mel p6) and in differentiated myoblasts (MB) after 7 days in DMEM with 2% of FBS. DYS1 antibody detects a specific band in melanocyte samples at the same molecular weight of differentiated myoblasts. pMEL-17 was used as a marker of melanocytes, while GAPDH as loading control.

(D) RT-PCR analysis of mDp427 isoform in cultured melanocytes at passage 6 (Mel p6) and in skeletal muscle (skm) reveals the presence of muscle full length mDp427 in both samples. NC, negative control. M VI, molecular weight marker VI.

(E) Double-immunolabeling with α-DG (green) and Dys (red) shows that there is a colocalization of two signals (merge), confirming dystrophin membrane pattern specificity.

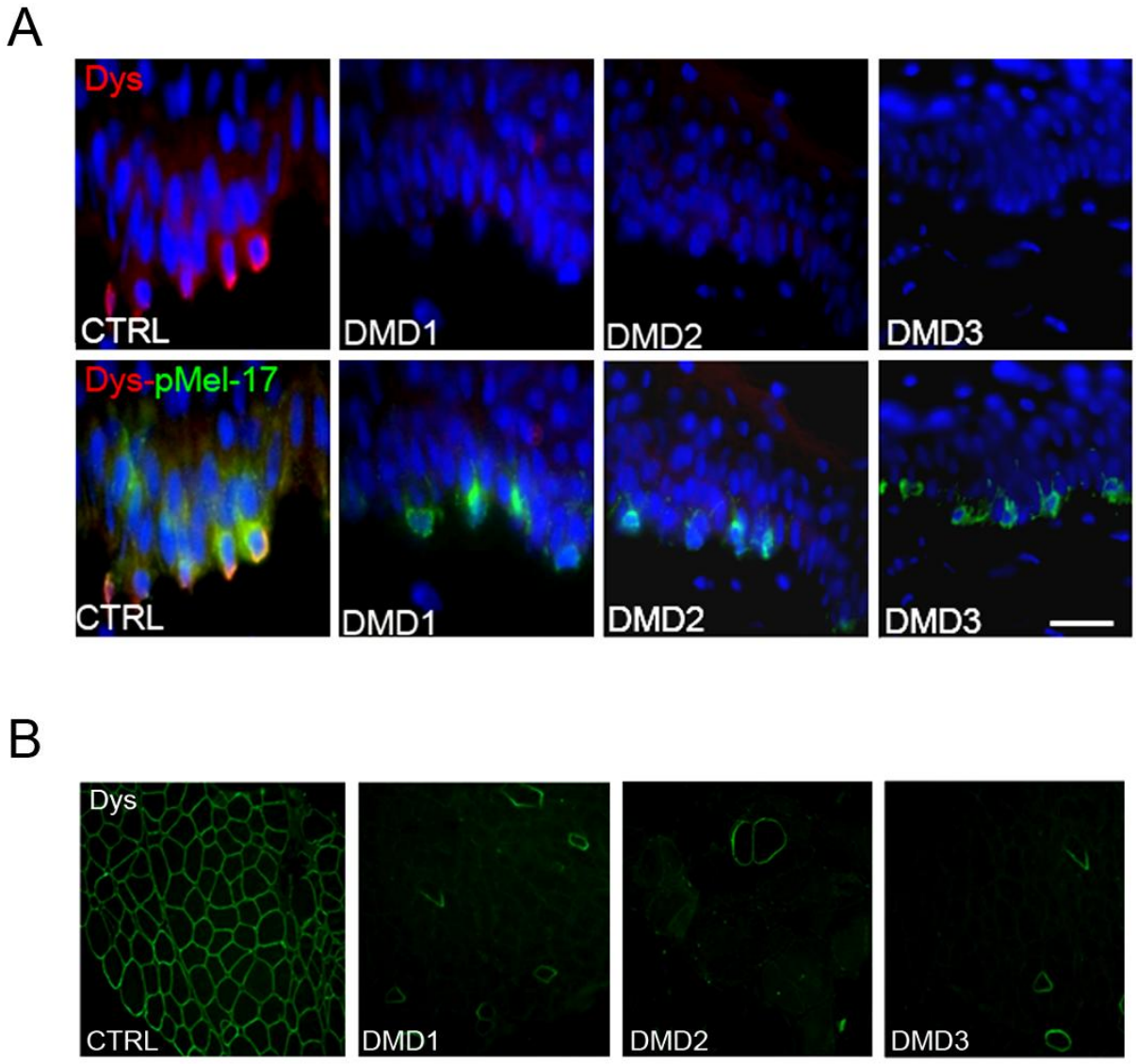

Fig 4, Immunofluorescence analysis of DMD patients.

(A) Dystrophin is not detectable at DEJ of DMD patients, despite the high number of epidermal melanocytes (p-MEL positive cells, green fluorescence). Nuclear staining, DAPI. Bar, 50 μm.

(B) Dystrophin immunolabeling in CTRL and DMD muscle skin sections. The same pattern was been identified in DMD skeletal muscle sections of three patients, where dystrophin is not expressed. Dystrophin positive fibers correspond to "revertant fibers".

