Expression Profiling in the Muscular Dystrophies: Identification of Novel Aspects of Molecular Pathophysiology✪

Yi-Wen Chen, Po Zhao, Rehannah Borup, and Eric P. Hoffman

Research Center for Genetic Medicine, Children's National Medical Center, George Washington University, Washington, DC 20010

Abstract. We used expression profiling to define the pathophysiological cascades involved in the progression of two muscular dystrophies with known primary biochemical defects, dystrophin deficiency (Duchenne muscular dystrophy) and α-sarcoglycan deficiency (a dystrophin-associated protein). We employed a novel protocol for expression profiling in human tissues using mixed samples of multiple patients and iterative comparisons of duplicate datasets. We found evidence for both incomplete differentiation of patient muscle, and for dedifferentiation of myofibers to alternative lineages with advancing age. One developmentally regulated gene characterized in detail, α-cardiac actin, showed abnormal persistent expression after birth in 60% of Duchenne dystrophy myofibers. The majority of myofibers (∼80%) remained strongly positive for this protein throughout the course of the disease. Other developmentally regulated genes that showed widespread overexpression in these muscular dystrophies included embryonic myosin heavy chain, versican, acetylcholine receptor α-1, secreted protein, acidic and rich in cysteine/osteonectin, and thrombospondin 4. We hypothesize that the abnormal Ca^{2+} influx in dystrophin- and α-sarcoglycan–deficient myofibers leads to altered developmental programming of developing and regenerating myofibers. The finding of upregulation of HLA-DR and factor XIIIa led to the novel identification of activated dendritic cell infiltration in dystrophic muscle; these cells mediate immune responses and likely induce microenvironmental changes in muscle. We also document a general metabolic crisis in dystrophic muscle, with large scale downregulation of nuclear-encoded mitochondrial gene expression. Finally, our expression profiling results show that primary genetic defects can be identified by a reduction in the corresponding RNA.

Key words: muscular dystrophy • microarray • dystrophin • α-sarcoglycan • expression profiling

Introduction

The dystrophin–glycoprotein complex of muscle fibers has emerged as a critical multiprotein complex that imparts structural integrity to the muscle fiber plasma membrane during the contraction of skeletal muscle. Identification of a principle component, dystrophin (Hoffman et al., 1987a), led to the identification of additional protein components of the protein complex (Ibraghimov-Beskrovnaya et al., 1992; Roberds et al., 1993; Bonnemann et al., 1995; Noguchi et al., 1995; McNally et al., 1996; Nigro et al., 1996). A major role of these proteins is to provide physical connections between intracellular cytoskeletons (actin filaments) and extracellular basal lamina (laminins). In brief, dystrophin attaches to the plasma membrane via the transmembrane protein β-dystroglycan using a cysteine-rich COOH-terminal domain. Physical connections between the plasma membrane and basal lamina are mediated by a multimeric chain involving dystrophin, β-dystroglycan, α-dystroglycan, and laminin α-2. The interaction of dystrophin with β-dystroglycan is stabilized by the heterotetrameric sarcoglycan complex (α, β, δ, and γ subunits) (Ervasti and Campbell, 1991; Cullen et al., 1996; Duclos et al., 1998). Interactions between dystrophin and intracellular actin filaments is mediated by a series of actin-binding sites, many of which are clustered in the NH_2-terminal 200 amino acids. Importantly, loss of dystrophin leads to loss of all actin–filament association with the plasma membrane (Rybakova et al., 2000). Utrophin is a paralogue of dystrophin, which can in part functionally replace dystrophin when overexpressed in muscle, though it is not able to restore actin–filament association with the membrane (Tinsley et al., 1998; Rybakova et al., 2000). The dystrophin/dystroglycan/laminin complex is partially redundant with the vinculin/integrin/laminin complex, though the dystrophin-based complex seems more important for homeostasis during muscle contraction, whereas the integrin-based cytoskeleton appears more important for the appropriate development of muscle (Hayashi et al., 1998; Burkin and Kaufman, 1999; Kaariainen et al., 2000).

✪The online version of this article contains supplemental material.

Address correspondence to Eric Hoffman, Research Center for Genetic Medicine, CNMC, 111 Michigan Ave. NW, Washington DC 20010. Tel.: (202) 884-6011. Fax: (202) 884-6014. E-mail: ehoffman@cnmc.org

In addition to the structural role for dystrophin and the associated proteins, there is clear evidence for signal transduction roles. The COOH-terminal domains of dystrophin bind a series of proteins, both directly and indirectly, each of which has been shown to have a role in muscle development or homeostasis. The dystrophin-binding syntrophins are a series of proteins with PDZ domains that in turn bind nNOS, ERK6, and other proteins (Yang et al., 1995; Chao et al., 1996; Hasegawa et al., 1999). Loss of dystrophin leads to the secondary loss of nNOS at the plasma membrane and defects in local blood flow regulation during exercise (Thomas et al., 1998). There are likely many additional directly or indirectly interacting proteins involved in signal transduction, and the delineation of these pathways is critical for understanding the pathophysiology of dystrophin deficiency.

Genetic abnormalities of dystrophin are the cause of the most common type of muscular dystrophies, Duchenne and Becker muscular dystrophy. The gene encoding dystrophin has a very high mutation rate (1 in 10,000 germline cells), due to the large size of the gene (2.5 million bp) and the presence of certain hotspots for rearrangements within introns (Gorospe and Hoffman, 1992; Hamed et al., 2001). As a result, the disease has a high incidence in all world populations, as well as in animals (Williams et al., 1983; Bulfield et al., 1984; Cooper et al., 1988; Carpenter et al., 1989; Roddie and Bundey, 1992). The presentation and progression of dystrophin deficiency varies dramatically between species, despite the shared biochemical feature of complete deficiency of the protein in muscle and heart from fetal life onward. Humans show evidence of myofiber membrane instability (high serum creatine kinase levels) from birth onwards, but do not show obvious clinical weakness until ~4 yr of age. The disease is then progressive, with patients gradually losing most of their skeletal muscle by 16 yr of age (Hoffman and Schwartz, 1991). Cats and mice lacking dystrophin show muscle hypertrophy as the predominant symptom, with little clinical evidence of weakness or muscle loss (Bulfield et al., 1984; Carpenter et al., 1989; Gaschen et al., 1992). Dogs lacking dystrophin show an even more rapid onset and progression than humans, though this can be variable from dog to dog (Cooper et al., 1988; Kornegay et al., 1988). Primary deficiencies of each of the sarcoglycan proteins have been documented in human patients, and the clinical symptoms are indistinguishable from primary dystrophinopathies (Duggan et al., 1997). Rodent models have been described for each of the four sarcoglycans, and, like dystrophin deficiency, these mouse and hamster models show muscle hypertrophy as the primary clinical symptom (Mizuno et al., 1995; Duclos et al., 1998; Hack et al., 1998; Durbeej et al., 2000).

The dramatic progressive nature of dystrophin deficiency and sarcoglycan deficiency in humans and dogs, but not cats and rodents, implies that there are important secondary, downstream effectors of muscle wasting and weakness. The clinical differences between the human diseases and their animal orthologues also support this hypothesis. Considerable data has recently accumulated concerning biochemical and environmental effectors of "acute" myofiber damage and necrosis, particularly in regards to membrane damage (plasma membrane fragility) and functional ischemia (nNOS deficiency) (Thomas et al., 1998). These "acute" features appear to be shared between dystrophin and α-sarcoglycan–deficient fibers of all species. Less well studied are the secondary "chronic" aspects of these disorders, which is particularly important as the downstream events appear responsible for the progressive weakness and early death in human patients. Histopathological correlations have provided descriptive associations with the progressive aspects of the human disease, such as proliferation of connective tissue (fibrosis), failure of myofiber regeneration, and infiltration of mast cells and other inflammatory cells (Hoffman and Schwartz, 1991; Gorospe et al., 1994a). However, no clear pathophysiological cascades have yet been defined and no molecular basis identified for any of the histological correlates.

We hypothesized that genome-wide expression profiling would provide insight into the downstream pathophysiological cascades in the muscular dystrophies. We felt that the muscular dystrophies would be an excellent disease system in which to apply emerging microarray technology for the following reasons. First, muscle is a relatively simple tissue, with the myofiber being the predominant cell type. Second, muscle is routinely biopsied from human patients and rapidly frozen, making tissue easily available for expression array studies. Third, the primary molecular defect in many cases of muscular dystrophy is known, such that it is possible to study groups of genetically homogeneous patients. To test this, we present expression profiling using Affymetrix HuGeneFL high-density oligonucleotide arrays (~6,000 full-length genes) on human muscle biopsies from dystrophin deficiency, α-sarcoglycan deficiency (α-SGD),[1] and normal controls.

Materials and Methods

Patient Materials

We used muscle biopsies from primary dystrophinopathy (Duchenne muscular dystrophy [DMD] and female manifesting carriers), other dystrophies (α-SGD, calpain deficiency, and merosin deficiency), and normal controls. All patients had documented mutations of the corresponding genes (α-sarcoglycan, merosin, and calpain III; see below) or showed primary biochemical deficiency at the protein level (dystrophinopathies: DMD, and female manifesting carriers).

For expression profiling, frozen muscle biopsies from five male patients with dystrophin deficiency (age 6–9 yr), four patients with α-SGD (two males and two females; age 8–11 yr), and five male controls (age 6–9 yr) were used. For dystrophin deficiency, all biopsies were shown to have marked dystrophin deficiency by immunoflouresence (60 kD and d10 polyclonal antibodies [Hoffman et al., 1987b; Koenig et al., 1988] and/or dys III COOH-terminal antibody monoclonal antibodies [Novocastro] [Nicholson et al., 1989]), immunoblot (30 kD [Hoffman et al., 1987b] or dys II rod domain [Novocastro] [Nicholson et al., 1989]), or both. All patients showed clinical symptoms consistent with the diagnosis of DMD. All patients showed a dystrophic myopathy by hematoxylin/eosin staining and dramatic elevations of serum creatine kinase levels. For α-SGD, all biopsies were shown to have normal dystrophin by immunostaining and immunoblot, but complete α-SGD by immunostaining. Patients used for expression array experiments had the following previously reported gene mutations in the α-sarcoglycan gene: I103T/L173P compound heterozygote, I124T, where the second allele was not expressed at the RNA level, R77C/D97G compound heterozygote, and ΔC166 homozygote (Duggan

et al., 1997). All patients showed a dystrophic myopathy by hematoxylin/eosin staining and elevation of serum creatine kinase levels. Control biopsies were from patients sent for diagnosis of a possible myopathy, but who showed normal muscle histology by hematoxylin/eosin staining, normal myofiber structure by electron microscopy, and normal mitochondria enzyme activity.

For immunostaining experiments, muscle biopsies from normal controls (7 males and 1 female; age fetus to 26 yr), DMD (17 males; age fetus to 9 yr), α-SGD (2 females, age 8 and 12 yr), female-manifesting carriers of Duchenne dystrophy (mosaic for dystrophin) (2 females, age 4 and 7 yr), calpain III deficiency (female, 15 yr), and partial-merosin deficiency (female, 23 yr) were studied. α-Sarcoglycan patients used for immunostaining had the following mutations: compound heterozygote I103T/L173P, and compound heterozygote R77C/A93V (Duggan et al., 1997). For calpain III deficiency and partial-merosin deficiency, biopsies were shown to have normal dystrophin by immunofluorecence and immunoblot. The partial-merosin deficient patient showed partial-merosin deficiency, by immunofluorecence, and a C862R missense mutation, with the second allele not expressed at the RNA level (Tezak et al., 2000). The calpain III–deficient patient showed a R572Q homozygous/hemizygous mutation in the calpain III gene as previously reported (Chou et al., 1999).

