Temporal gene expression profiling of dystrophin-deficient (mdx) mouse diaphragm identifies conserved and muscle group-specific mechanisms in the pathogenesis of muscular dystrophy

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Mutations in dystrophin are the proximate cause of Duchenne muscular dystrophy (DMD), but pathogenic mechanisms linking the absence of dystrophin from the sarcolemma to myofiber necrosis are not fully known. The muscular dystrophies also have properties not accounted for by current disease models, including the temporal delay to disease onset, broad species differences in severity, and diversity of skeletal muscle responses. To address the mechanisms underlying the differential targeting of muscular dystrophy, we characterized temporal expression profiles of the diaphragm in dystrophin-deficient (mdx) mice between postnatal days 7 and 112 using oligonucleotide microarrays and contrasted these data with published hindlimb muscle data. Although the diaphragm and hindlimb muscle groups differ in severity of response to dystrophin deficiency, and exhibited substantial divergence in some transcript categories including inflammation and muscle-specific genes, our data show that the general mechanisms operative in muscular dystrophy are highly conserved. The two muscle groups principally differed in expression levels of differentially regulated genes, as opposed to the non-conserved induced/repressed transcripts defining fundamentally distinct mechanisms. We also identified a postnatal divergence of the two wild-type muscle group expression profiles that temporally correlated with the onset and progression of the dystrophic process. These findings support the hypothesis that conserved disease mechanisms interacting with baseline differences in muscle group-specific transcriptomes underlie their differential responses to DMD. We further suggest that muscle group-specific transcriptional profiles contribute toward the muscle targeting and sparing patterns observed for a variety of metabolic and neuromuscular diseases.

INTRODUCTION

Skeletal muscles are not created equal. While some craniofacial muscles are adapted to novel functional roles (1,2), these specialized groups are often viewed as exceptions to an otherwise highly stereotyped muscle phenotype. Existing concepts of muscle role specificity contend that the percentage content of four conserved muscle fiber types (I, IIA, IIX/D and IIB) is paramount as a functional determinant. However, the broad adaptations of role-specific muscle groups, such as extraocular and masticatory, provide compelling evidence that muscle specialization is not as simple as variance in content of conserved fiber types. Recent gene expression profiling studies support this view by establishing that specializations in muscle

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group-specific transcriptomes extend beyond traits traditionally associated with fiber type contraction speed and fatigability and involve more than the craniofacial muscles (3–8).

The oversimplification that skeletal muscle is generic is an impediment to recognition of muscle group-specific traits as factors in disease. Different muscle groups do not respond in the same ways to metabolic and neuromuscular disease (9). For example, most myopathies have an unexplained predilection for proximal muscles, while rare distal myopathies have distinctive patterns of muscle targeting (e.g. hand or leg onset and anterior or posterior compartment bias). Examples of restricted disease patterns also are found among the limb girdle muscular dystrophies and metabolic myopathies. Although differential targeting in neuromuscular disease is well documented, little is known of the mechanisms underlying muscle group-specific responses.

The muscular dystrophies have properties not accounted for by current disease models, including the temporal delay from birth to disease onset, the broad species differences in severity, and the diversity of skeletal muscle responses. Both muscle fiber type- and group-specificity have been reported for Duchenne muscular dystrophy (DMD) and its animal models. Fast-twitch glycolytic (IIB) fibers exhibit heightened sensitivity to the primary dystrophin deficiency (10), but, by contrast, dystrophin appears to function in an oxidative fiber-type signaling cascade (11). In the mdx mouse model of DMD, skeletal muscles exhibit a heterogeneous response to muscular dystrophy: extraocular muscles are completely spared, hindlimb muscles undergo a progressive dystrophic process throughout the mdx lifespan, and the diaphragm shows more severe pathology at all stages (12–24). Although extraocular muscle sparing provides support for the concept that phenotypic differences play a key role in disease (25), the mechanisms responsible for the diverse muscle group response in muscular dystrophy are only partially understood. Fiber type content alone does not fully explain muscle targeting patterns and the phenotypic range seen in models of DMD.

To address the mechanisms underlying the differential targeting of muscular dystrophy, we have characterized temporal expression profiles of the diaphragm in dystrophin-deficient (mdx) and wild-type mice using oligonucleotide microarrays and contrasted these data with our published patterns from hindlimb muscle (26). These findings represent an important first step toward explaining the heterogeneity of muscle group responses to a variety of neuromuscular diseases. Specifically, characterization of conserved and muscle group-specific disease mechanisms will help refine a consensus model of the pathogenesis of muscular dystrophy.

RESULTS
Our objectives were to characterize the transcriptional expression signature of mdx diaphragm versus wild-type diaphragm and to identify both consensus and skeletal muscle group-specific profiles that may be responsible for muscular dystrophy phenotypes. To this end, diaphragm microarray data compiled here were compared with our prior findings from hindlimb muscles of dystrophin-deficient mice (26).

