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Proteomic Analysis of Signalling Pathway Deregulation in Dystrophic Dog Muscle

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1. Introduction

During recent years, considerable effort has been made to develop proteomics technologies, with the aim of providing a complementary approach to the genomics tools already used in biomedical settings. This development has been extremely fast, and a number of emerging methodological proteomics tools now allow scientists to study the variable aspects of proteins in particular cell types, tissues or disease states. These tools include antibody arrays, two-dimensional-gel electrophoresis (2D-GE) and mass spectrometry (MS), the latter knowing an increasing use. In particular, candidate or non-candidate-based analyses of cell signalling represent powerful approaches for the investigation of the answers developed by cells in response to genetic modifications. Signalling molecules are key players in the regulation of the numerous and various biological processes occurring in a cell, and the alteration of signalling pathways has been associated with multiple diseases. Alterations in individual signalling pathways have been described in neuromuscular disorders, however, little information is available regarding their putative implication in Duchenne Muscular Dystrophy (DMD).

DMD is an X-linked neuromuscular disorder that affects 1 newborn in 3500. This recessive disease represents the most common and severe form of muscular dystrophy. Although the genetic basis of the disease is well resolved, the cellular mechanisms associated with the physiopathology remain largely unknown. Increasing evidence suggests that mechanisms secondary to the dystrophin deficiency at the basis of the disease, such as alterations in key signalling pathways, may play an important role. Proteomic profiling of dystrophic *vs* healthy skeletal muscle can help to generate a DMD-specific proteomic signature. Understanding which particular signal transduction pathways are involved in muscular dystrophy might provide a basis for new target and therapeutic agents discovery. This chapter examines signalling pathways status in skeletal muscles from the Golden Retriever Muscular Dystrophy (GRMD) dog, the only clinically relevant animal model for DMD (Valentine et al., 1988; Cooper et al., 1988). More specifically, we will describe how proteomic studies were successfully used to identify reliable biomarkers of the disease in animal models.

2. Signalling pathways and DMD

In dystrophic muscles, the absence of dystrophin, and the consequent destabilization of the dystrophin-glycoprotein associated complex DGC (a multiprotein transmembrane complex), lead to the loss of sarcolemma integrity, calcium overload, calpains activation and finally, necrosis of the myofibers (Muntoni et al., 2003). Besides providing mechanical stability, the DGC interacts with several proteins, including growth factor receptor-bound protein 2 (Grb2) (Yang et al., 1995), neuronal nitric oxide synthase (nNOS) (Brenman et al., 1995), calmodulin (Madhavan et al., 1992), focal adhesion kinase (FAK) (Cavaldesi et al., 1999) and caveolin-3 (Crosbie et al., 1998), that play a role in cell signalling. Grb2 has been identified as a component of the Ras/ mitogen-activated protein kinases (MAPK) signalling pathway and both FAK and Grb2 function as mediators of survival signalling in various cell types, often working through the phosphatidyl inositol 3-kinase (PI3K)/Akt pathway (Langenbach&Rando, 2002). Even if this mechanism can, in part, account for the degenerative phenotype observed in DMD, it seems increasingly obvious that the deregulation of intracellular signalling pathways also plays a role. These pathways, which are implicated in the regulation of crucial processes such as the balance between apoptosis and cell survival or the equilibrium between atrophy and hypertrophy, involve cascades of phosphorylation/dephosphorylation events. Protein kinases represent key enzymes responsible for the phosphorylation of specific targets. Moreover, altered cell signalling is thought to increase the susceptibility of muscle fibers to secondary triggers, such as functional ischemia and oxidative stress, and free-radical scavengers can have a direct impact on the activity/phosphorylation of some components of the MAPK cascades (Hnia et al., 2007). In progressive muscular dystrophy, muscles are characterized by hypertrophy in the early phase, while atrophic changes are observed with aging (Noguchi, 2005).

Studies of the X chromosome-linked muscular dystrophy (*mdx*) mouse model of DMD (Bulfield et al., 1984) revealed modulations in MAPK signalling cascades, as dystrophic animals exhibited increased phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (Kumar et al., 2004; Lang et al., 2004) and c-jun N-terminal kinases 1 and 2 (JNK1/2) (Kolodziejczyk et al., 2001; Nakamura et al., 2005; St-Pierre et al., 2004), and decreased phosphorylation of p38 (Lang et al., 2004). The PI3K/Akt signalling pathway has also been shown to be affected in the *mdx* mouse, with an increased synthesis and phosphorylation of Akt observed (Dogra et al., 2006; Peter&Crosbie, 2006). Studies finally demonstrated that directly modulating signalling pathways activity could improve *mdx* muscle function (Kim et al., 2010; Tang et al., 2010). More specifically, increasing Akt activity by transgenic overexpression of the activated kinase itself has been shown to be able to reverse the dystrophic phenotype (Blaauw et al., 2009; Peter et al., 2009).