Expression Profiling

Five dystrophin deficient, four α-sarcoglycan deficient, and five control muscle biopsies were used to extract total RNA by using TRI$_{ZOL}$® reagent (GIBCO BRL). Each biopsy was divided into two fragments, and RNA was isolated from each fragment independently (28 biopsy fragments total). 10 μg of total RNA from each biopsy fragment (28 muscles total) was converted into double-stranded cDNA by using SuperScript Choice system (GIBCO BRL) with an oligo-dT primer containing T7 RNA polymerase promoter (Genset). The double-stranded cDNA was purified by phenol/chloroform extraction, and then used for in vitro transcription using ENZO BioArray RNA transcript labeling kit (Affymetrix). Biotin-labeled cRNA was purified by RNeasy kit (QIAGEN), and fragmented randomly to ~200 bp (200 mM Tris-acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc). cRNA samples of each group were pooled in duplicate before hybridizing to Affymetrix HuGeneFL microarray for 16 h. The microarray was washed and stained on the Affymetrix Fluidics Station 400, using instructions and reagents provided by Affymetrix. This involves removal of nonhybridized material, and then incubation with phycoerythrin–streptavidin to detect bound cRNA. The signal intensity was amplified by second staining with biotin-labeled anti-streptavidin antibody and followed phycoerythrin–streptavidin staining. Fluorescent images were read using the Hewlett-Packard G2500A Gene Array Scanner.

Data Analysis

Data analysis of Affymetrix microarrays was done using GeneChip® software (version 3.3), as described previously (Lockhart et al., 1996). In brief, each gene is queried by 20 perfect match (PM) and 20 mismatch (MM) 25-base probes; the latter has a single base change in the center of the 25-bp probe. Comparison of the hybridization signal from the PM and MM probes allows a specificity measure of signal intensity, and elimination of most nonspecific cross-hybridization from the data analysis. Values of intensity difference, as well as ratios of each probe pair, are used for determining whether a gene is called "present" or "absent." For comparison of different datasets (e.g., dystrophin deficiency versus normal control), each probe pair in an experimental GeneChip® assay is compared with control groups, and four matrixes were used to determine the difference calls that indicate whether transcription level of a gene is changed.

Iterative comparisons of different datasets were done by spreadsheet analysis (Microsoft Excel). In brief, each dystrophin deficiency chip ($n = 2$) and α-SGD chip ($n = 2$) was compared with each control chip ($n = 2$) to determine the expression difference between each muscular dystrophy and the control. Difference calls that showed consistent results in all four pairwise comparisons of each disease were extracted for further analysis.

Immunohistochemistry

Polyclonal antibodies against complement component 3 (C3) and thrombospondin-4 were provided by Dr. Fernando Vivanco (Fundacion Jimenez Diaz, Madrid, Spain) (Alberti et al., 1996) and Dr. Jack Lawler (Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA) (Lawler et al., 1995). Sheep-anti–human factor XIIIa polyclonal antibody was from Cedarlane. Monoclonal antibody against HLA-DR was
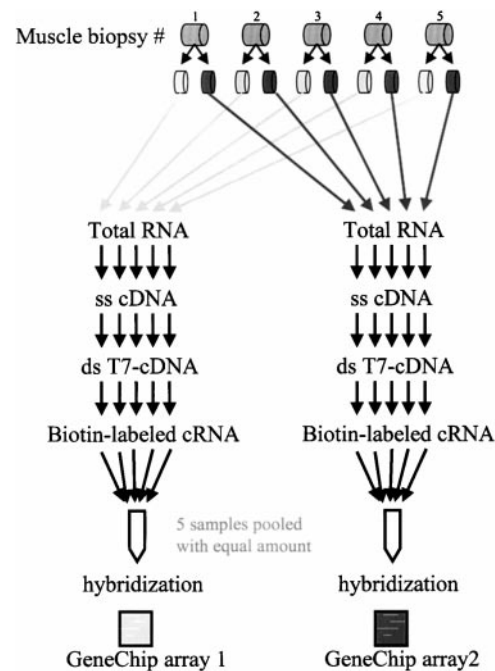

Figure 1. Experimental protocol employed for expression array analyses.

from Biomeda. Monoclonal antibody against secreted phospholipase A2 was from Cayman Chemical. Monoclonal antibodies against secreted protein, acidic and rich in cysteine (SPARC)/osteonectin and versican were from USBiological. A monoclonal antibody against α-cardiac actin was from Maine Biotechnology Services. All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, including FITC-conjugated donkey anti–mouse IgG, Cy3-conjugated goat anti–mouse IgG, Cy3-conjugated donkey anti–sheep IgG, and Cy3 conjugated donkey anti–rabbit IgG.

Serial 4-μm-thick frozen muscle sections were cut with an IEC Minotome cryostat, mounted to Superfrost Plus Slides (Fisher Scientific,), and fixed in cold anhydrous acetone. Sections were then blocked for 30 min in 10% horse serum and 1× PBS, and incubated with primary antibody for 3 h at room temperature. Antibody dilutions were as follows: (a) 1:500 for C3, thrombospondin-4, and factor XIIIa, (b) 1:200 for PLA2, (c) 1:10,000 for SPARC/osteonectin (d) 1:2,000 for versican, (e) 1:1,000 for embryonic myosin heavy chain, (f) 1:20 for HLA-DR, and (g) 1:10 for α-cardiac actin. Washes were done with 10% horse serum and 1X PBS, and sections then incubated with secondary antibody for 1 hour. FITC-conjugated donkey anti-mouse IgG was diluted 1:100. All other Cy3-conjugated secondary antibodies were diluted 1:500.
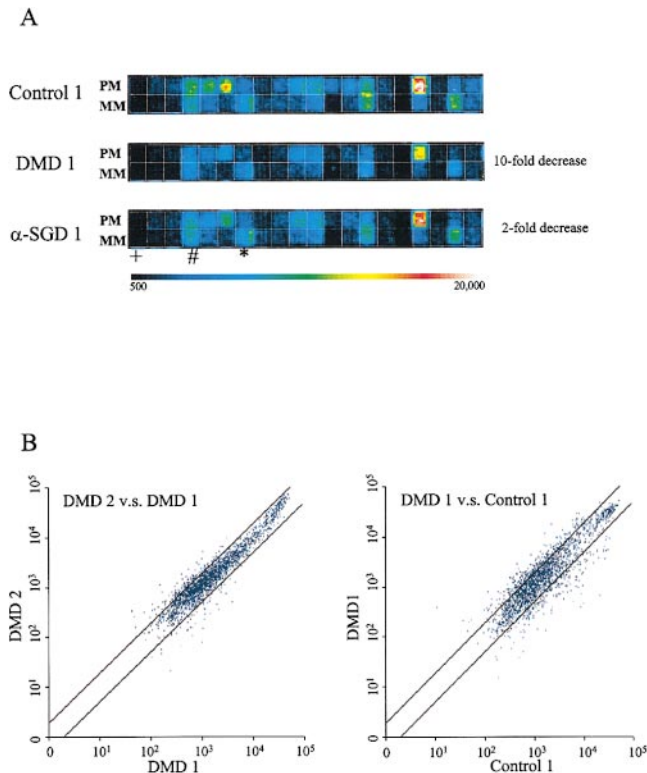
Online Supplemental Materials

Affymetrix image files for the six chip hybridizations and the absolute analysis results of each chip are available at http://www.jcb.org/cgi/content/full/151/6/1321/DC1.

Results

### Expression Profiling of Dystrophin Deficiency and α-SGD

The goal of this study was to determine downstream gene expression changes resulting from known primary biochemical defects in muscle. However, other sources of gene expression changes include variability in cell-type content of patient muscle biopsies and genetic background differences between individuals. These variables can complicate interpretation. To minimize the effect of these vari-

A

Control 1 PM / MM

DMD 1 PM / MM — 10-fold decrease

α-SGD 1 PM / MM — 2-fold decrease

+ # *

500   20,000

B

DMD 2 v.s. DMD 1

DMD 1 v.s. Control 1

Figure 2. Expression profile data for pooled muscular dystrophy patient biopsies. (A) Example of raw data of probe sets for ERK6 in normal pooled controls (Control 1), DMD (DMD1), and α-sarcoglycan–deficient (α-SGD1) patient biopsies. Shown are 20 probe pairs for the ERK6 gene on the GeneChip® HuGeneFL array, with the top feature of each pair showing hybridization of RNA to the perfect match probe (PM), and the lower feature showing the mismatched control probe (MM). Relative quantitation of averaged features is provided, as well as the difference call (e.g., increase or decrease) comparing DMD1 and α-SGD1 to the control 1 dataset. This analysis shows a 10-fold reduction of ERK6 mRNA specific for DMD. Probe pairs whose performance is inconsistent with the rest of probe set are indicated, with one showing equal hybridization with match and mismatch (#), one showing likely cross-reactivity to a more abundant RNA sequence (*), and one showing lack of hybridization (+). The color bar indicates intensity ranged from 500 to 20,000. (B) Scatter graph representation of comparative data, with axes showing relative expression levels of probe sets for each gene. Shown are single comparisons of expression profiling data sets (DMD1 versus DMD2; Control 1 versus DMD1). Lack of concordance between DMD1 and DMD2 represents combined experimental error, including tissue heterogeneity in muscle biopsy. Only "present" calls are shown, which represent ∼2,000 of the 6,000 genes tested. Comparison of control 1 and DMD1 data sets shows considerably more spread in expression levels throughout the intensity range, indicating gene expression relevant to dystrophin deficiency in muscle. The solid lines indicate twofold difference cutoff.

ables, we used the following experimental strategy (Fig. 1). First, each patient muscle biopsy to be studied was split and processed in duplicate. The duplication of each biopsy sample would be expected to control for all sources of both tissue and experimental variability, including cell-type heterogeneity within the tissue, variables in RNA isolation and biotinylated cRNA production, and variability in hybridization to GeneChip® microarrays. Second, to

A

DMD v.s. control

B

α-SGD v.s. control
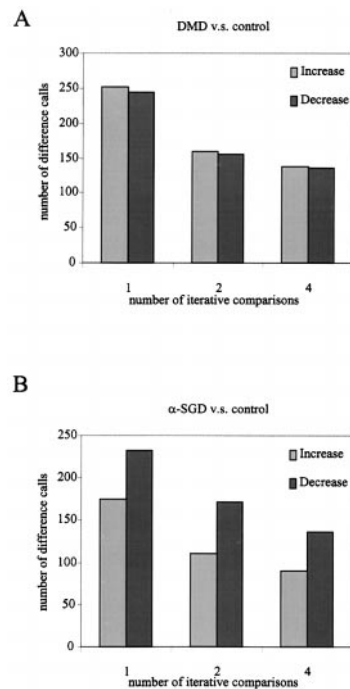
Figure 3. Iterative comparisons of duplicate data sets result in stringent determination of differentially expressed genes. (A) The effect of sequential iterative comparisons between DMD and control gene expression profiles is shown. A single data set comparison shows ∼250 increase and 250 decrease calls. Iterative comparisons of two independent data sets result in a decreasing number of difference calls that survive all comparisons (158 increase and 155 decrease calls after two comparisons; 137 increase and 135 decrease calls after four comparisons). (B) The same iterative analysis of α-sarcoglycan–deficient muscle versus normal controls is shown. A similar decline in surviving difference calls is seen.

minimize genetic polymorphic variation in expression patterns between different individuals, we studied four or five patient biopsies simultaneously, with equal amounts of cRNA mixed for each of the groups, and the resulting cRNA pools were then hybridized to a single GeneChip® array. Polymorphic variations in expression profiles should be normalized by this approach, whereas gene expression changes correlating with the primary biochemical defect should be retained. The mixing protocol also reduced the cost of the analysis, requiring substantially less microarrays to carry out the experiments. This protocol resulted in six datasets (normal1, normal2, DMD1, DMD2, α-SGD1, and α-SGD2) (Fig. 1). An example of raw image data showing hybridization of cRNA to 20 probe pairs of a single gene is shown in Fig. 2 A.