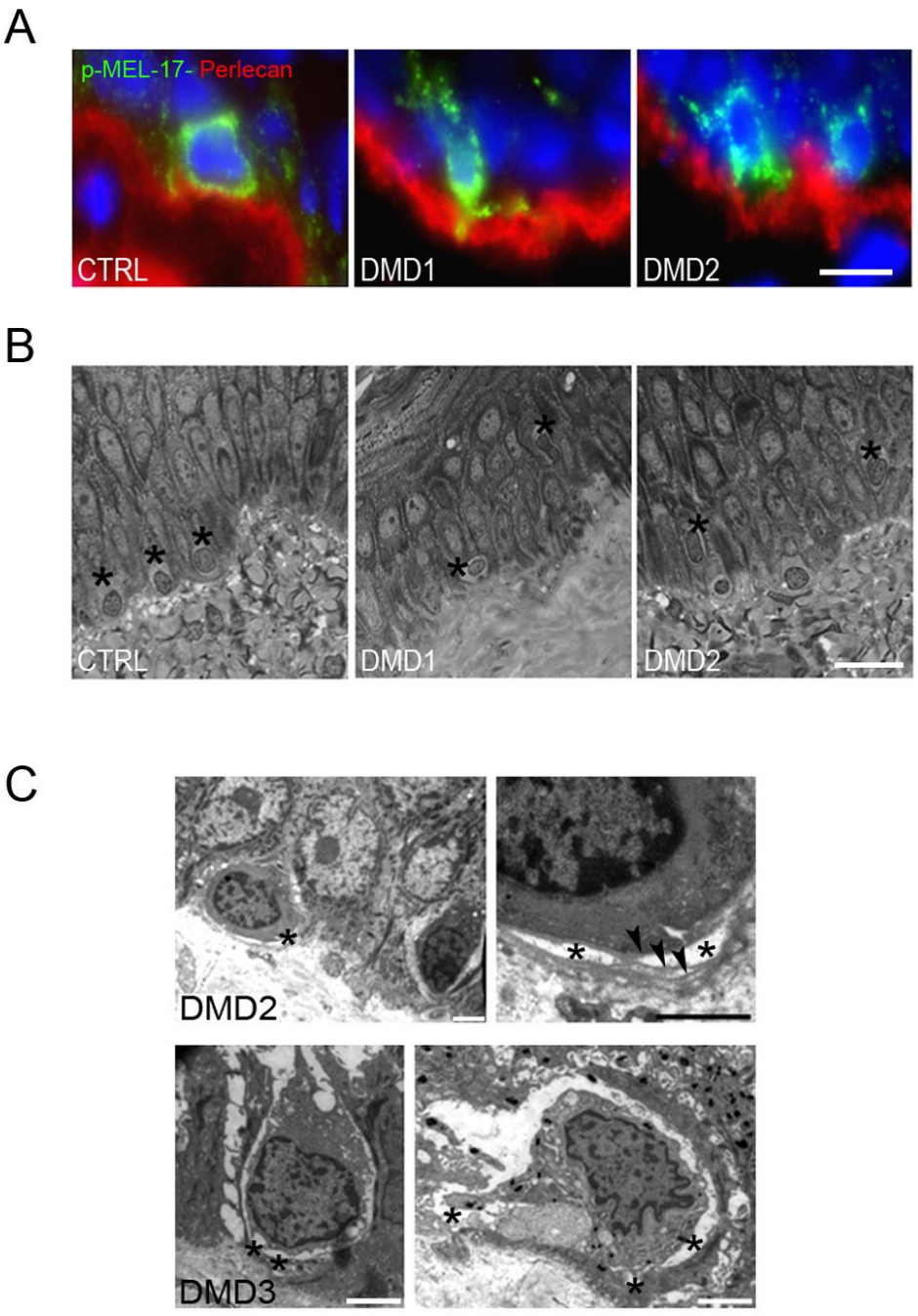

Fig 5, Immunofluorecence and ultrastructural analysis of DMD skin.

(A) Double labelling with perlecan and P-MEL 17 shows melanocytes are not fully attached on the basal lamina compare to control skin.

(B) Semithin sections toluidine blue stained display attachment defects of DMD melanocytes as indicated by asterisks.

(C) Ultrastructural analysis of DMD 2 and DMD3 patients better shows abnormalities of melanocytes as lamina duplication (arrow) and plasmamembrane detachment (asterisks).