Moreover, the phosphorylation status of Akt was shown to be altered in human and canine dystrophic biopsies (Peter&Crosbie, 2006; Feron et al., 2009). Enhanced expression and activity of the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) has been observed in dystrophin-deficient dog muscle, and proposed to be at the origin of Akt inactivation (Feron et al., 2009). Indeed, PTEN opposes PI3K action by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate (PtdIns(3,4,5)P$_3$) (Maehama&Dixon, 1998) and the increased activity detected in GRMD muscle would presumably lead to a decreased level of the phosphoinositide, which should limit the recruitment and activation of Akt (see Figure 1 for a schematic representation of the signalling pathway deregulation in dystrophic dog muscle).
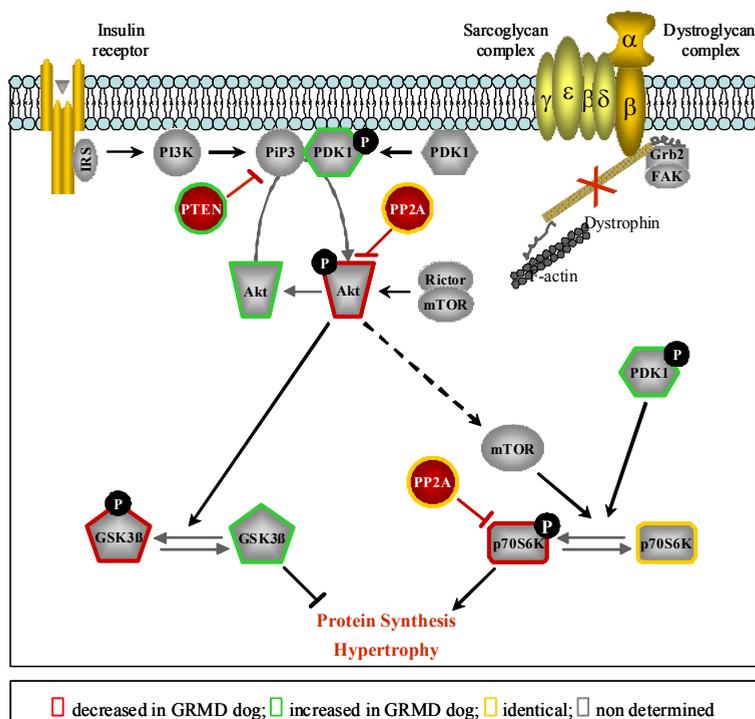
Fig. 1. Schematic diagram of signalling in the PI3K/Akt pathway and deregulations detected in GRMD skeletal muscle. Proteins shown in red, green and yellow indicate decreased, increased and unchanged level of expression or activity of these enzymes in GRMD *vs.* healthy muscle, respectively. As protein phosphatase 2A (PP2A) activity was not statistically modulated in GRMD muscle, the protein is shown in yellow. The deregulations detected in GRMD skeletal muscle could lead to decreased protein synthesis and block compensatory hypertrophy.

Akt directly phosphorylates glycogen synthase kinase-3 (GSK3β) at Ser9, thereby repressing its activity (Cross et al., 1995), and catalyses, *via* the mammalian target of rapamycin (mTOR), 70-kDa ribosomal protein S6 kinase (p70S6K) phosphorylation and activation (Chung et al., 1992; Glass, 2005; Inoki et al., 2002; Inoki et al., 2005; Price et al., 1992) (Figure 1). The PI3K/Akt/GSK3β and PI3K/Akt/mTOR/p70S6K pathways have been implicated in the regulation of skeletal muscle mass. Akt/mTOR signals were found to be upregulated during hypertrophy and downregulated during atrophy and the activation of Akt or p70S6K (or inactivation of GSK3β) appeared to be sufficient to induce hypertrophy. Moreover, in addition to acting as an inductive cue for hypertrophy, activation of the Akt/mTOR pathway could also prevent muscle atrophy *in vivo* (Bodine et al., 2001; Rommel et al., 2001). Furthermore, it has been shown *in vitro* that the overexpression of Src homology 2 (SH2) domain-containing inositol-5'-phosphatase 2 (SHIP-2), which, like PTEN, decreases PIP3 level, led to atrophy whereas the overexpression of a dominant negative mutant, which increases PIP3 level, induced hypertrophy (Rommel et al., 2001). The overexpression of

SHIP-2 in healthy mice muscle had no effect on fiber size but the overexpression of the phosphatase in a model of compensatory hypertrophy completely blocked the hypertrophy response (Bodine et al., 2001). It is thereby likely that the overexpression and increased activity of PTEN detected in GRMD muscle (by decreasing Akt activity and p70S6K phosphorylation, and by activating GSK3β) could prevent compensatory muscle hypertrophy. More recently, the peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC-1α) and PTEN inhibitor DJ-1/Parkinson disease (autosomal recessive early-onset) 7 (PARK7) appeared substantially reduced in GRMD *vs* healthy muscle (Feron et al., 2009; Guevel et al., 2011). Given the role of DJ-1 in the regulation of PTEN, this suggests that PTEN activation in GRMD dog muscle may originate from the under-expression of DJ-1. Noteworthy, in addition to its role in PTEN's regulation, DJ-1 also promotes the activity of PGC-1α (Zhong&Xu, 2008). As such, DJ-1 sensitive signalling pathways may provide high priority targets for the development of novel drug therapies for DMD.

Thus, compelling evidence suggest that alterations in signal transduction pathways may represent significant contributing factors to the progression of DMD. Proteomic profiling performed on the *mdx* mouse (Doran et al., 2006; Lewis et al., 2009; Ohlendieck, 2011) and GRMD dog models (Feron et al., 2009; Guevel et al., 2011) identified signalling proteins and reliable biomarkers of the secondary changes taking place in dystrophic muscles.

3. Proteomic analysis of dystrophic dog muscle

Proteomic approaches have been developed in order to try to identify putative biomarkers of DMD. The proteome-wide investigation of proteins requires technological efforts in three essential steps: the separation, the identification and the quantification of multiple proteins. Reversible protein phosphorylation is arguably the most common and significant mechanism for the dynamic control of biological processes. Phosphorylation can dramatically alter a protein's biological location and/or activity and recent studies clearly highlighted the involvement of phosphoproteins and kinases in DMD (Kolodziejczyk et al., 2001; Kumar et al., 2004; Lang et al., 2004; Peter&Crosbie, 2006; Feron et al., 2009). Although up to one-third of the total proteome might be phosphorylated, the absolute levels of any single protein specie might be very low. In contrast to the traditional biochemical study of single proteins or isolated pathways in the context of muscular dystrophy, technical advances in the high-throughput screening by MS and array-based technology have established new ways of identifying entire cellular proteins populations in one shift analytical approach.