Description of genes tested on the GeneChip® HuGeneFL array is listed on our site (http://www.cnmcresearch.org; link to microarray). Among the 7,095 probe sets (∼280,000 oligonucleotide features) on the Affymetrix HuGeneFL microarray, we found a consistent number of "present" calls for each of the six cRNA pools tested (control 32 and 37%; DMD 36 and 32%; α-SGD 30 and 36%). Data from each experiment is posted on a web site for public access, and comparison to other HuGeneFL datasets (http://microarray.CNMCResearch.org/resources.htm; link to "muscle, human"). Included on the web site is the raw im-

A

B
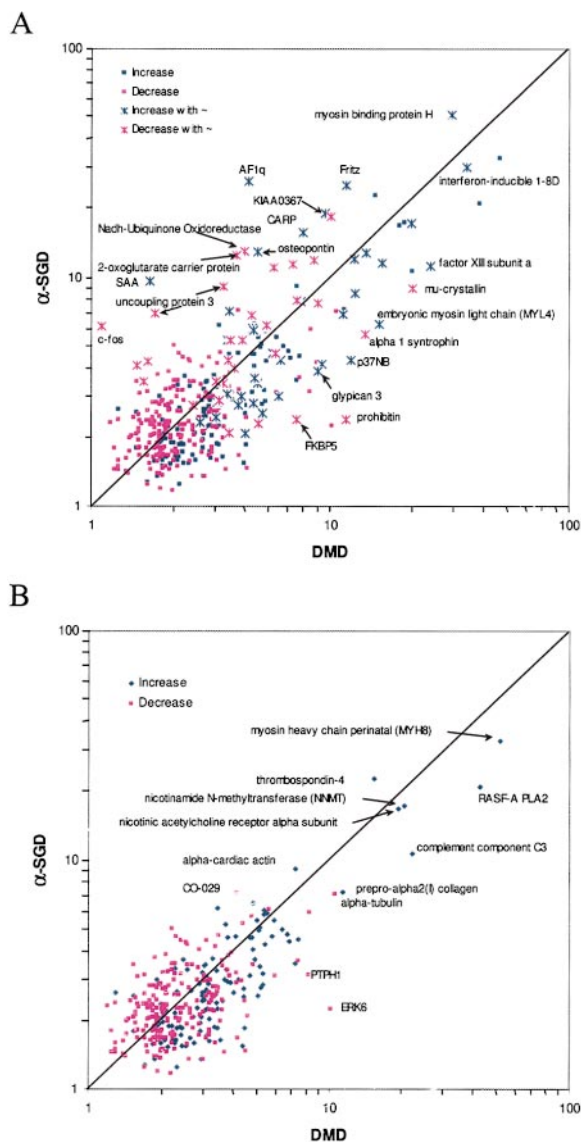
Figure 4. Comparison of differentially expressed genes in dystrophin deficiency and α-SGD expressed as fold changes compared with normal muscle. Both graphs show average fold changes >2 on a log scale. Those spots on the diagonal represent genes that show a similar expression change in both dystrophin deficiency and α-SGD, whereas those lying off the horizontal show greater differences in one or the other dystrophy. (A) The graph includes all difference calls that included one or more tilda values (~). This includes difference values where the expression level in any single dataset approaches the noise level, leading to an absent or marginal call. This often leads to a very large value for the ratio (e.g., dividing by zero) for determination of fold change, with a resulting exaggeration of the difference, and a higher probability of lying off the diagonal. Many of these tilda genes are those that show little or no expression in normal muscle, and are increased in the muscular dystrophies. (B) The graph shows removal of all data showing tildas. These genes have a strong confidence level with regards to the extent of the difference with normal muscle, and also the relative difference with regards to the two muscular dystrophies. Of particular note is the disease-specific decrease of ERK6 in dystrophin deficiency, and the very large increases in many developmentally regulated muscle genes that are shared by both muscular dystrophies.

age files for each of the six microarrays, text files containing absolute analyses of each chip ("present" calls; GeneChip® software output), and comparison analyses between different chips (difference calls; GeneChip® software output).

There was high concordance (88%) of "present" calls between duplicated datasets. However, the level of RNA found for each gene showed some variability between datasets; consistent with most microarray data published to date, the highest variability in levels was in genes showing low levels of cRNA hybridization (Fig. 2 B). The variability in levels of specific genes is likely a combination of tissue variability and experimental variability.

Genes that are consistently increased or decreased in all four possible iterative comparisons were determined (e.g., control 1 versus DMD1, control 1 versus DMD2, control 2 versus DMD1, and control 2 versus DMD2) (Fig. 3). Only ~40–60% of difference calls from a single comparison typically survived all four iterative comparisons of datasets. For this analysis, "marginal" difference calls assigned by GeneChip® software analysis were retained in the data sets. The cutoff used for difference calls was a twofold change in expression (either increase or decrease). The four iterative comparisons gave four values for "fold change," which were then averaged.

It is important to note that we focused on difference calls that satisfied all four iterative comparisons of data. This can be considered a very stringent selection of data, as genes that showed significant changes in expression in three data comparisons, but not the fourth, would be excluded from further study. We studied a series of genes that showed difference calls in three comparisons, but not the fourth; in each case the fold changes were close to the twofold cutoff used (data not shown). All data is presented on the web site, with selected data presented in Tables I and II.

From the four pairwise comparisons for each disease to normal controls, we identified 275 differentially regulated genes for dystrophin deficiency, and 233 differentially regulated genes for α-SGD. Thus, ~30% of probe sets tested were expressed in muscle, and ~10% of these showed differential regulation in dystrophin deficiency and/or α-SG deficiency. Expression of 138 genes was upregulated, and 137 genes downregulated in dystrophin deficiency versus control, 90 were upregulated and 143 genes downregulated in α-SGD versus control (Fig. 3). These data are also presented as a log scale graph of fold changes, with and without "tilda" values (Fig. 4). As explained in the figure legend, "tildas" are assigned when the denominator approaches zero (e.g., "absent" call), leading to possible exaggeration of the resulting ratio (Fig. 4).

Gene Expression Changes Shared by Dystrophin Deficiency and α-SGD

We expected many pathological processes to be involved in muscular dystrophy patient muscle, including degeneration cascades, regeneration programs, and fibrotic proliferation genes. We expected these changes to be shared between the two closely related primary biochemical defects. Consistent with this, we found 144 genes to show greater than twofold up and downregulation in both dystrophin deficiency and α-SGD. We then clustered these genes by pathological processes (Table I). The largest functional group of upregulated genes were genes of cell surface and

Table I. List of Shared Expression Changes of Genes in Dystrophin Deficiency and α-SGD

Accession no.	Gene name	Average-fold changes	
		Dystrophin deficiency	α-Sarcoglycan deficiency
Cell surface and extracellular matrix			
S77094	Nicotinic acetylcholine receptor α subunit 1	19	17
Z19585	Thrombospondin-4	15	23
HG2994-HT4850	Elastin, alt. splice 2	13	12
Z74616	Prepro-α2(I) collagen	11	7
U24488	Tenascin-X	11	7
U16306	Chondroitin sulfate proteoglycan versican	8	8
X53331	Matrix Gla protein	7	4
X52022	Type VI collagen α3 chain	7	5
HG3044-HT3742	Fibronectin, alt. splice 1	6	6
M55998	α-1 Collagen type I	6	5
J03040	SPARC/osteonectin	5	4
X15880	Collagen VI α-1 COOH-terminal globular domain	5	7
U21128	Lumican	5	5
X02761	Fibronectin (FN precursor) 1	4	3
Z74615	Prepro-α1(I) collagen	3	3
L02950	μ-Crystallin	−22	−9
Intracellular signaling and cell–cell communication			
M38591	S100 calcium-binding protein A10	5	6
M62403	Insulin-like growth factor–binding protein 4 (IGFBP4)	5	4
D13666	Osteoblast-specific factor 2 (OSF-2os)	4	7
M80563	S100 calcium-binding protein A4	4	5
HG2788-HT2896	Calcyclin	4	5
HG3543-HT3739	Insulin-like growth factor 2	3	3
U09578	MAPKAP kinase (3pK)	−9	−4
U50360	Calcium, calmodulin-dependent protein kinase II γ	−9	−12
L21993	Adenyl cyclase	−6	−3
U07225	P2U nucleotide receptor	−5	−6
M72885	G0S2	−3	−4
Immune response			
M22430	Phospholipase A2, group IIA	42	21
X57351	1-8D gene from interferon-inducible gene family	37	30
K02765	Complement component C3, α and β subunits	22	11
X83703	Cytokine-inducible nuclear protein	8	16
M14058	Complement C1r	5	4
J04080	Complement component C1s	5	3
M65292	Factor H homologue	5	3
Energy metabolism and mitochondria function			
HG4747-HT5195	NADH-ubiquinone oxidoreductase, 51 kD subunit	−6	−5
U65579	Mitochondrial NADH dehydrogenase-ubiquinone Fe-S protein 8	−5	−4
M69177	Monoamine oxidase B (MAOB)	−5	−7
J05073	Phosphoglycerate mutase (PGAM-M)	−4	−2
X66114	Solute carrer family 25,member 11	−4	−13
L77701	COX17	−4	−4
M37400	Glutamate oxaloacetate transaminase	−4	−4
X05409	Mitochondrial aldehyde dehydrogenase I ALDH I	−4	−3
M32598	Glycogen phosphorylase (PYGM)	−3	−2
J04444	Cytochrome c-1	−3	−3
X99728	NADH-ubiquinone oxidoreductase flavoprotein 3	−3	−2
X73874	Phosphorylase kinase,muscle,α1subunit	−3	−3
Z68204	Succinyl CoA synthetase	−3	−4
M91463	Glucose transporter (GLUT4)	−3	−4
S69232	Electron transfer flavoprotein-ubiquinone oxidoreductase	−3	−4
L16842	Cytochrome b of complex III	−3	−2
M22760	Cytochrome c oxidase subunit Va (COX5A)	−2	−2
M22632	Glutamate oxaloacetate transaminase	−2	−2
U80040	Aconitase	−2	−2
M15856	Lipoprotein lipase	−2	−5
L24774	Δ3, Δ2-CoA-isomerase	−2	−3
U49352	Liver 2,4-dienoyl-CoA reductase 1	−2	−5
M22877	Somatic cytochrome c (HCS)	−2	−2

(continues)

Table I. (continued)

Accession no.	Gene name	Average-fold changes	
		Dystrophin deficiency	α-Sarcoglycan deficiency
U17886	Succinate dehydrogenase complex subunit B	−2	−3
HG4334-HT4604	Glycogenin	−2	−2
Z14244	Cytochrome c oxidase subunit VIIb	−2	−2
Muscle structure and developmental genes			
X13100	Embryonic myosin heavy chain	140	124
Z38133	Skeletal muscle perinatal myosin heavy chain (MYH8)	51	33
L05606	Myosin-binding protein H	32	51
J00073	α-cardiac actin	7	9
L03785	Regulatory myosin light chain (MYL5)	5	5
U34301	Nonmuscle myosin heavy chain IIB gene	3	4
S81737	α-1 Syntrophin	−14	−6
X06956 (probe set 1)	α-Tubulin	−11	−7
HG2259-HT2348(probe set 2)	α-Tubulin	−8	−6
L34355	α-Sarcoglycan	−3	−5
Others and unknown			
M14539	Factor XIII subunit a	26	11
U51010 (probe set 1)	Nicotinamide N-methyltransferase (NNMT)	22	17
U08021 (probe set 2)	Nicotinamide N-methyltransferase (NNMT)	20	17
X68733	α1-Antichymotrypsin	6	4
M19045	Lysozyme	6	6
X14008	Lysozyme	5	5
X96719	Activation-induced C-type lectin (AICL)	3	4
X82209	Meningioma 1	−9	−8
L11672	Zinc finger protein 91	−5	−6
HG3344-HT3521	Ubiquitin-conjugating enzyme Ubch5	−4	−5
U03056	Tumor suppressor (LUCA-1)	−4	−5
M35252	Transmembrane 4 superfamily member 3	−4	−7
Y00339	Carbonic anhydrase II	−4	−4
Z49878	Guanidinoacetate N-methyltransferase	−4	−3
S83364	Putative Rab5-interacting protein	−4	−3
U52960	RNA polymerase II complex component SRB7	−3	−4
U48707	Protein phosphatase-1, regulatory subunit 8	−3	−4
U05861	Hepatic dihydrodiol dehydrogenase	−3	−3
U91903	Fritz	12	25
AB002365	KIAA0367 gene	9	19
U61374	Sushi-repeat–containing protein, X chromosome (SRPX)	7	5
X67698	Epididymal secretory protein (19.5 kD)	5	3
U79253	Clone 23893	−7	−8
D80010	KIAA0188	−6	−6
D14660	KIAA0104	−4	−4

extracellular proteins (42%). Additional functional groups included genes involved in immune responses (20%) and cell growth, differentiation, and signaling (15%).