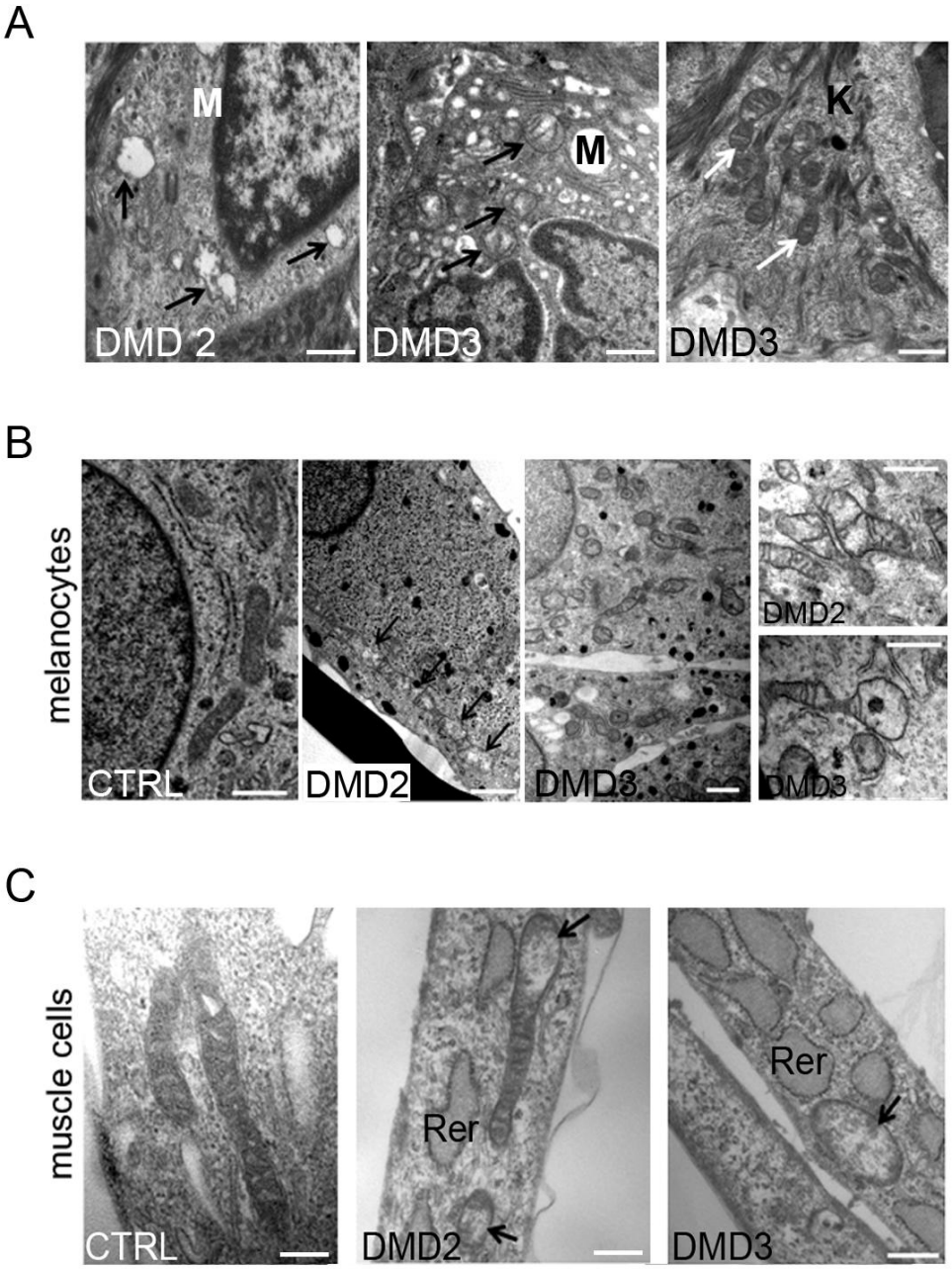

Fig 6, Morphological alteration of DMD melanocyte mitochondria.

(A) Transmission electron microscopy analysis of DMD melanocytes (M) in skin sections show mitochondrial changes, such as increased size, reduced matrix density and disrupted cristae, (black arrows). On the contrary, mitochondria appears normal (white arrows) in DMD keratinocytes (K).

(B) Ultrastructural analysis of normal and DMD cultured melanocytes. Normal melanocytes (CTRL) display elongated mitochondria with well-preserved cristae. Mitochondria of patients DMD2 and DMD3 appear enlarged with reduced matrix density and swelling (arrows). Bar, 600 nm.

(C) Ultrastructural analysis of normal and DMD myoblasts. Normal myoblasts (CTRL) show long mitochondria with a regular short axis. DMD2 and DMD3 cultured myoblasts show altered matrix density and disrupted cristae (arrows). Rer, rough endoplasmic reticulum. Bar, 600 nm.