Global identification of signalling proteins can be done by a dedicated approach using antibody arrays. Antibody arrays also serve as an attractive option to carry out phosphoproteomic profiling in disease (Feron et al., 2009; Gembitsky et al., 2004; Kingsmore, 2006). Phospho-specific antibody arrays commercially available facilitate the investigation of specific activated pathways in muscular disorders. On the other hand, a considerable number of proteomic studies have employed unbiased technology such as 2D-GE and stable isotope-labelling techniques combined with MS. To construct an accurate model of the proteome variations occurring in dystrophic dog muscle, complementary proteomic screenings have been done (Figure 2).
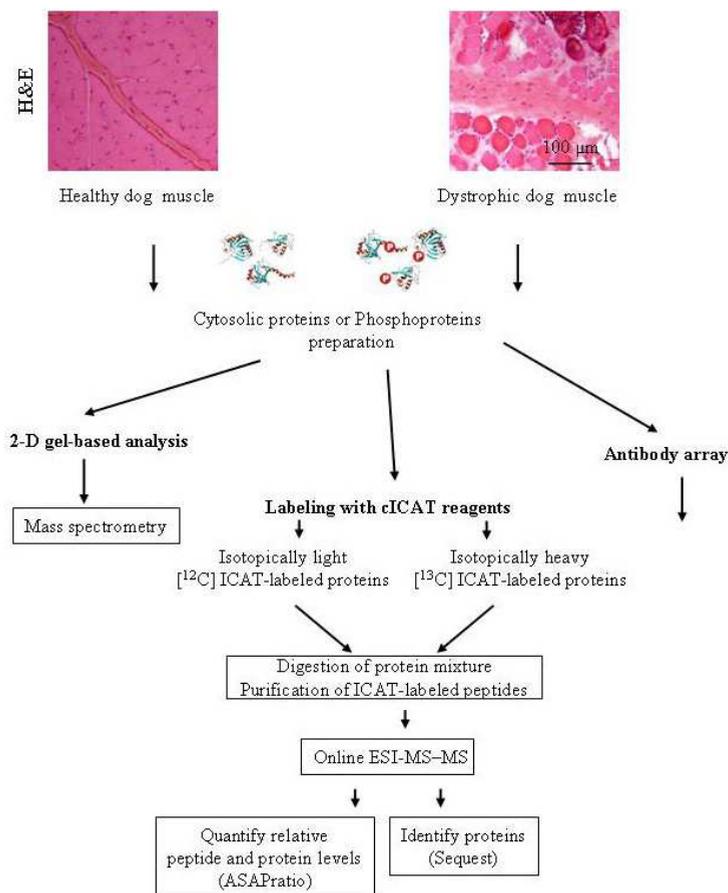
Fig. 2. Schematic diagram of the proteomic analysis of dystrophic dog muscle. H&E (hematoxylin and eosin) staining showing classical pathological changes of DMD, including fiber size variation, fiber splitting, and central nucleation in skeletal muscle. 2D-GE, isotope-coded affinity tag (ICAT) quantitative proteomic analysis and antibody array of proteins purified from 4-month-old healthy and GRMD dog muscle.

3.1 Protein array profiling

Biomedical research of the 21st century will largely be based on the results of studies focusing on the evaluation of gene expression and performed in order to develop molecular tools for the diagnosis and treatment of human diseases. Currently, DNA arrays represent the most commonly used means to follow gene expression in health and disease, and in muscular dystrophies in particular (Chen et al., 2000; Rouger et al., 2002). However, gene expression studies are limited by several aspects: i) gene expression levels do not necessarily reflect the level of proteins (that can also be regulated by degradation), ii) the activity of some proteins is regulated by posttranslational modifications (PTM) such as phosphorylation, glycosylation, carbonylation, acetylation and ubiquitylation, or by allosteric modifications

and iii) localization changes can also play roles in this regulation. On the other hand, a lot of studies have been focusing on single proteins, protein complexes or isolated pathways, limiting the understanding of the pathogenesis of DMD at the organism level. In order to obtain this information and to be able to measure in parallel the expression and the state of activation of several hundreds of proteins, it was important to develop new approaches. Protein array-based approaches can provide not only data complementary to DNA microarrays but also provide unique information about the functional state of proteins under normal and pathological conditions (Hanash, 2003; MacBeath, 2002). Miniaturized protein array technology has opened a new chapter in biotechnology due to its ability to compare, characterize and quantify simultaneously a large number of proteins in the form of spots, thus replacing numerous individual protein by protein tests.It also allows parallel evaluation of several parameters in complex biological solutions. Moreover, a minute spot with immobilized protein sample on an array slide provides greater sensitivity for the detection of molecular interactions compared to other binding assays (Ekins&Chu, 1999). For the first time, antibody arrays were used by Anderson group to look for protein expression changes in spinal muscular atrophy (Anderson&Davison, 1999). A relatively small number of differences were found within a group of proteins that function as both RNA binding proteins and transcription factors. A second group used microarrays to profile the level of proteins associated with calcium regulation in sarcoplasmic reticulum isolated from muscle (Schulz et al., 2006). They used a reverse-phase protein array printed with proteins from genotyped animals and probed with seven target proteins important in calcium regulation. Reverse-phase arrays have been used for profiling phosphorylated proteins in various cancers (Grubb et al., 2003; Sheehan et al., 2005), and it was hoped that these arrays would become a powerful clinical tool for diagnosis and therapy guidance in different diseases.

More recently, the antibody array technology was used to assess the phosphorylation status of key proteins of the MAPK and PI3K/Akt signalling pathways in the *Vastus lateralis* muscle from 4-month old GRMD *vs* healthy dogs. The antibody array technology represents a powerful tool for the semi-quantitative comparison of the expression and/or phosphorylation level of a high number of proteins in a limited number of samples (Sakanyan, 2005; Yeretssian et al., 2005). The main advantage resides in the gain of time that it provides, as a high number of proteins can be studied in just one experiment. Moreover (and in contrary to the ICAT technology for example), antibody arrays give access to information about PTM, such as phosphorylation, which is of course crucial in the context of cell signalling. Though it represents a biased technique (data is obtained only for the antibodies initially spotted on the membrane) but, as hundreds of different antibodies can be spotted onto the same membrane, it can easily be used for screening purposes such asfor disease diagnosis using disease biomarkers. This study indicated that Akt1, GSK3β and p70S6K, as well as ERK1/2 and the p38δ and γ kinases all displayed a decreased phosphorylation level in canine dytrophic muscle (Figure 3). Antibody arrays allow the detection of the presence of specific proteins, and the level of expression of phospho-proteins in disease tissue (Cahill, 2001), thus having a potential for biomedical and diagnostic applications. However, it had not been possible to address the systematic analysis of proteins using this dedicated approach.