Among 80 downregulated genes, 36% of them were involved in mitochondria function and energy metabolism (Table I). Importantly, this data suggests that there is a widespread disorder of both aerobic and anaerobic energy metabolism in patient muscle. Approximately 12% of downregulated genes were involved in cell growth, differentiation, and signaling. Specific genes were selected for verification of expression changes by immunostaining of patient muscle biopsies. As described below, all tested expression changes were confirmed by immunostaining data.

Gene Expression Changes Specific for Dystrophin Deficiency and α-SGD

We then tested for gene expression changes that were specific for either dystrophin deficiency or α-SG deficiency. We expected that this analysis would provide valuable tran-

scriptional information regarding well documented secondary protein deficiencies in each disorder, and would help determine whether these secondary biochemical abnormalities were due to reduced RNA levels or protein instability. In addition, we hypothesized that such disease-specific changes might point to genes or proteins that could have a functionally significant association with dystrophin or α-sarcoglycan at the protein level or gene transcription level. Such biochemical or genetic partners could provide insights into novel pathways or pathophysiological cascades.

For dystrophin deficiency, 131 genes showed significant expression changes relative to normal muscle, yet were assigned as "no change" in α-sarcoglycan deficient muscle. Similarly, α-sarcoglycan deficient muscle showed 89 genes with difference calls that were not seen in dystrophin deficiency. However, the large majority of these genes, in both dystrophin deficiency and α-SGD, showed relatively small changes near the twofold cutoff for statistical acceptance of a difference call. Thus, we felt it was likely that many, if not most, of these potential disease-specific expression

Table II. List of Disease Specific Changes in Dystrophin Deficiency and α-SGD

Accession no.	Gene name	Average-fold changes
Dystrophin deficiency		
U09579	Melanoma differentiation associated protein(mda-6)	16
J03909	γ-Interferon–inducible protein (IP-30)	13
U32907	p37NB	12
U04285	Lysosomal acid lipase, cholesteryl ester hydrolase (LIPA)	9
Z37987(probe set 1)	Glypican 3 (GPC3)	9
L47125(probe set 2)	Glypican 3 (GPC3)	7
J03801	Lysozyme	6
L13391	Helix-loop-helix basic phosphoprotein (G0S8)	6
K03430	Complement C1q B-chain	6
X52947	Cardiac gap junction protein	5
X76534	NMB	5
L33799	Procollagen C-proteinase enhancer protein (PCOLCE)	5
X79483	ERK6	−10
M64572	Protein tyrosine phosphatase	−8
L37127	RNA polymerase II	−7
U42031	54-kD progesterone receptor–associated immunophilin FKBP54	−7
α-SGD		
U16954	AF1q	26
X51441(probe set 1)	Serum amyloid A (SAA) protein	18
J03474	Serum amyloid A	16
U20758	Osteopontin	13
M12529	Apolipoprotein E	13
X51441(probe set 2)	Serum amyloid A (SAA) protein	10
X15882	Collagen VI α-2	6
M21119	Lysozyme	6
X63578	Parvalbumin	6
X78565	Tenascin-C	6
M34309	Epidermal growth factor receptor (HER3)	5
U80184	Nuclear aconitase mRNA, encoding mitochondrial protein	−18
HG3141-HT3317	NADH-ubiquinone oxidoreductase, 39-kD subunit	−13
X17644	GST1-Hs mRNA for GTP-binding protein	−12
D25304	KIAA0006	−11
AF001787	Uncoupling protein 3	−9
X06956	HALPHA44 gene for α-tubulin	−7
U82818	UCP3S	−7
V01512	c-fos	−6
D13635	KIAA0010	−5

changes represented experimental and biological "noise" and not biochemical or genetic "partners" for dystrophin or α-sarcoglycan.

To focus on those gene expression changes most likely to represent biochemical or genetic "partners," we selected only those genes showing fivefold or greater changes specifically for either dystrophin deficiency or α-SGD (Table II). Using these criteria, only a relatively few genes showed disease-specific changes in gene expression (12 upregulated and 4 downregulated genes specific for dystrophin deficiency, and 11 upregulated and 9 downregulated genes specific for α-SGD).

Dystrophin-deficient patient biopsies showed a specific decrease in dystrophin mRNA (fourfold) that was not seen in α-SGD, suggesting that primary genetic defects can potentially be identified by expression profiling. However, there were other genes showing similar or greater disease-specific differences. Two disease-specific changes were an extracellular signal regulated kinase (ERK6; 10-fold decrease) and a protein tyrosine phosphatase (8-fold decrease). For α-SGD, nine genes showed disease-specific decreases. For example, distinct sets of probes for the uncoupling protein3 gene detected nine- and sevenfold decreases in α-sarcoglycan-deficient, but not dystrophin-deficient patients.

Importantly, there were no significant gene expression changes of well documented secondary protein deficiencies, such as dystroglycan, sarcoglycans (β, δ, and γ), or nNOS. Also, there was not a change in utrophin RNA levels in either Duchenne or α-sarcoglycan dystrophies. On the other hand, another dystrophin-binding protein, α1-syntrophin, showed dramatic reductions in RNA levels in both dystrophin deficiency (14-fold) and α-SGD (6-fold), and α-sarcoglycan showed 3–5-fold reductions in both diseases as well.

A recent report has shown that ERK6 associates with α1-syntrophin, and it is also known that the PDZ domain of α1-syntrophin interacts directly the COOH terminus of dystrophin (Hasegawa et al., 1999). Thus, some of the biochemical partners of dystrophin (α1-syntrophin and ERK6) are also seen to be part of a coordinately regulated transcriptional group, as all three genes show dramatically reduced levels of RNA. Our data suggests this coordinately regulated gene cluster may also include a protein tyrosine phosphatase as a similar disease-specific mRNA reduction is seen.

Table III. Gene Expression Changes Confirmed by Immunofluorescence

Gene name	Accession no.	-fold change DMD	-fold change α-SGD	Normal muscle localization	Dystrophic muscle localization
Factor XIIIa	M14539	+26	+11	None	Cellular infiltrates
HLA-DR α heavy chain	X00274	+3	+3	Small amount endomysium	Cellular infiltrates
Complement component 3	K02765	+22	+11	None	Necrotic muscle fibers
Secreted phospholipase A2	M22430	+42	+21	None	Necrotic muscle fibers
Thrombospondin-4	Z19585	+15	+23	Endomysium	Focal increases
α-Cardiac actin	J00073	+7	+9	None	Majority of myofibers
Embryonic myosin heavy chain	X13100	+141	+124	None	Regenerating muscle fibers
Osteonectin/SPARC	J03040	+5	+4	Rare, punctate staining endomysium	Increase endomysium
Versican	U16306	+8	+8	Endomysium	Diffuse increase endomysium

Confirmation of Gene Expression Changes, and Cellular Localization of Differentially Regulated Gene Products

To confirm our expression array findings, we chose a series of differentially regulated genes to study by immunostaining patient muscle biopsies. This approach also allowed us to identify the localization of the differentially regulated gene product.

Serial 4-μm-thick frozen muscle sections were processed for immunostaining with antibodies against 15 protein products of differentially expressed genes. All were tested on both unfixed and acetone-fixed sections of normal controls, DMD, and α-sarcoglycan–deficient patient muscles. A subset of antibodies were also tested on female manifesting carriers of DMD (somatic mosaic for dystrophin expression in muscle) and unrelated muscular dystrophies of known causes (calpain III deficiency and partial merosin deficiency). Of the 15 antibodies tested, 9 provided adequate signal/noise ratios for interpretation of protein localization and amount. Results of antibody studies are summarized in Table III.

Factor XIIIa, HLA-DRα Heavy Chain. Factor XIIIa is a protein known to be involved in blood coagulation as a fibrin cross-linker. We found upregulation of this gene by 11–26-fold in both muscular dystrophies, though the expression in normal muscle was undetectable by GeneChip® array studies, hence possibly exaggerating the extent of upregulation. Immunolocalization of this protein showed positive cells in both epimysial and endomysial connective tissue in dystrophic muscle (Fig. 5). Double immunostaining with a marker for endothelium (laminin α1) showed that the staining for factor XIIIa did not colocalize with this protein, though the factor XIIIa–positive cells were often in close proximity to blood vessels (data not shown).

HLA-DR is a histocompatibility antigen highly expressed in antigen presenting cells. HLA-DRα was upregulated in both dystrophin-deficient (threefold) and α-sarcoglycan–deficient (threefold) patient muscle. Immunostaining for HLA-DRα showed strong immunolocalization to a subset of cells that resembled those immunostained by factorXIIIa. Indeed, double immunostaining for both factor XIIIa and HLA-DRα showed that most positively stained cells coexpressed these two proteins (Fig. 5).

This data suggested that these cells represented tissue dendritic cells (Sueki et al., 1993). To test this, immunostaining was carried out with markers for circulating dendritic cell subtypes (CD1a, CD1b, and CD1c). Many of the factorXIIIa/HLADR-positive cells also stained with CD1a, and less frequently with CD1b and CD1c (data not shown). The data suggests that the infiltrating cells responsible for expression of factorXIIIa and HLA-DRα are related to tissue (dermal) dendritic cells. This is the first report of factor XIIIa+ and HLA-DR+ dendritic cell infiltration in dystrophic muscles.

Thrombospondin 4, SPARC, and Versican. Thrombospondin 4 is an extracellular matrix calcium-binding protein particularly abundant in tendon and early osteogenic tissues. It has been shown to be upregulated in denervated muscle, though its function is poorly understood. Thrombospondin 4 was 15-fold increased in dystrophin deficiency, and 23-fold increased in α-SGD. Immunostaining of dystrophic patient muscle biopsies showed thrombospondin 4 to be localized to areas of macrophage infiltration, though the areas showing very strong staining for thrombospondin 4 extended beyond frankly necrotic regions (Fig. 6, D–G). This data suggests that thrombospondin 4 is expressed by interstitial cells in response to macrophage infiltration, denervation, and/or cellular damage of neighboring myofibers (Arber and Caroni, 1995).

SPARC/osteonectin is an extracellular glycoprotein that is strongly expressed during development and tissue regeneration, where it functions to mediate connections between cells and the extracellular matrix (Lane and Sage, 1994). SPARC showed fivefold upregulation in dystrophin deficiency, and fourfold elevation in α-SGD. Immunolocalization showed punctate immunostaining that was dramatically increased in the endomysial and perimysial connective tissue (data not shown).

Versican is a chondroitin sulfate proteoglycan that, like SPARC and thrombospondin, is prevalent in myogenesis of muscle (Carrino et al., 1999). This gene showed eightfold upregulation in both dystrophies. Immunolocalization identified diffusely increased amounts of the protein in endomysial, but not perimysial, connective tissue of dystrophic muscle (Fig. 6, A–C).