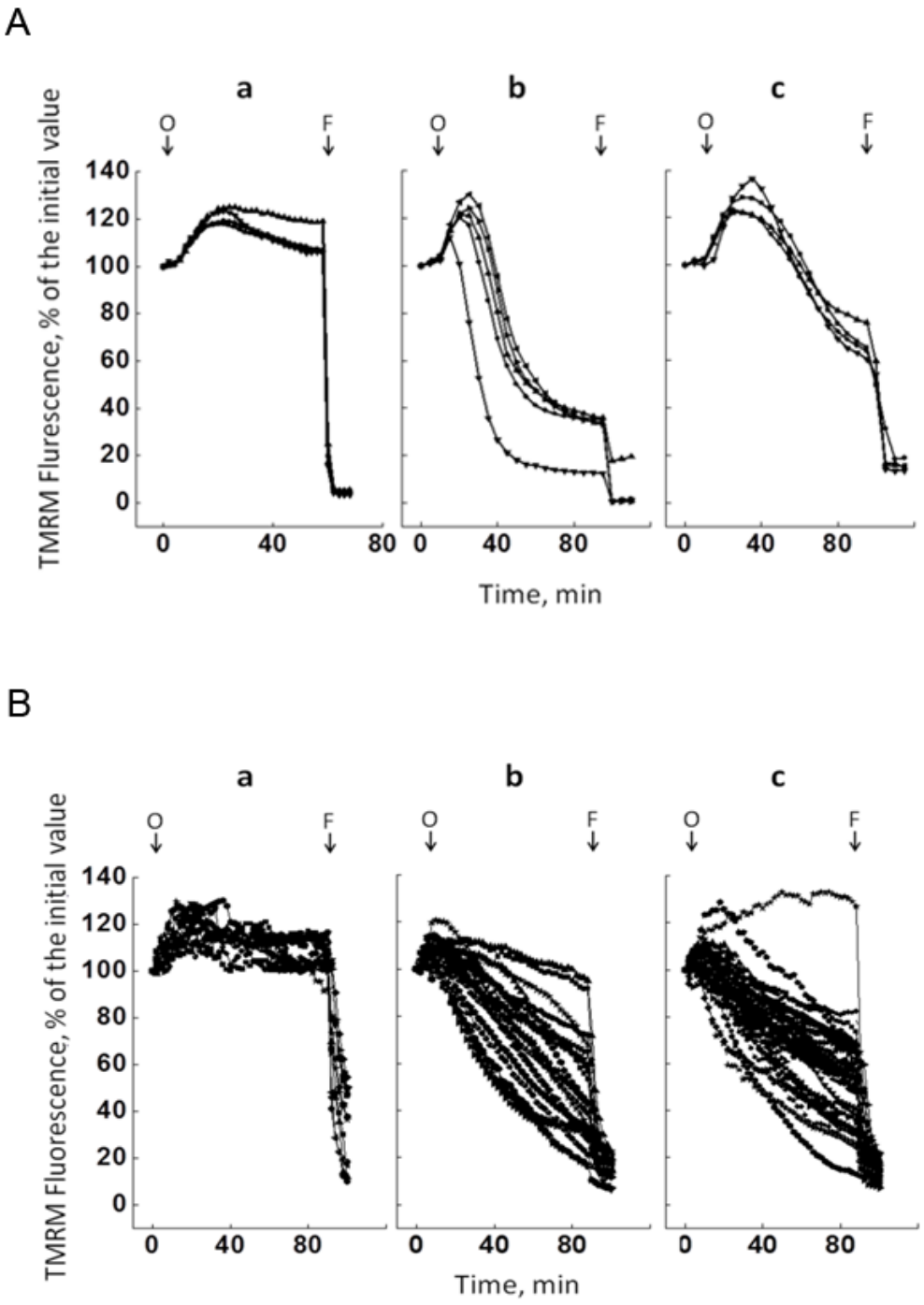

Fig 7, Functional alteration of mitochondria in DMD cultured melanocytes.

(A) Analysis of the effect of oligomycin on mitochondrial potential in melanocytes from healthy donor (panel a) and from patients DMD2 (panel b) and DMD3 (panel c). When indicated by arrows 4μM of oligomycin (O) and 4μM of FCCP (F) were added. Traces report one representative experiments of eight (panel a), five (panel b) and four (panel c). Each line corresponds to one individual cell. Note that all cells from DMD patients but not cells from the normal donor depolarized with oligomycin.

(B) Analysis of the effect of oligomycin (O) on mitochondrial membrane potential in myoblasts from healthy donor (panel a) and from patients DMD2 (panel b) and DMD3 (panel c). When indicated by arrows 4mM of oligomycin (O) and 4mM of FCCP (F) were added. T Each trace reports the response of one individual cell from four (panel a), nine (panel b) and twelve (panel c) experiments. If a threshold is set at 90% of the initial fluorescence, the fraction of myoblasts with depolarizing mitochondria is 0/12 (0%) for the normal donor, 18/20 (90%) for patient DMD2 and 33/35 (95%) for patient DMD3.

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PUBBLICATIONS

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