A lot of evidence now indicates that various signalling and metabolic pathways are altered in DMD, and a global, unbiased, proteomics study was necessary to identify these

perturbations. In order to characterize the complete dystrophic proteome, the use the recent 2D-GE technology coupled to MS became favorable.
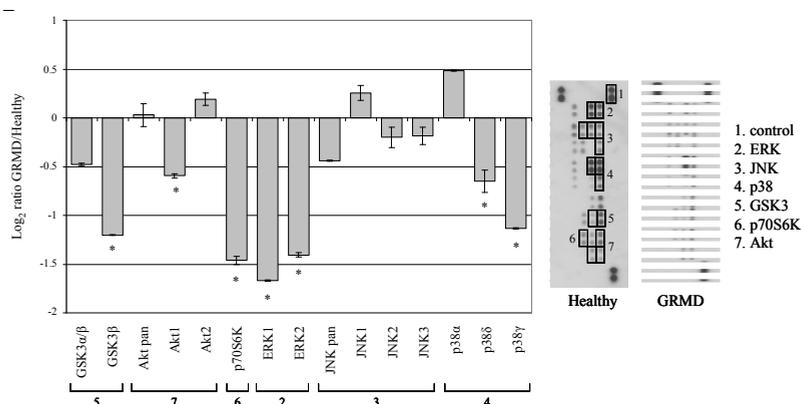

Fig. 3. Antibody array analysis revealed PI3K/Akt and MAPK signalling pathway modulation in GRMD skeletal muscle. Healthy and GRMD muscle extracts from 4-month-old dogs were incubated with two antibody arrays. A ratio of signal intensity (GRMD/healthy) was calculated, and log transformed (base2). A cutoff value was determined by ANOVA analysis at 95% confidence level ($p < 0.05$). * - significantly different from healthy muscle.

3.2 Mass spectrometry-based proteomic analyses

MS-based proteomics represents an unbiased approach allowing the comprehensive cataloging of the whole protein alterations associated with a specific disease. This chapter outlines the findings from recent applications of MS-based proteomics for studying alterations in dystrophic dog muscle, and examines novel strategies to establish DMD-specific biomarkers.

3.2.1 Separation of muscle proteins by two-dimensional gel electrophoresis

2D-GE represents a highly reproducible and discriminatory technique that allows the analysis of the accessible (meaning soluble and abundant) muscle proteins. The proteins that appear differentially expressed between the different samples analyzed are then identified by high-throughput MS (matrix-assisted laser desorption/ionization time-of-flight - MALDI-ToF or electrospary ionization - ESI). Proteomics dataset are finally interlinked with international web-based gel electrophoretic and protein sequence databanks for comparative analysis. Modern mass spectrometers produce and separate ions according to their mass-to-charge ratio (m/z) with an extraordinary resolving power. MS-based analysis of the skeletal muscle proteome has already been successfully used in the context of muscle development, fiber type specification, fast-to-slow transformation, muscle growth and aging, and in the context of denervation-induced fiber damage, atrophy, obesity, diabetes and muscular dystrophies (Ohlendieck, 2010). Detailed 2D maps of the major soluble muscle proteome, including proteins involved in actomyosin apparatus, regulation of contraction, ion homeostasis, signalling, cytosolic and mitochondrial metabolism and stress response,

have been established for various mammals (Doran et al., 2009a). The results obtained by this high-throughput technology should then be confirmed by more classical techniques such as western immunoblotting and immunohistochemistry. For example, the *mdx* mouse model was employed in recent proteomics profiling studies which revealed new disease markers in dystrophin-deficient fibres (Doran et al., 2004; Doran et al., 2006a, 2006b; Gardan-Salmon et al., 2011). A differential in-gel analysis (DIGE) analysis of *mdx vs* normal diaphragm muscle revealed a drastic differential expression pattern of 35 proteins, with 21 proteins being decreased (including the F-box only protein 11 - Fbxo11, adenylate kinase 1 – AK1, and the calcium-binding protein regucalcin) and 14 proteins being increased, including the small cardiovascular heat shock protein cvHSP and muscle proteins such as vimentin, desmin and myosin heavy chain (MHC) (Doran et al., 2006a).

More recently, the GRMD dog model was used to profile changes in protein abundance associated with DMD using 2D-GE. To eliminate the structural and contractile proteins that are over-abundant in crude protein extracts prepared from skeletal muscle, and to enrich the samples in signalling proteins, the study restricted the analysis to the cytosolic and phospho-enriched proteins of the *Vastus lateralis* muscle removed from 4-month-old healthy and dystrophic dogs. Among the differentially expressed proteins , 8 were chosen, according to their high level of dysregulation, for further identification by MS. This led to the identification of skeletal muscle markers involved in the contractile function and mitochondrial proteins involved in energy metabolism (Guevel et al., 2011). Although 2D-GE analysis represents an efficient technique to identify relative changes in protein expression, it is not well suited for studying low-abundant proteins, which are often important regulators of cell signalling. The high abundance of cytoskeletal, contractile and chaperone proteins identified in the phospho-enriched sample combined with dynamic range issues associated with the 2D-GE approach hampered studies on skeletal muscle analysis. Recently, Hojlund and colleague used 1D-GE and high-performance liquid chromatography (HPLC)-ESI-MS/MS to characterize the proteome of human skeletal muscle (Hojlund et al., 2008). The proteins identified in this study provide a representation of the major biological function of healthy human skeletal muscle. To elucidate changes in the proteome associated with DMD, and to overcome disadvantages of 2D-GE, peptide-centric approach can be used, which allows quantitative comparison of two samples. Numerous stable isotope-labelling techniques have been employed in quantitative shotgun proteomics, including isobaric tag for relative and absolute quantification (iTRAQ); isotope-coded affinity tag (ICAT); and stable isotope labelling by amino-acids in cell culture (SILAC) (Ohlendieck, 2011). In dystrophic skeletal muscle, the ICAT labelling approach has been used for the quantitative proteomic profiling of healthy and GRMD dog muscles.