α-Cardiac Actin, Embryonic Myosin Heavy Chain. Both α-cardiac actin and embryonic myosin heavy chain are
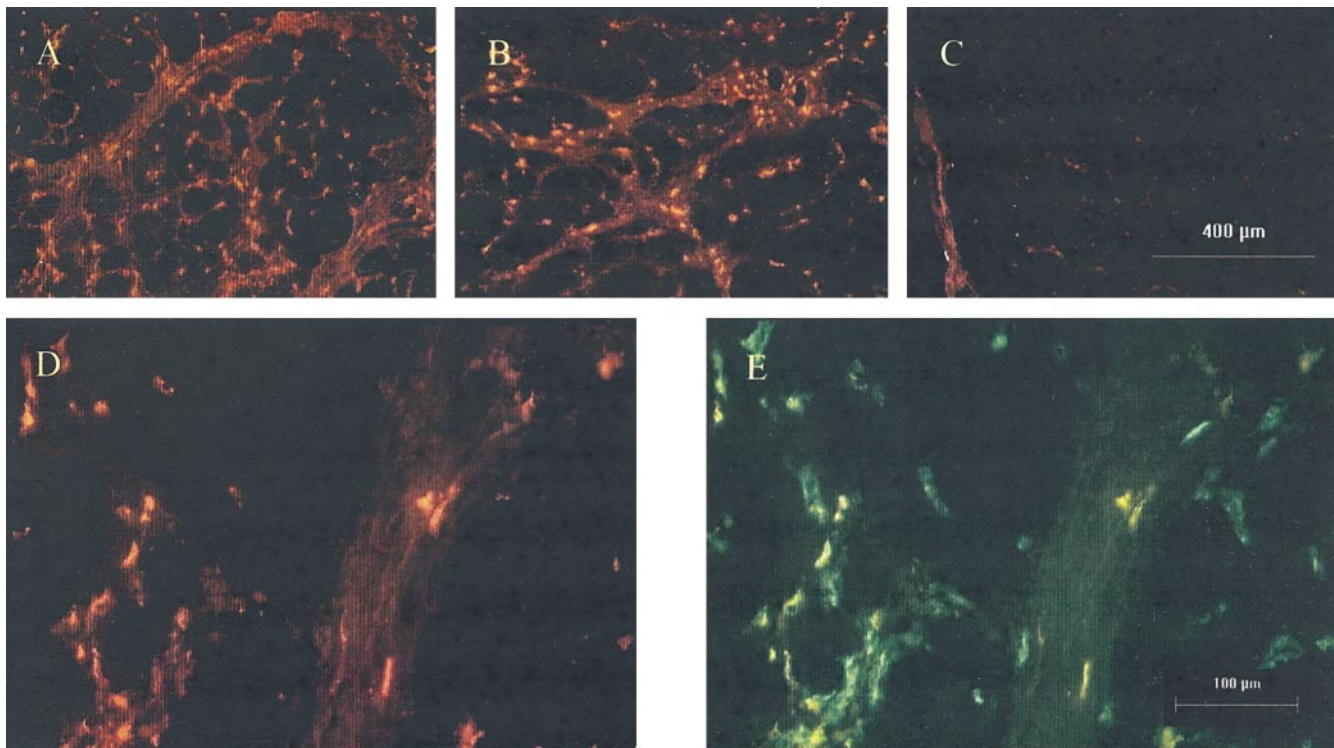
Figure 5. Immunolocalization of factor XIIIa shows colocalization with HLA-DR in tissue dendritic cells. (A–C) Immunofluorescent visualization of factor XIIIa in frozen muscle biopsy sections from DMD (A), α-SGD (B), and normal control (C) are shown. Factor XIIIa localizes to infiltrating connective tissue cells in both dystrophin- and α-sarcoglycan–deficient muscle; these cells are absent in control muscle. Double staining of dystrophin-deficient muscle with factor XIIIa (D) and HLA-DR (E) shows colocalization of these proteins in the majority of cells, suggesting that these are infiltrating dendritic cells. These cells were variably positive for other dendritic cell markers, with subsets also positive for CD1a and CD1b in the epimysial connective tissue (dermal dendritic cells), and other subsets positive for CD14 in the endomysial connective tissue (stationary macrophages) (data not shown). Macrophages infiltrating necrotic fibers were not stained for factor XIIIa (data not shown).

specific isoforms of proteins that are transiently expressed during normal muscle development and regeneration (Whalen et al., 1979; Toyofuko et al., 1992). Both of these proteins showed upregulation in dystrophic muscle: α-cardiac actin was increased 7–9-fold and embryonic myosin 124–140-fold. Immunolocalization of these proteins in muscle biopsies, similar to those used for expression profiling, showed high-level expression of embryonic myosin in ~20% of dystrophic myofibers, and of α-cardiac actin in ~80% of fibers (Fig. 7).

Overt degeneration/regeneration of myofibers is a relatively rare event by histological assays (Fig. 6), and the large proportion of myofibers positive for these developmentally specific isoforms did not seem to be justified by the limited amount of regeneration in the dystrophic muscle biopsies. To test the association of myofiber regeneration with the amount of α-cardiac–actin positive myofibers, we studied both female mosaics for dystrophin deficiency (Fig. 7, D and E) and a series of patient muscle biopsies from DMD patients (fetal, neonate, 3-, 5-, and 8-yr old), normal controls, merosin deficiency, and calpain deficiency (Fig. 7 F). In the manifesting carrier of dystrophin deficiency (Fig. 7, D and E), both dystrophin-positive and dystrophin-negative myofibers were strongly positive for α-cardiac actin, and most appeared to be fully developed myofibers, suggesting that α-cardiac–actin expression persisted beyond the point of complete myofiber regeneration.

Immunostaining of α-cardiac actin in both normal and dystrophin-deficient fetal muscle (~18–22-wk gestation) showed 100% of myofibers to be positive for α-cardiac actin. By birth, the proportion of α-cardiac–actin positive myofibers in normal muscle declined to 0%, whereas in dystrophin-deficient muscle, ~60% of fibers remained strongly positive (Fig. 7 F). This high level was maintained throughout the disease process. Two unrelated dystrophic controls, partial merosin deficiency and calpain III deficiency, showed lower levels of α-cardiac actin (~15–20% positive fibers). As dystrophin-deficient neonatal muscle shows relatively little evidence of degeneration/regeneration by histopathology, we conclude that the overexpression of α-cardiac actin shows persistent expression beyond the normal windows of development and regeneration.

Discussion

Expression Profiling As a Means of Understanding Pathological Processes

Here, we report expression profiling as an experimental approach to define the biochemical cascades underlying the progressive pathophysiology of the inherited muscular dystrophies. A critical aspect of expression profiling studies is to limit the number of variables under study. This becomes highly problematic in the study of human pathologi-
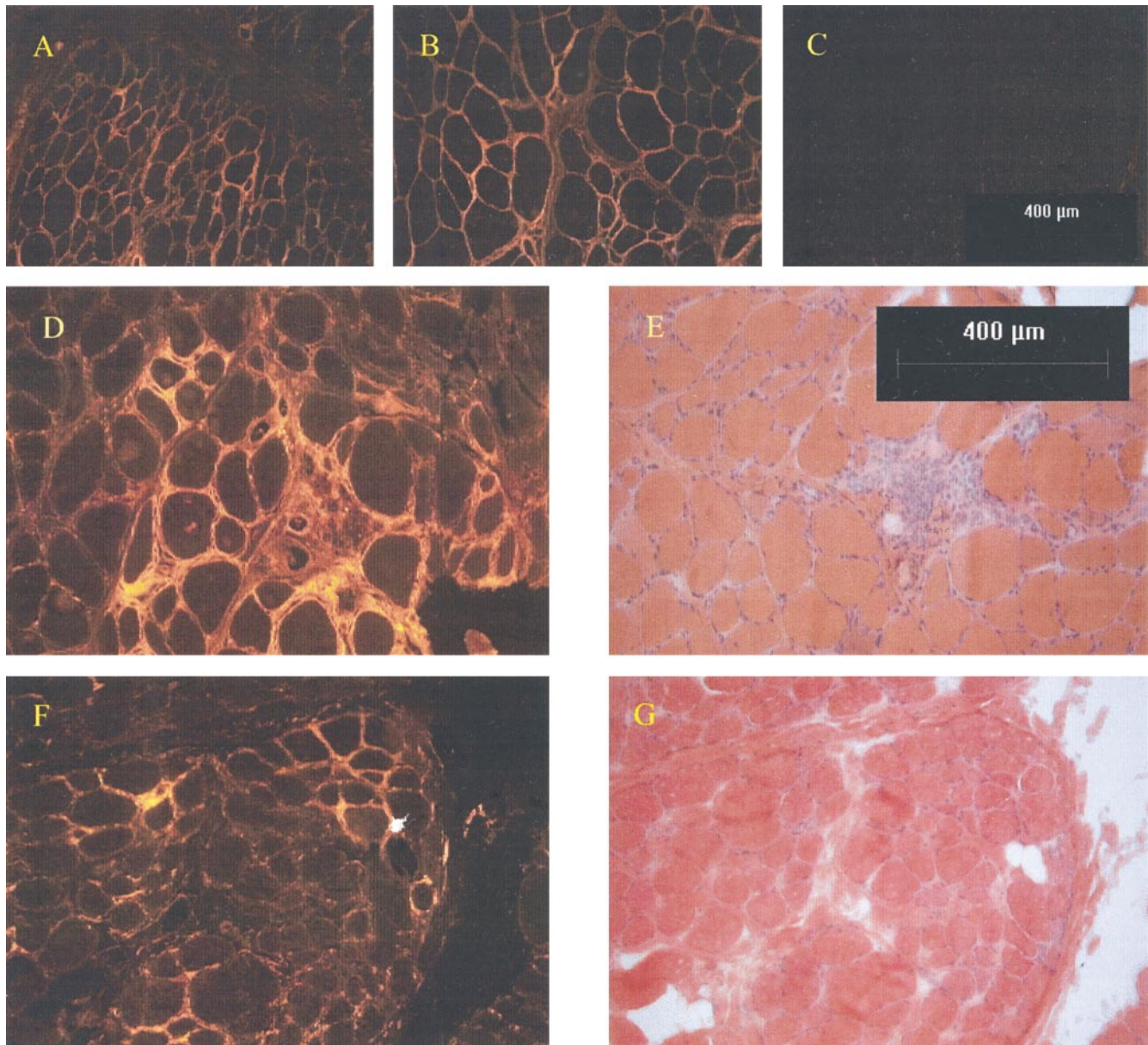
Figure 6. Versican and thrombospondin IV show upregulation in dystrophin- and α-sarcoglycan–deficient muscle. (A–C) Shown are sections from dystrophin-deficient (A), α-sarcoglycan–deficient (B), and normal control (C) muscle biopsies immunostained for the large chondroitin sulfate proteoglycan, versican. Upregulation of versican is seen in the endomysial connective tissue in both dystrophin- and α-sarcoglycan–deficient muscle. (D–G) Shown are adjacent sections immunostained for thrombospondin IV (D and F), and stained with hematoxylin and eosin (E and G). A region of grouped necrosis of myofibers is seen in the α-sarcoglycan–deficient muscle (E). This same region shows a large increase in thrombospondin IV protein production (D). However, in the dystrophin-deficient muscle, regions with upregulation of thrombospondin IV (F) do not show obvious necrosis in the adjacent sections stained with hematoxylin and eosin (G).

cal tissue in that there is considerable heterogeneity within tissue biopsies, differences in the genetic background between unrelated patients (genetic noise), and many other uncontrolled variables. One method to control for these variables is to study large numbers of patient samples, and then use elaborate statistical analyses to parse specific variables. This approach requires large numbers of analysis, which is costly and can be subject to statistical artifacts.