3.2.2 Quantitative proteomic analysis

ICAT labelling followed by LC-MS/MS was used to analyze the quantitative variations of the proteome in both a cytoplasmic and a phospho-enriched fraction prepared from the *Vastus lateralis* muscle of 4-month old healthy and GRMD dogs (Guevel et al., 2011). A total of 84 proteins appeared significantly altered (61 proteins from the cytosolic fraction and 36 proteins from the phospho-enriched fraction, with an overlap of 13 proteins). These proteins were classified into 7 major categories including: i) muscle development and contraction, ii) glycolytic metabolism, iii) oxidative metabolism, iv) calcium ion homeostasis, v) intracellular signalling, vi) regulation of apoptosis, and vii) other functions. Gene Ontology

(GO) annotation of the altered proteome led to several key findings which might reflect the ongoing muscle regeneration taking place in dystrophic muscle. Among the proteins altered in the intracellular signalling category in the dystrophic muscle, protein phosphatase 1 (PP1) and DJ-1 appear particularly interesting. PP1, which is present in skeletal muscle, is known to regulate both glycogen and fatty metabolism, while promoting the dephosphorylation of myosin. The protein DJ-1 (also called PARK7) was recently described as a negative regulator of PTEN (Kim et al., 2005; Villa-Moruzzi et al., 1996). Interestingly, the under-expressed proteins primarily composed of metabolic proteins, many of which have been shown to be regulated by PGC-1α. Interestingly, among the several PGC-1α targets identified to be under-expressed in dystrophic dog muscle, 5 (namely 6-phosphofructokinase, phosphoglucomutase-1, aconitase 2, cytochrome c1 and fatty acid binding protein 3) have already been identified in a different transcriptomic study as under-expressing in DMD compared to healthy human biopsies (Pescatori et al., 2007). PGC-1α has been described as a potent regulator of mitochondrial biogenesis and oxidative metabolism in skeletal muscle (Wu et al., 1999; Lin et al., 2002). In addition, activation of the peroxysome proliferator-activated receptor (PPAR)/PGC-1α pathway has been shown, by preventing the bioenergetic deficit observed, to efficiently improve a mitochondrial myopathy phenotype (Wenz et al., 2008), suggesting that PGC-1α mediated improvement of dystrophic muscle may rely (in part) on the restoration of PGC-1α mitochondrial targets (Handschin et al., 2007). Interestingly, a recent study has shown that pharmacologic activation of PPARβ/δ also leads to an upregulation in the expression of utrophin A, which was concurrent with a partial correction of the dystrophic phenotype (Miura et al., 2009). Taken together, these results provide compelling new evidence that defects in PTEN and PGC-1α contribute to profound signalling pathway deregulation in the canine model of DMD as well as to the disease progression. In addition, they demonstrate that proteomics tools are of particular interest for the study of muscular disorders. Recently, the combination of proteomics, metabolomics and fluoximics has confirmed the existence in the *mdx* mouse of perturbations that reflect mitochondrial energetic alterations (Griffin&Des Rosiers, 2009). The broad aim of these studies has been two-fold, first the identification of co-founding factors that promote or limit the disease progression and second, the identification of new biomarkers that could be used to more accurately define the disease status.

4. Reliable biomarkers of DMD, with a special focus on signalling proteins

As previously mentioned, the absence of a single protein (dystrophin) in muscle has devastating consequences. Despites the tremendous efforts that have been made for more than 20 years in order to try to understand how this initial genetic defect could lead to the progressive and irreversible muscle wasting observed, the pathogenesis of DMD has not been fully characterized. Furthermore, no curative treatment is yet available and DMD patients are still dying during early adulthood. The identification, at the proteome level, of the alterations associated with DMD is important for at least five reasons. By providing a better understanding of the pathogenesis of DMD, they should i) improve diagnosis, ii) enable a better monitoring of disease progression, iii) lead to the proposal of new therapeutic targets (in the perspective of a pharmacological treatment – alone or in combination with a gene or cell therapy approach), iv) enable the fast and efficient evaluation of the benefits provided by the treatments currently under study (are they able to

reverse the secondary changes associated with the absence of dystrophin?) and v) in some cases, they could even enable the improvement of a given therapy. Ideally, serum biomarkers should be identified (Cacchiarelli et al., 2011) allowing an easy and non invasive analysis, but a muscle muscular biopsy could always be used if necessary.

Skeletal muscle proteomics represents a new and powerful analytical tool for the swift separation and identification of new biomarkers and, in the recent years, several reviews written mainly by Doran, Ohlendieck and their colleagues focused on the proteomics analysis, by 2D-GE and high-throughput MS, of skeletal muscle during aging or disease (Lewis et al., 2009; Griffin&Des Rosiers, 2009; Doran et al., 2009a; Doran et al., 2007a, 2007b). Although being extremely powerful for the identification of new biomarkers, the proteomics analysis of skeletal muscle encounters some limitations. In the particular case of DMD, some complications are also due to the increase in endomysial fat and connective tissue, changes in the interstitial volume, infiltration by immune cells, residual blood components or drastic transformations in contractile fiber types. However, MS is so sensitive that it can differentiate these effects in heterogeneous cell mixtures. On the other hand, it is not always easy to distinguish between a DMD-specific biomarker and a biomarker that is more linked to muscle degeneration in general. The detailed analysis of the overlapping results obtained in the studies of DMD *vs* dysferlinopathies *vs* age-induced muscle wasting may help to distinguish between common and more specific biomarkers. In parallel to 2D-GE, we successfully used the antibody array technology to compare cell signalling in dystrophic *vs* healthy dog skeletal muscle (Feron et al., 2009). Finally, metabolomics and fluxomics (metabolic flux anlysis) studies have been successfully performed on skeletal muscle (Griffin&Des Rosiers, 2009).

In 2003, Ge and colleagues published the results of a proteomic analysis, using 2D-GE/MS, of *mdx* hindlimb skeletal muscle (Ge et al., 2003). Among the 60 proteins identified as differentially expressed in dystrophic *vs* healthy muscle (40 in the cytosolic fraction and 20 in the microsomal one), AK1 (cytosolic fraction) appeared to be of particular interest, because of its dramatic decrease (> four-fold) and because of its role, along with creatine kinase (CK), in the regulation of nucleotide ratios and energy metabolism. The expression and activity of AK1 was reduced in *mdx* muscle at different stages (one, three and six months), suggesting a direct link with the deficiency in dystrophin. Decreased AK1 activity in *mdx* muscle could contribute to the energetic defect, the decreased force and the increased fatigability exhibited. At the same time, the redistribution of energy flow through the alternative and compensatory CK phosphotransfer system could limit cellular energy failure. In DMD patients, lower ATP levels and impaired energy metabolism had been reported very early, and several studies suggest that defects in energy metabolism could contribute to DMD pathogenesis.

Ge and colleagues, in 2004, also published a study in which they compared by 2D-GE the proteome of *mdx vs* control hindlimb muscles at different stages of the disease (Ge et al., 2004). Among the 46 differentially expressed cytosolic proteins detected at three months (10 down- and 36 up-regulated proteins), 24 could be identified by MS. These proteins belong to five different functional categories, and illustrate the increase in protein turnover caused by the cycles of degeneration/regeneration characteristic of *mdx* muscles. Concerning metabolism and energy production (i), the reduction in AK1 was confirmed and an increase in the expression level of the CK, ATP synthase, ATP succinyl-CoA synthetase and

pyrophosphatase enzymes was detected, highlighting the general mitochondrial dysfunction and metabolism crisis taking place in dystrophic muscles. Concerning the serine protease inhibitor family (ii), an up-regulation was detected for protease inhibitor member 1a and serine protease inhibitors 3, 6 and 1-5, suggesting a partial inhibition of proteolysis in *mdx* muscles. Concerning growth and differentiation (iii), an increase was detected in the expression level of PP1, cofilin 2 (CFL2) and ε 14-3-3, indicating active proliferation and differentiation. Of interest, an increase in CFL2 had also been observed in human DMD biopsies. As far as calcium homeostasis is concerned (iv), the up-regulation of PP1 can also be cited, as the phosphatase binds to the ryanodine-sensitive calcium release channel protein to regulate calcium flux. The calcium-binding protein annexin V was also increased. Finally, and concerning cytoskeleton reorganization and biogenesis (v), RhoGDI-1, γ-actin and tropomyosin 1 were found to be up-regulated in *mdx* muscles, indicating cytoskeleton remodeling. At one and six months, 62 and 48 differentially expressed proteins were detected, respectively. At one month, most of the proteins detected were down-regulated whereas at six months (as it was the case at three months), most of them were up-regulated. These results confirmed the specificity of the one month stage ("DMD-like" crisis) in the evolution of the disease in the *mdx* mouse. Six proteins were detected as differentially expressed in *mdx vs* healthy muscles at the three stages tested: AK1 (down-regulated), CK (up-regulated), myosin light chain 2 (MLC2, up-regulated), annexin V, tropomyosin and ε 14-3-3 (all down-regulated at one month and up-regulated at three and six months). Some of these changes thus appear directly linked to the absence of dystrophin whereas some others appear more dependent of the phenotype on the muscle. The elevation detected in MLC2 levels could reflect the proliferation/differentiation processes occurring in *mdx* muscles, as the protein is involved in muscle differentiation, and its consistent elevation during the progression of the disease suggests that *mdx* muscles may assume a chronic or abnormal differentiation state.

In another study, Doran and colleagues performed a 2D-GE/MS-based subproteomics analysis of calcium-binding proteins by using the cationic carbocyanine dye 'Stains-All' (Doran et al., 2004). Among the 8 dye-positive proteins identified as greatly reduced in *mdx vs* healthy skeletal muscle, calsequestrin was present. Calsequestrin represents the main luminal sarcoplasmic reticulum calcium reservoir protein. It is a terminal cisternae constituent with high-capacity and medium-affinity, and acts as a mediator of the excitation-contraction-relaxation cycle, both as a luminal ion trap and an endogenous regulator of the ryanodine receptor. The authors could also confirm the reduction in sarcalumenin, a calcium-shuttle element of the longitudinal tubules. These results of course confirm the calcium hypothesis of DMD. The reduction in calsequestrin could explain the impaired calcium buffering capacity of dystrophic sarcoplasmic reticulum, which is known to cause an increase in free cytosolic calcium level and thus in proteolysis. Previous microsomal study had not detected any change in the expression level of calsequestrin, highlighting the power of the technique used here.