We present a novel approach for expression profiling of human patient pathological tissues, which we show is successful in reducing the effect of many uncontrolled variables on the resulting data, and thereby uncovering significant gene expression changes. This method employs tissue biopsies from groups of patients with known primary genetic defects that are age matched; the primary biochemical defect is held constant, and, thus, the major variable under study is controlled. All sources of experimental variability, including genetic background differences and tissue heterogeneity, were attenuated through the use of two different regions of each biopsy and mixing equal amounts of target cRNA from unrelated patients (Fig. 1). We show that it is critical to have iterative comparisons of data replicates; ~50% of expression changes identified by one pairwise comparison were not consistently detected in comparisons of multiple
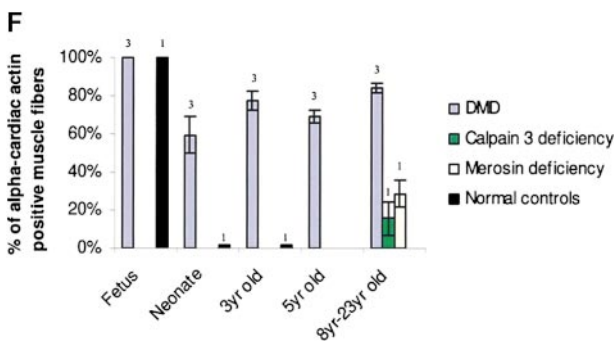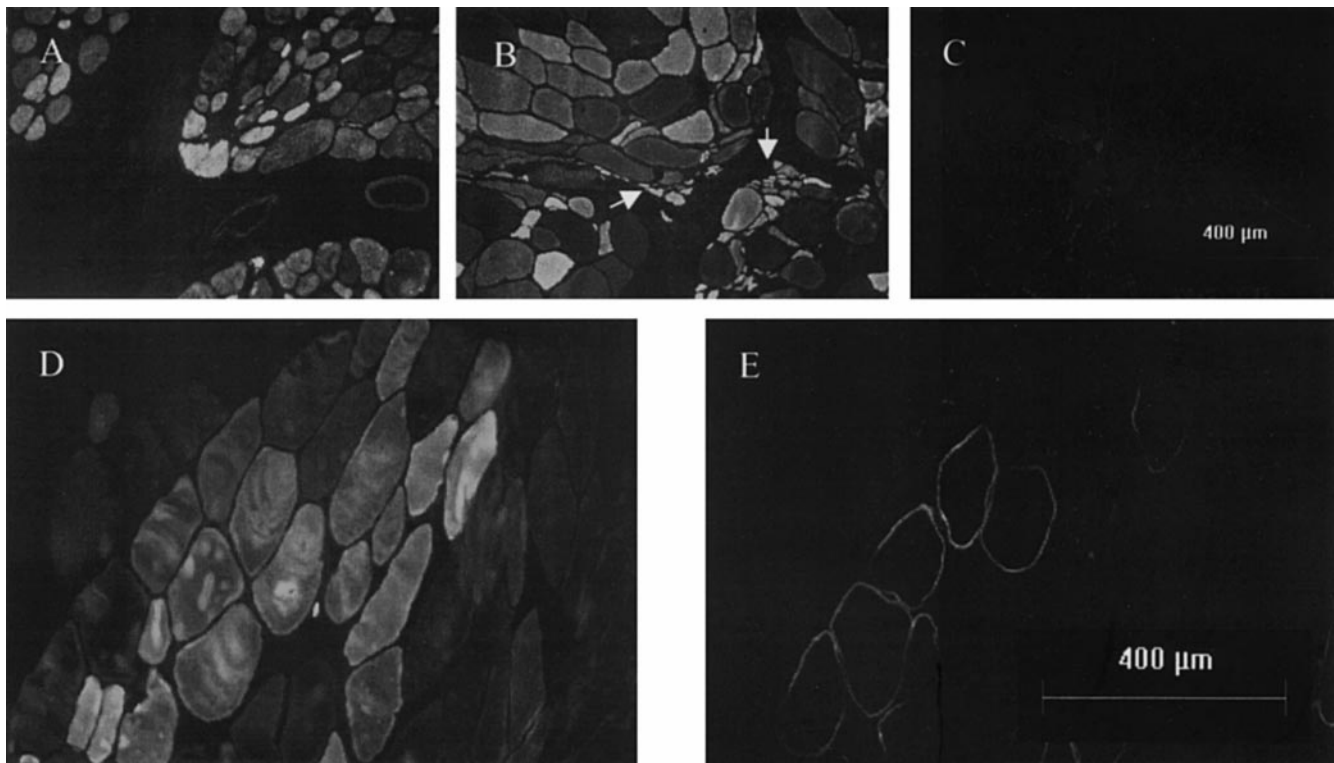
Figure 7. Developmentally regulated myogenic proteins are persistently upregulated in dystrophin- and α-sarcoglycan–deficient muscle. (A–E) Shown are immunostainings for α-cardiac actin, with quantitation of the percent of α-cardiac–actin positive myofibers in a series of muscle biopsies (F). Muscles from patients with dystrophin deficiency (A), α-SGD (B), normal control (C), and a female manifesting carrier for dystrophin deficiency (D) are shown. Immunostaining for dystrophin (E) shows mosaic pattern of immunostaining in the female carrier, with both dystrophin-positive and dystrophin-negative cells. For quantitation, the number of patients employed is shown on the top of the column at each time point (F). For each patient, three different fields were counted. The bars indicate the standard deviation derived from three individuals when three patients were studied, or three fields when only one individual was included. At the 8–23-yr time point, three 8-yr-old dystrophin-deficiency patients, one 15-yr-old calpain 3–deficiency patient, and one 23-yr-old merosin-deficiency patient were counted. α-Cardiac actin is developmentally regulated during early muscle development, but shows little or no expression in normal muscle from birth onwards (C and F). Both show dramatic upregulation in dystrophic muscle, despite little evidence of actively regenerating fibers in the dystrophin deficiency (A) and α-sarcoglycan–deficient (B) muscles. The α-sarcoglycan–deficient muscle also shows atrophic, angulated fibers (arrows) suggestive of either failed regeneration or denervation; these fibers are also positive for α-cardiac actin (B). In the female mosaic for dystrophin deficiency, immunostaining for α-cardiac actin shows both dystrophin-positive and -negative cells to stain positively.

data sets. We showed this iterative method to be accurate in detecting differentially expressed genes; all differentially expressed genes tested by protein immunostaining of patient tissues validated the expression profiling data.

We also present a novel application of expression profiling where two biochemically related primary genetic defects are compared to identify potential members of transcriptional pathways. In this case, patients with primary genetic defects of two associated proteins, dystrophin and α-sarcoglycan, were compared; these disorders show nearly identical clinical, histological, and functional defects. Some of the differentially expressed genes are indeed biochemical partners, and our data shows that these corresponding genes are associated in a transcriptional regulatory pathway (ERK6, α-syntrophin, and dystro-

phin). Moreover, our data provides the first proof of principle that primary genetic defects may be able to be identified by expression profiling. We found a specific fourfold decrease in dystrophin RNA in Duchenne dystrophy biopsies. We did not find a similar specific decrease in α-sarcoglycan gene expression in α-sarcoglycan–deficient patients, however, this is likely due to the fact that these patient had missense mutations that typically do not show nonsense-mediated decay. On the other hand, nearly all Duchenne dystrophy patients show frameshift mutations or stop codons that result in nonsense-mediated decay of the dystrophin mRNA (Hamed et al., 2000).

We provide insights into the disease process of these related muscular dystrophies at multiple levels: (a) a general metabolic crisis in patient muscle, (b) novel proteins in-

volved in early stages of myofiber necrosis, (c) novel cells and proteins involved in local inflammatory and bystander responses, and (d) persistent expression of developmentally regulated genes suggesting that muscle may assume a chronically dedifferentiated state. Whereas expression profiling is widely felt to provide the parallel data generation needed to understand many complex biological processes, previous reports of expression profiling have been limited to physiological responses of microorganisms to environmental change and the subclassification of human cancers (Alon et al., 1999; Golub et al., 1999; Jelinsky and Samson, 1999). To our knowledge, we present the first report of expression profiling in patient tissues with monogenic defects. Below, we briefly discuss some of the more dramatic changes we identified in the dystrophies, focusing on those that are cell autonomous and those that involve tissue microenvironment (non-cell autonomous). Many additional interpretations of the data obtained are possible, however, all data is deposited on a web site for public access for further analyses (http://microarray. CNMCResearch.org/resources.htm).

Cell Autonomous Features of the Pathophysiology of Dystrophin and α-Sarcoglycan Deficiencies

Many genes involved in mitochondrial function and energy metabolism were found to be reduced by twofold or more (26 genes), suggesting generalized mitochondrial dysfunction and metabolic crisis. Mitochondrial dysfunction has been reported previously, using a variety of assays in both human dystrophy patients and animal models, including 31P-NMR studies (Barbiroli et al., 1992) and biochemical assays (Gannoun-Zaki et al., 1995; Kuznetsov et al., 1998). It is widely felt that the chronic calcium influx due to the poor integrity of the plasma membranes of dystrophic muscle leads to calcium overloading of the mitochondrial matrix, as well as decreased mitochondrial function. Our data shows that much of this decreased function is due to reduced transcription of nuclear-encoded mitochondrial genes. The finding of a metabolic defect agrees with the beneficial effect of performance-enhancing metabolic agents such as creatine, coenzyme Q, carnitine, and others on dystrophic muscle function (Granchelli et al., 2000) (J.A. Granchelli, personal communication).

We noted that many important calcium-regulated signaling molecules were downregulated at the transcriptional level, such as adenylate cyclase (sixfold), calmodulin-dependent protein kinase (fivefold), and phospholipase C (twofold). Presumably, this reflects a negative-feedback loop where the chronic calcium influx from membrane instability over stimulates calcium-sensitive pathways, leading to a compensatory reduction in transcription of the corresponding genes. The downregulation of these genes might also downregulate down stream protein kinase A and C that are known to regulate expression of mitochondria genes and energy metabolism.

We found very high expression of many developmentally regulated genes, including α-cardiac actin (8-fold), embryonic myosin heavy chain (100-fold), versican (8-fold), perinatal myosin heavy chain (80-fold), acetylcholine receptor α 1 (12-fold), embryonic myosin light chain (10-fold), and others (Fig. 4). At first, we suspected that these simply reflected regenerative processes in the muscle, as it is well known that myofiber regeneration recapit-

ulates much of the developmental program of muscle fibers. However, a variety of observations suggested that expression of these developmentally regulated genes was not limited to actively regenerating myofibers, and, instead, reflected persistent overexpression of these genes. First, overexpression of α-cardiac actin was present in 60–80% of DMD muscle fibers, whereas only a small subset of these showed histological evidence of regeneration (Fig. 7). Second, neonatal myofibers from normal muscle showed no α-cardiac actin, whereas 60–80% of DMD myofibers were positive. Third, mature dystrophin-positive myofibers in female mosaics also showed high expression of α-cardiac actin, suggesting that the protein was diffusing from adjacent dystrophin negative or regenerating regions, or that signals resulting in overexpression were trammsmitted within mosaic fibers. A previous study of α-cardiac–actin gene transcription in rat muscle showed that denervated muscle induced α-cardiac actin mRNA to levels which were sixfold higher than normal, and, thereafter, maintained these levels at a constant level (Toyofuko et al., 1992). Regenerating muscle from an autograft resulted in mRNA levels that were initially increased by 40-fold, but then dropped to normal levels within 40 d. Taking these observation together, it is possible that degeneration/regeneration cycles in dystrophic muscle leads to high levels of α-cardiac actin mRNA and protein that diffuse within syncytial myofibers. However, our finding of the majority of neonatal fibers from preclinical DMD patient biopsies to be positive for α-cardiac actin suggests that these may be a persistent overexpression of this protein in dystrophin-deficient and α-sarcoglycan–deficient myofibers. We propose a model where this persistent expression is due to a chronic state of undifferentiation induced by altered Ca^{2+} signaling within the myofiber. Consistent with this model, it has been recently shown that altering the pattern of Ca^{2+} influx in differentiating myogenic cells can determine the developmental pathway that the cells will take (Naya et al., 2000).

A second result suggesting dedifferentiation of dystrophic muscle was the upregulation of versican (eightfold). Versican is one of the large chondroitin sulfate proteoglycans that has been shown to be important during the early development of muscle (Carrino et al., 1999). Muscle tissue switches from large proteoglycans, early in development, to small proteoglycans later. Versican is expressed during regeneration, but our immunostaining results showed high level, persistent expression of versican protein throughout the endomysial connective tissue in both dystrophin deficiency and α-sarcoglycan–deficient muscle, consistent with the dramatic upregulation of RNA levels shown by the expression array studies (Fig. 6). Importantly, versican has been shown to stimulate the proliferation of chondrocytes (Zhang et al., 1999); we also found upregulation of other chondrocyte- and bone-related transcripts, such as OSF-2 (4-fold), matrix Gla protein (7-fold), osteopontin (13-fold), and serine protease 11 (3-fold). Our results suggest that persistent expression of developmentally regulated genes, coupled with chronic calcium influx in dystrophin-deficient myofibers, leads to altered development and regeneration of myofibers in dystrophic muscles.