More recently, Doran and colleagues could also show, using 2D-GE/MS again, that another protein involved in calcium homeostasis, regucalcin, was reduced in young and aged *mdx vs* healthy diaphragm, limb and heart muscles (Doran et al., 2006a). Regucalcin represented the most interesting hit in respect to the calcium hypothesis of DMD as its reduced level could render *mdx* fibers more susceptible to necrosis. Regucalcin is a cytosolic calcium-handling

protein involved in signalling. By enhancing the calcium-pumping activity in the plasma membrane, endoplasmic reticulum and mitochondria, regucalcin appears as an important regulator that maintains low cytosolic calcium levels. Its reduced expression level could be confirmed by immunoblotting in the diaphragm muscle from 3-week-, 9-week-, 11-month- and 20-month-old *mdx* mice. At 9 weeks, a reduced level of the protein could also be observed in hindlimb and heart muscles. Doran and colleagues finally used the powerful DIGE technique and identified 2398 proteins among which 35 exhibited a differential expression level in *mdx vs* healthy diaphragm muscle (Doran et al., 2006b). These proteins are involved in muscle contraction, cytoskeleton formation, mitochondrial function, metabolism, ion homeostasis and chaperone function. The most interesting finding concerned the dramatic increase in the expression level of the small heat shock protein cvHSP (highest fold change). This drastic increase could be observed in 9-week- and 11-month-old *mdx* diaphragm muscles (it increases with the age – in correlation with the severity of the phenotype). Whereas the protein was concentrated in subsarcolemmal regions in healthy muscle, it was shown to be present throughout the cytoplasm of *mdx* fibers, with a typical striated appearance suggesting an association with contractile elements and/or cytoskeletal components and a role in the stress response developed by *mdx* damaged fibers. This increase in cvHSP was associated with the differential expression of others key heat shock proteins (HSP20, GRP75, HSP90 and HSP110), emphasizing stress response as an important mechanism in DMD pathogenesis, and suggesting that it could be targeted by new pharmacological treatments. Heat shock chaperon proteins can be activated, besides by heat shock *per se*, by other stress factors such as inflammation, ischemia, oxidative stress, exposure to heavy metals or certain amino acids analogs. They prevent the aggregation of misfolded proteins as well as they influence the transport of mature proteins. The up-regulation of cvHSP observed in *mdx* diaphragm indicates an attempt of damaged muscle fibers to repair their cytoskeletal network. The change observed in the localization of the protein also suggests a protective role in muscle fiber degeneration. The up-regulation of chaperones probably represents an autoprotective mechanism, whereby the stress response can be considered as a reaction to the pathological increase in abnormally folded muscle proteins.

More recently, a magnetic bead fractionation and MS-based serum protein profiling was performed in the *mdx* mouse and described coagulation Factor XIIIa, previously identified in human serum, as a potential biomarker of muscular dystrophy (Alagaratnam et al., 2008). Factor XIIIa plays roles in coagulation and cardiovascular biology, possibly through macrophage activation, and macrophages are known to infiltrate dystrophic muscles. Because blood serum analysis is fast, economical and non invasive, this type of study is of high interest, and a study with serum from DMD patients should be performed. However, this factor alone may not be sufficient to distinguish between DMD and other inflammatory context.

Finally, our lab identified two signalling molecules (PTEN and PGC1-α) as biomarkers in GRMD dog model, strongly reinforcing the hypothesis that signalling pathways alteration could play a role in DMD pathogenesis. In a first study (Feron et al., 2009), we were able to show that an increase in the activity of PTEN, a phosphatase that counteracts Akt activation by dephosphorylating the PIP3 generated by PI3K (Maehama&Dixon, 1998), in dystrophin-deficient dog muscle leads to a profound and long-term deregulation of the PI3K/Akt

signalling pathway. All the GSK3β⁺ fibers observed in dystrophic muscle appeared to exhibit a strong accumulation of PTEN, whereas the fibers with weak PTEN labelling were systematically GSK3β⁻. In order to see if the alterations initially detected at 4-months were specific of this age, the double labelling experiment was repeated at 3 months (when the morphological features of muscular dystrophy are yet very few) and 36 months (which corresponds to a very advanced stage). PTEN⁺/GSK3β⁺ fibers could be observed at all stages, and we demonstrated that the alteration of the pathway could not be attributed only to a feature or regeneration or to a consequence of inflammatory changes. In conclusion, increased PTEN activity revealed to be a signature of muscular dystrophy pathogenesis in dog, leading to long-term and deep PI3K/Akt signalling pathway alteration. This dysregulation probably limits compensatory hypertrophy, thus exacerbating muscle degeneration, and these results could open the door to new potential therapeutic targets for the treatment of DMD.

In a second analysis, a quantitative proteomic analysis of dystrophic *vs* healthy dog muscle was performed using the ICAT technology coupled to LC/MS/MS (Guevel et al., 2011). This study, performed on both a cytoplasmic and a phospho-enriched fractions, identified 84 proteins as being differentially represented in GRMD *vs* healthy dog muscle. Interestingly, many of the under-expressed proteins detected have been previously shown to be regulated by PGC-1α, and we were able to show that PGC1-α expression was indeed dramatically reduced in GRMD *vs* healthy muscle. These results confirmed that defective energy metabolism is a central hallmark of the disease in the canine model, and reinforced once more the hypothesis that secondary changes may play an active role in DMD pathogenesis.

In conclusion, proteomics studies performed in the recent years on dystrophic *vs* healthy muscles led to the identification of new biomarkers of DMD, such as AK1 (nucleotide metabolism), calsequestrin, regucalcin (calcium homeostais) and cvHSP (cellular stress response) in the *mdx* mouse and PTEN and PGC-1α (cell signalling: atrophy/hypertrophy and energy metabolism) in the GRMD dog. Other putative biomarkers were also identified in the following processes: nucleotide metabolism (CK, Atp5b), calcium homeostasis (sarcalumenin), cellular stress response (αBC, chaperonins), muscle contraction (MHC and MLC, troponin, actin), intermediate filament formation (vimentin, desmin), glycolysis (glyceraldehyde-3-phosphate dehydrogenase, aldolase), polyol pathway of glucose metabolism (sorbitol dehydrogenase), citric acid cycle (isocitrate dehydrogenase), fatty acid oxidation (electron transferring flavoprotein), aldehyde metabolism (aldehyde reductase, aldehyde dehydrogenase), formation of acetyl-coenzyme A (dihydrolipoamide dehydrogenase), remethylation pathway of homocysteine homeostasis (betaine-homocysteine methyltransferase), acid-base balance (carbonic anhydrase), oxygen transport (β-haemoglobin, α-globin), protein ubiquitination (Fbxo11) and transcriptional control (Jmjd1a). Taken together, these results indicate a drastic reduction in key metabolic regulators and a compensatory up-regulation of structural elements.