The acetylcholine receptor α1 subunit was also dramatically upregulated in patient muscle (19-fold). This protein is known to increase at extrajunctional sites during both
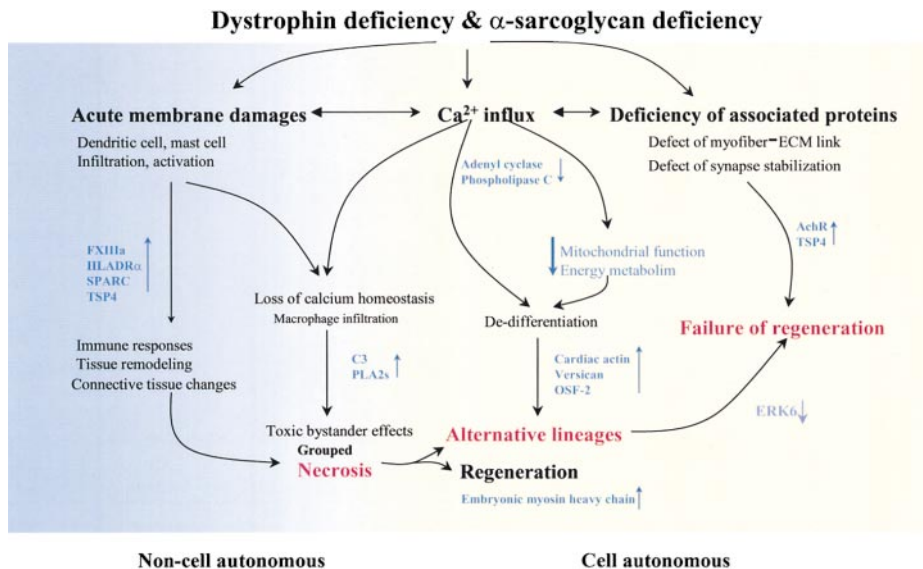
Figure 8. Pathophysiological flow chart of the muscular dystrophies.

normal development and denervation atrophy (Fischer et al., 1999), however, similar to α-cardiac actin, the extent of induction of the acetylcholine receptor seemed too large to explain by the small amount of regenerating fibers and the few atrophic fibers seen in the biopsies. Several studies have demonstrated that deficiency of dystrophin and the sarcoglycan complex disturb the muscle fiber–laminin interaction and the stability of neuromuscular synapse (Brown et al., 1999; Grady et al., 2000), and that this leads to morphological changes of the junction (Zaccaria et al., 2000). These findings are consistent with two possible models: either myofibers are in a persistent undifferentiated state, or motor neurons are unable to maintain strong attachments to myofibers, leading to a constant stimulation of denervation signal pathways.

Non-cell Autonomous Features of the Pathophysiology of Dystrophin and α-Sarcoglycan Deficiencies

This is the first report of factor XIIIa+ and HLA-DR+ dendritic cell infiltration in dystrophic muscles. MHC class II+ dendritic cells have been reported at low level in normal and regenerating rodent muscles (Pimorady-Esfahani et al., 1997), and muscle dendritic cells have been shown to be important in immune aspects of gene delivery to muscle (Jooss et al., 1998). However, the expression profiling and immunophenotyping we present here shows that the dendritic cell infiltrate in dystrophic muscle is that of activated dermal dendritic cells. In skin, similar subpopulations of XIIIa+ and HLA-DR+ dermal dendritic cells have been shown to be closely associated with tissue mast cells, and increased factor XIIIa protein expression in dendritic cells has been observed in response to mast cell degranulation (Sueki et al., 1993). We have shown previously that in muscle, extensive mast cell proliferation and degranulation in dystrophin-deficient human, dog, and mouse muscle (Gorospe et al., 1994a,b). Also, we have shown that dystrophin-deficient myofibers are acutely sensitive to mast cell inflammatory mediators and proteases, showing widespread grouped necrosis when exposed to mast cell granules; this is presumably due to exacerbation of the membrane defect by the mast cell mediators (Gorospe et al., 1996). Our new results suggest that dendritic cells and mast cells may act coordinately to mediate chronic and acute microenvironmental changes in dystrophic muscles. These findings may have relevance to current treatment of Duchenne dystrophy patients with steroids. Prednisone is well documented to slow the progression of DMD and α-SGD, yet the inflammatory cell target of prednisone has not been identified, as drugs which inhibit T and B cell function do not seem to benefit DMD patients (Griggs et al., 1993; Connolly et al., 1998). Prednisone recently has been shown to prevent activation of dendritic cells (Matasic et al., 1999). Thus, our findings suggest that prednisone may improve patient muscle function by acting on dendritic cell pathways.

We identified a series of novel upregulated proteins in the endomysial connective tissue of dystrophic muscle that are traditionally considered developmentally specific isoforms, namely thrombospondin IV, SPARC, and versican. Each of these proteins are also known to be important in regeneration and/or denervation of muscle. However, we found that expression of these proteins was not limited to regions of regenerating myofibers, suggesting that these, like α-cardiac actin and acetylcholine receptor α1, show a persistent and/or chronic overexpression in dystrophic muscle. Importantly, each of these three proteins have been found to have a role in tissue remodeling and cell migration; these microenvironmental changes could exacerbate the effects of dystrophin deficiency in muscle. The thrombospondins are a family of extracellular calcium-binding proteins that are involved in cell proliferation, adhesion, and migration (Arber and Caroni, 1995; Newton et al., 1999). SPARC/osteonectin is a secreted glycoprotein that contains modular domains that can function independently to bind cells and matrix components. Because SPARC can selectively disrupt cellular contacts with matrix and, thereby, effect changes in cell shape, it has been referred to as an anti-adhesin (Lane and Sage, 1994). Versican is involved in stimulating cell proliferation and migration in both smooth muscle and chondrocytes (Evanko et al., 1999; Zhang et al., 1999).

Conclusion

The global gene expression analyses presented here add critical new information to existing pathophysiological models of dystrophin and α-SGD (Fig. 8). Our results suggest that developmental reprogramming, both in the form of failure to deactivate developmentally regulated genes and the persistent activation of proteins involved in development and regeneration, leads to a series of both cell autonomous, and non-cell autonomous (microenvironmental) changes. We hypothesize that these changes lead to the progressive aspects of the human disease and phenotypic differences in the orthologous animal models. Future expression profiling experiments will focus on the correlation of specific pathological cascades with patient age and the degree of disability, as well as cross-species experiments in animal models. These will help distinguish between regeneration cascades, chronic undifferentiated status, and abnormal differentiation pathways in dystrophic muscle.

Our results also show that it is possible to identify primary genetic defects in patient tissue through analysis of carefully controlled expression profiling experiments. In this instance, we were able to show a disease-specific reduction of dystrophin mRNA in Duchenne dystrophy patient muscle (fourfold reduction) compared with α-sarcoglycan deficient muscle. In the future, molecular diagnostics is likely to be possible with expression profile "fingerprints" of patient muscle. These profiles would reflect both generic responses to pathological processes in muscle and specific changes reflecting the primary biochemical defect. Critical in the accuracy and sensitivity of such testing will be the public availability of large numbers of expression profiles of patients with known genetic defects.

References

Alberti, S., D. Alvarez, S. Merino, M.T. Casado, F. Vivanco, J.M. Tomas, and V.J. Benedi. 1996. Analysis of complement C3 deposition and degradation on *Klebsiella pneumoniae*. *Infect. Immun.* 64:4726–4732.

Alon, U., N. Barkai, D.A. Notterman, K. Gish, S. Ybarra, D. Mack, and A.J. Levine. 1999. Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc. Natl. Acad. Sci. USA.* 96:6745–6750.

Arber, S., and P. Caroni. 1995. Thrombospondin-4, an extracellular matrix protein expressed in the developing and adult nervous system promotes neurite outgrowth. *J. Cell Biol.* 131:1083–1094.

Barbiroli, B., R. Funicello, S. Iotti, P. Montagna, A. Ferlini, and P. Zaniol. 1992.

31P-NMR spectroscopy of skeletal muscle in Becker dystrophy and DMD/BMD carriers. Altered rate of phosphate transport. *J. Neurol. Sci.* 109:188–195.

Bonnemann, C.G., R. Modi, S. Noguchi, Y. Mizuno, M. Yoshida, E. Gussoni, E.M. McNally, D.J. Duggan, C. Angelini, and E.P. Hoffman. 1995. Beta-sarcoglycan (A3b) mutations cause autosomal recessive muscular dystrophy with loss of the sarcoglycan complex. *Nat. Genet.* 11:266–273.

Brown, S.C., A. Fassati, L. Popplewell, A.M. Page, M.D. Henry, K.P. Campbell, and G. Dickson. 1999. Dystrophic phenotype induced in vitro by antibody blockade of muscle alpha-dystroglycan-laminin interaction. *J. Cell Sci.* 112:209–216.

Bulfield, G., W.G. Siller, P.A. Wight, and K.J. Moore. 1984. X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci. USA.* 81:1189–1192.

Burkin, D.J., and S.J. Kaufman. 1999. The alpha7beta1 integrin in muscle development and disease. *Cell Tissue Res.* 296:183–190.

Carpenter, J.L., E.P. Hoffman, F.C. Romanul, L.M. Kunkel, R.K. Rosales, N.S. Ma, J.J. Dasbach, J.F. Rae, F.M. Moore, M.B. McAfee, et al. 1989. Feline muscular dystrophy with dystrophin deficiency. *Am. J. Pathol.* 135:909–919.

Carrino, D.A., J.M. Sorrell, and A.I. Caplan. 1999. Dynamic expression of proteoglycans during chicken skeletal muscle development and maturation. *Poult. Sci.* 78:769–777.

Chao, D.S., J.R. Gorospe, J.E. Brenman, J.A. Rafael, M.F. Peters, S.C. Froehner, E.P. Hoffman, J.S. Chamberlain, and D.S. Bredt. 1996. Selective loss of sarcolemmal nitric oxide synthase in Becker muscular dystrophy. *J. Exp. Med.* 184:609–618.

Chou, F.L., C. Angelini, D. Daentl, C. Garcia, C. Greco, I. Hausmanowa-Petrusewicz, A. Fidzianska, H. Wessel, and E.P. Hoffman. 1999. Calpain III mutation analysis of a heterogeneous limb-girdle muscular dystrophy population. *Neurology.* 52:1015–1020.

Connolly, A.M., A. Pestronk, S. Mehta, and M. Al-Lozi. 1998. Primary alpha-sarcoglycan deficiency responsive to immunosuppression over three years. *Muscle Nerve.* 21:1549–1553.

Cooper, B.J., N.J. Winand, H. Stedman, B.A. Valentine, E.P. Hoffman, L.M. Kunkel, M.O. Scott, K.H. Fischbeck, J.N. Kornegay, R.J. Avery, et al. 1988. The homologue of the Duchenne locus is defective in X-linked muscular dystrophy of dogs. *Nature.* 334:154–156.

Cullen, M.J., J. Walsh, S.L. Roberds, and K.P. Campbell. 1996. Ultrastructural localization of adhalin, alpha-dystroglycan and merosin in normal and dystrophic muscle. *Neuropathol. Appl. Neurobiol.* 22:30–37.

Duclos, F., V. Straub, S.A. Moore, D.P. Venzke, R.F. Hrstka, R.H. Crosbie, M. Durbeej, C.S. Lebakken, A.J. Ettinger, J. van der Meulen, et al. 1998. Progressive muscular dystrophy in α-sarcoglycan–deficient mice. *J. Cell Biol.* 142:1461–1471.

Duggan, D.J., J.R. Gorospe, M. Fanin, E.P. Hoffman, and C. Angelini. 1997. Mutations in the sarcoglycan genes in patients with myopathy. *N. Engl. J. Med.* 336:618–624.

Durbeej, M., R.D. Cohn, R.F. Hrstka, S.A. Moore, V. Allamand, B.L. Davidson, R.A. Williamson, and K.P. Campbell. 2000. Disruption of the beta-sarcoglycan gene reveals pathogenetic complexity of limb-girdle muscular dystrophy type 2E. *Mol. Cell.* 5:141–151.

Ervasti, J.M., and K.P. Campbell. 1991. Membrane organization of the dystrophin–glycoprotein complex. *Cell.* 66:1121–1131.

Evanko, S.P., J.C. Angello, and T.N. Wight. 1999. Formation of hyaluronan- and versican-rich pericellular matrix is required for proliferation and migration of vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 19:1004–1013.

Fischer, U., S. Reinhardt, E.X. Albuquerque, and A. Maelicke. 1999. Expression of functional alpha7 nicotinic acetylcholine receptor during mammalian muscle development and denervation. *Eur. J. Neurosci.* 11:2856–2864.

Gannoun-Zaki, L., S. Fournier-Bidoz, G. Le Cam, C. Chambon, P. Millasseau, J.J. Leger, and C.A. Dechesne. 1995. Down-regulation of mitochondrial mRNAs in the mdx mouse model for Duchenne muscular dystrophy. *FEBS Lett.* 375:268–272.

Gaschen, F.P., E.P. Hoffman, J.R. Gorospe, E.W. Uhl, D.F. Senior, G.H.D. Cardinet, and L.K. Pearce. 1992. Dystrophin deficiency causes lethal muscle hypertrophy in cats. *J. Neurol. Sci.* 110:149–159.

Golub, T.R., D.K. Slonim, P. Tamayo, C. Huard, M. Gaasenbeek, J.P. Mesirov, H. Coller, M.L. Loh, J.R. Downing, M.A. Caligiuri, C.D. Bloomfield, and E.S. Lander. 1999. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science.* 286:531–537.

Gorospe, J.R., and E.P. Hoffman. 1992. Duchenne muscular dystrophy. *Curr. Opin. Rheumatol.* 4:794–800.

Gorospe, J.R., M.D. Tharp, J. Hinckley, J.N. Kornegay, and E.P. Hoffman. 1994a. A role for mast cells in the progression of Duchenne muscular dystrophy? Correlations in dystrophin-deficient humans, dogs, and mice. *J. Neurol. Sci.* 122:44–56.

Gorospe, R.J., M.D. Tharp, T. Demitsu, and E.P. Hoffman. 1994b. Dystrophin-deficient myofibers are vulnerable to mast cell granule-induced necrosis. *Neuromuscul. Disord.* 4:325–333.

Gorospe, R.J., B.K. Nishikawa, and E.P. Hoffman. 1996. Recruitment of mast cells to muscle after mild damage. *J. Neurol. Sci.* 135:10–17.

Grady, R.M., H. Zhou, J.M. Cunningham, M.D. Henry, K.P. Campbell, and J.R. Sanes. 2000. Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin–glycoprotein complex. *Neuron.* 25:279–293.

Granchelli, J.A., C. Pollina, M.S. Hudecki. 2000. Pre-clinical screening of drugs using the mdx mouse. *Neuromuscul. Disord.* 10:235–239.

Griggs, R.C., R.T.D. Moxley, J.R. Mendell, G.M. Fenichel, M.H. Brooke, A. Pestronk, J.P. Miller, V.A. Cwik, S. Pandya, J. Robison, et al. 1993. Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). *Neurology.* 43:520–527.

Hamed, S.A., J.R. Gorospe, and E.P. Hoffman. 2001. Complete sequence screening of the dystrophin cDNA in Duchenne dystrophy: the most common mutation of the most mutable gene is a novel recurring duplication of exon 2. *Hum. Mutat.* In press.

Hack, A.A., C.T. Ly, F. Jiang, C.J. Clendenin, K.S. Sigrist, R.L. Wollmann, and E.M. McNally. 1998. γ-Sarcoglycan deficiency leads to muscle membrane defects and apoptosis independent of dystrophin. *J. Cell Biol.* 142:1279–1287.

Hasegawa, M., A. Cuenda, M.G. Spillantini, G.M. Thomas, V. Buee-Scherrer, P. Cohen, and M. Goedert. 1999. Stress-activated protein kinase-3 interacts with the PDZ domain of alpha1-syntrophin. A mechanism for specific substrate recognition. *J. Biol. Chem.* 274:12626–12631.

Hayashi, Y.K., F.L. Chou, E. Engvall, M. Ogawa, C. Matsuda, S. Hirabayashi, K. Yokochi, B.L. Ziober, R.H. Kramer, S.J. Kaufman, et al. 1998. Mutations in the integrin alpha7 gene cause congenital myopathy. *Nat. Genet.* 19:94–97.

Hoffman, E.P., and L. Schwartz. 1991. Dystrophin and disease. *Mol. Aspects Med.* 12:175–194.

Hoffman, E.P., A.P. Monaco, C.C. Feener, and L.M. Kunkel. 1987a. Conservation of the Duchenne muscular dystrophy gene in mice and humans. *Science.* 238:347–350.

Hoffman, E.P., R.H. Brown, Jr., and L.M. Kunkel. 1987b. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. *Cell.* 51:919–928.

Ibraghimov-Beskrovnaya, O., J.M. Ervasti, C.J. Leveille, C.A. Slaughter, S.W. Sernett, and K.P. Campbell. 1992. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature.* 355:696–702.

Jelinsky, S.A., and L.D. Samson. 1999. Global response of *Saccharomyces cerevisiae* to an alkylating agent. *Proc. Natl. Acad. Sci. USA.* 96:1486–1491.

Jooss, K., Y. Yang, K.J. Fisher, and J.M. Wilson. 1998. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J. Virol.* 72:4212–4223.

Kaariainen, M., J. Kaariainen, T.L. Jarvinen, L. Nissinen, J. Heino, M. Jarvinen, and H. Kalimo. 2000. Integrin and dystrophin associated adhesion protein complexes during regeneration of shearing-type muscle injury. *Neuromuscul. Disord.* 10:121–132.

Koenig, M., A.P. Monaco, and L.M. Kunkel. 1988. The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. *Cell.* 53:219–226.

Kornegay, J.N., S.M. Tuler, D.M. Miller, and D.C. Levesque. 1988. Muscular dystrophy in a litter of golden retriever dogs. *Muscle Nerve.* 11:1056–1064.

Kuznetsov, A.V., K. Winkler, F.R. Wiedemann, P. von Bossanyi, K. Dietzmann, and W.S. Kunz. 1998. Impaired mitochondrial oxidative phosphorylation in skeletal muscle of the dystrophin-deficient mdx mouse. *Mol. Cell. Biochem.* 183:87–96.

Lane, T.F., and E.H. Sage. 1994. The biology of SPARC, a protein that modulates cell–matrix interactions. *FASEB J.* 8:163–173.

Lawler, J., K. McHenry, M. Duquette, and L. Derick. 1995. Characterization of human thrombospondin-4. *J. Biol. Chem.* 270:2809–2814.

Lockhart, D.J., H. Dong, M.C. Byrne, M.T. Follettie, M.V. Gallo, M.S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton, and E.L. Brown. 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* 14:1675–1680.

Matasic, R., A.B. Dietz, and S. Vuk-Pavlovic. 1999. Dexamethasone inhibits dendritic cell maturation by redirecting differentiation of a subset of cells. *J. Leukoc. Biol.* 66:909–914.

McNally, E.M., D. Duggan, J.R. Gorospe, C.G. Bonnemann, M. Fanin, E. Pegoraro, H.G. Lidov, S. Noguchi, E. Ozawa, R.S. Finkel, et al. 1996. Mutations that disrupt the carboxyl-terminus of gamma-sarcoglycan cause muscular dystrophy. *Hum. Mol. Genet.* 5:1841–1847.

Mizuno, Y., S. Noguchi, H. Yamamoto, M. Yoshida, I. Nonaka, S. Hirai, and E. Ozawa. 1995. Sarcoglycan complex is selectively lost in dystrophic hamster muscle. *Am. J. Pathol.* 146:530–536.

Naya, F.J., B. Mercer, J. Shelton, J.A. Richardson, R.S. Williams, and E.N. Olson. 2000. Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *J. Biol. Chem.* 275:4545–4548.

Newton, G., S. Weremowicz, C.C. Morton, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, and J. Lawler. 1999. The thrombospondin-4 gene. *Mamm. Genome.* 10:1010–1016.

Nicholson, L.V., K. Davison, M.A. Johnson, C.R. Slater, C. Young, S. Bhattacharya, D. Gardner-Medwin, and J.B. Harris. 1989. Dystrophin in skeletal muscle. II. Immunoreactivity in patients with Xp21 muscular dystrophy. *J. Neurol. Sci.* 94:137–146.

Nigro, V., E. de Sa Moreira, G. Piluso, M. Vainzof, A. Belsito, L. Politano, A.A. Puca, M.R. Passos-Bueno, and M. Zatz. 1996. Autosomal recessive limb-girdle muscular dystrophy, LGMD2F, is caused by a mutation in the delta-sarcoglycan gene. *Nat. Genet.* 14:195–198.

Noguchi, S., E.M. McNally, K. Ben Othmane, Y. Hagiwara, Y. Mizuno, M. Yoshida, H. Yamamoto, C.G. Bonnemann, E. Gussoni, P.H. Denton, et al. 1995. Mutations in the dystrophin-associated protein gamma-sarcoglycan in chromosome 13 muscular dystrophy. *Science.* 270:819–822.

Pimorady-Esfahani, A., M.D. Grounds, and P.G. McMenamin. 1997. Macrophages and dendritic cells in normal and regenerating murine skeletal muscle. *Muscle Nerve.* 20:158–166.

Roberds, S.L., R.D. Anderson, O. Ibraghimov-Beskrovnaya, and K.P. Campbell. 1993. Primary structure and muscle-specific expression of the 50-kDa dystrophin-associated glycoprotein (adhalin). *J. Biol. Chem.* 268:23739–23742.

Roddie, A., and S. Bundey. 1992. Racial distribution of Duchenne muscular dystrophy in the West Midlands region of Britain. *J. Med. Genet.* 29:555–557.

Rybakova, I.N., J.R. Patel, and J.M. Ervasti. 2000. The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. *J. Cell Biol.* 150:1209–1214.

Sueki, H., D. Whitaker, M. Buchsbaum, and G.F. Murphy. 1993. Novel interactions between dermal dendrocytes and mast cells in human skin. Implications for hemostasis and matrix repair. *Lab. Invest.* 69:160–172.

Tezak, Z., J. Devaney, M. Marino, E. Pegoraro, R. Finkel, and E.P. Hoffman. 2000. Missense mutations: expansion of the phenotype of laminin a2 deficiency. *Annual Meeting of the American Society of Human Genetics.* In press.

Thomas, G.D., M. Sander, K.S. Lau, P.L. Huang, J.T. Stull, and R.G. Victor. 1998. Impaired metabolic modulation of alpha-adrenergic vasoconstriction in dystrophin-deficient skeletal muscle. *Proc. Natl. Acad. Sci. USA.* 95:15090–15095.

Tinsley, J., N. Deconinck, R. Fisher, D. Kahn, S. Phelps, J.M. Gillis, and K. Davies. 1998. Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat. Med.* 4:1441–1444.

Toyofuko, T., J.R. Hoffman, R. Zak, and B.M. Carlson. 1992. Expression of α-cardiac and α-skeletal actin mRNAs in relation to innervation in regenerating and non-regenerating rat skeletal muscles. *Dev. Dyn.* 193:332–339.

Whalen, R.G., K. Schwartz, P. Bouveret, S.M. Sell, and F. Gros. 1979. Contractile protein isozymes in muscle development: identification of an embryonic form of myosin heavy chain. *Proc. Nat. Acad. Sci. USA.* 76:5197–5201.

Williams, W.R., M.W. Thompson, and N.E. Morton. 1983. Complex segregation analysis and computer-assisted genetic risk assessment for Duchenne muscular dystrophy. *Am. J. Med. Genet.* 14:315–333.

Yang, B., D. Jung, J.A. Rafael, J.S. Chamberlain, and K.P. Campbell. 1995. Identification of alpha-syntrophin binding to syntrophin triplet, dystrophin, and utrophin. *J. Biol. Chem.* 270:4975–4978.

Zaccaria, M.L., M.E. De Stefano, C. Gotti, T.C. Petrucci, and P. Paggi. 2000. Selective reduction in the nicotinic acetylcholine receptor and dystroglycan at the postsynaptic apparatus of mdx mouse superior cervical ganglion. *J. Neuropath. Exp. Neurol.* 59:103–112.

Zhang, Y., L. Cao, C. Kiani, B.L. Yang, W. Hu, and B.B. Yang. 1999. Promotion of chondrocyte proliferation by versican mediated by G1 domain and EGF-like motifs. *J. Cell. Biochem.* 73:445–457.