5. Proteomic profiling of experimental therapy

As previously mentioned, no curative treatment is yet available for DMD patients that can benefit only from palliative care and generally die during early adulthood. Two therapeutic strategies can be envisaged to treat, or at least to alleviate the symptoms of DMD: try to

restore dystrophin expression in dystrophic muscle fibers (through gene or cell therapy approaches), or target the molecular pathways lying downstream of dystrophin (through pharmacological treatments). Several strategies have been recently set up in order to rescue dystrophin synthesis in animal models of DMD and some of them have now entered clinical trials (Kinali et al., 2009; van Deutekom et al., 2007). One of the major problems in comparing the benefit of different therapeutic treatments is to find common outcome measurements. This paragraph does not aim at describing in details the therapies currently under study [for a review, please see (Sugita&Takeda, 2010; Guglieri&Bushby, 2010; Zhang et al., 2007)], but rather to show how proteomic profiling could be used to evaluate the efficiency of therapeutic treatments. Recently, Doran and colleagues (Doran et al., 2009b) used DIGE analysis to evaluate the efficiency of an exon skipping-based strategy in the *mdx* mouse.

The idea behind the study is that, as secondary mechanisms such as abnormal signalling, energy metabolism defects, alterations in ion homeostasis or in excitation-contraction coupling, probably play a crucial role in DMD pathogenesis, any novel therapeutic strategy should be evaluated on several aspects: re-expression of dystrophin (except for therapeutic treatments targeting downstream events), muscle function tests, but also correction of the secondary changes previously detected in the above mentioned processes.

Proteomic profiling of exon skipping-treated *mdx* muscles showed that the re-expression of dystrophin led to the correction of the previously detected alterations in calcium handling, nucleotide metabolism, bioenergetic pathways, acid-base balance and cellular stress response. More precisely, the re-expression of dystrophin was associated with the restoration of β-dystroglycan and nNOS (two proteins associated with the DGC at the sarcolemma), and with a normal expression level of some biomarkers previously identified (namely calsequestrin, adenylate kinase, aldolase, mitochondrial creatine kinase and cvHsp).

Both the primary and secondary abnormalities provoked by the absence of dystrophin were reversed, reinforcing the interest of the exon skipping strategy for the treatment of DMD. This study reinforced the role ofsecondary mechanisms in DMD pathogenesis, and it demonstrated for the first time the utility of evaluation of the effect of any novel therapeutic approach on both the re-expression of dystrophin and the indirect alterations associated with its absence. AK1, that had previously been reported as down-regulated in *mdx* diaphragm muscle (Ge et al., 2003), was restored after the antisense-induced exon skipping. Also, the down-regulation of the mitochondrial isozyme of CK was partially reversed. Accordingly, the known down-regulation of two others metabolic enzymes, namely aldolase and isocitrate dehydrogenase (Doran et al., 2006b) was also partially reversed. Conversely, the major increase previously reported in the level of cvHSP in *mdx* diaphragm was significantly reduced after treatment. As far as the acid-base balance is concerned, the expression level of carbonic anhydrase was also restored after treatment. For calsequestrin, an immunoblotting experiment (as DIGE is not well suited for the analysis of membrane proteins) was performed that revealed that exon skipping treatment was again able to restore its expression (Doran et al., 2004), suggesting a partial abolishment of the secondary changes in calcium homeostasis associated with DMD. The exon skipping strategy presented here effectively reverses both the metabolic crisis and the compensatory up-regulation of different chaperones and enzymes associated with muscular dystrophy in the mouse.

Lastly, miRNAs specifically expressed in muscle cells and known to be released in the blood of DMD patients in a way proportional to the extent of muscle degeneration, could be used as biomarkers in the evaluation of therapeutic strategies (Cacchiarelli et al., 2011).

To sum up, even though many different DMD therapeutic approaches are now entering clinical trials, a unifying method for assessing the benefit of different treatments is still lacking.

6. Conclusion

In conclusion, proteomics analysis plays a significant role in our ability to understand molecular mechanisms associated with DMD. Various technological platforms are now available for proteomic studies enabling us to address different aspects of dystrophic muscle governed by signalling pathways. We foresee proteomics emerging as a vital technique in clinical research to assist us in understanding which particular signal transduction pathways are involved in muscular dystrophy and to evaluate the benefit of clinical trials.

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8. References

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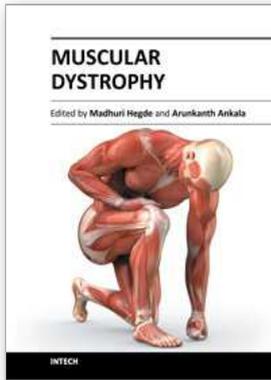
Muscular Dystrophy

Edited by Dr. Madhuri Hegde

With more than 30 different types and subtypes known and many more yet to be classified and characterized, muscular dystrophy is a highly heterogeneous group of inherited neuromuscular disorders. This book provides a comprehensive overview of the various types of muscular dystrophies, genes associated with each subtype, disease diagnosis, management as well as available treatment options. Though each different type and subtype of muscular dystrophy is associated with a different causative gene, the majority of them have overlapping clinical presentations, making molecular diagnosis inevitable for both disease diagnosis as well as patient management. This book discusses the currently available diagnostic approaches that have revolutionized clinical research. Pathophysiology of the different muscular dystrophies, multifaceted functions of the involved genes as well as efforts towards diagnosis and effective patient management, are also discussed. Adding value to the book are the included reports on ongoing studies that show a promise for future therapeutic strategies.

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