Comparison of temporal expression patterns of mdx diaphragm and hindlimb muscle
Quality control measures were satisfactory for all arrays: the percent of probes detected as present/array [44.57 ± 6.38 (SD) overall; 44.75 ± 6.34 and 44.39 ± 6.59 for wild-type and mdx arrays, respectively] and 3′/5′ signal ratios for β-actin and GAPDH (<2 for all arrays) were within normal limits. A total of 702 transcripts met stringent selection criteria (consistent increase/decrease call in three of three replicates in pairwise comparisons of mdx versus wild-type; see Methods) for differential regulation in mdx diaphragm versus wild-type diaphragm between P7 and P112 (Supplementary Table 1). This was nearly identical to our prior report for hindlimb (n = 719) (26). Both muscle groups exhibited an age-dependent increase in the number of differentially expressed genes until a peak at P56 and then a decline (Fig. 1A and B). Except at P7, up-regulated transcripts exceeded those down-regulated for both muscles. P7 was a unique time point in disease progression; many transcripts repressed in the mdx P7 diaphragm were later either unchanged or up-regulated relative to age-matched controls (e.g. Spp1, Caenog1 and Kche1l, see Supplementary Table 1). P56 diaphragm also was unique in having nearly equal numbers of up-regulated and down-regulated transcripts [the expressed sequence tag (EST), metabolism, and other transcript categories contained 86% of the down-regulated transcripts; see Supplementary Table 1 and below]. Hierarchical cluster analysis showed a progressive pattern among induced and repressed transcripts in diaphragm and hindlimb muscle (averaged mdx/wild-type expression ratios are shown in Fig. 1C and D; individual replicate ratios are shown in Supplementary Fig. 1). By contrast, only 50% of transcripts were shared by mdx diaphragm and hindlimb (Fig. 1E). Conserved patterns are putative consensus disease mechanisms, while muscle group-specific patterns may explain phenotypic divergence.

Aggregate disease load index (DLI) analysis of mdx skeletal muscle
Since up- and down-regulated transcripts contribute to disease, we used the aggregate DLI (sum of fold change absolute values for all differentially regulated transcripts at each age) as an indicator of disease progression [see Materials and Methods and Porter et al. (26)]. The diaphragm aggregate DLI showed a difference between mdx and wild-type at P7, then decreased in value by P14, exhibited a non-linear rise to a peak at P56, and then a slight decline between P56 and P112 (Fig. 2A). The diaphragm aggregate DLI plot was similar to that of hindlimb.

Functional classification of differentially expressed transcripts
Approximately 79% of all transcripts detected as differentially expressed in mdx diaphragm with the MG-U74Av2 arrays were known genes (Supplementary Table 1). After functional annotation, including replacement of Affymetrix-supplied annotations by official gene nomenclature, the majority of known genes (63.5%) were classified into nine categories relevant to muscular dystrophy (Supplementary Table 1 and
altered metabolism (Fig. 2B). Some known genes (n = 202) could not be classified as dystrophy-related based upon knowledge of their functions or of the pathogenesis of DMD, and 149 were ESTs. Compared with age-matched wild-type, the mdx diaphragm exhibited altered expression of transcripts for virtually all adaptive and/or pathogenic mechanisms that have been linked to muscular dystrophy (Fig. 2B), including apoptosis (n = 14), cytoskeletal adaptations (n = 29), calcium homeostasis (n = 16), extracellular matrix (ECM) biology (n = 39), inflammation (n = 122), muscle regeneration (n = 54), proteolysis (n = 37), reactive oxygen species or antioxidant mechanisms (n = 9) and altered metabolism (n = 76). Most of the individual functional categories contained similar numbers of transcripts in diaphragm and hindlimb. Inflammation was the most prominent functional category, and also differed the most (hindlimb was 34% greater than diaphragm), for the two muscles.

Dissecting disease mechanisms by functional category-specific DLIs

To visualize interactive mechanisms in mdx versus wild-type muscle and to contrast the diaphragm and hindlimb data (26), we generated DLI plots for specific functional categories (Fig. 3). Sub-classification of transcripts beyond these nine major categories allowed further dissection of disease processes (see Supplementary Figs 2 and 3). The DLI plots fit two patterns. Group I exhibited a tight correlation of diaphragm and hindlimb throughout the P7 to P112 time frame (ECM, Ca\(^{2+}\) homeostasis, metabolism, cytoskeleton and ROS/antioxidant categories). Group II showed initial correlation of DLIs for P7 to P23, but then divergence (inflammation, muscle-specific, proteolysis/lysosomal, and apoptosis categories).

Inflammation is thought to participate in the pathogenesis of muscular dystrophy (see 27) and is a major component of the transcriptional response of mdx hindlimb (26,28). The overall inflammation DLI plots (Fig. 3A) for the two muscle groups was nearly identical at early ages, but the diaphragm did not follow the substantial increase in hindlimb after P23. At peak DLI value for hindlimb (P56), the diaphragm inflammation index was 75% less. Cytokine-specific DLI plots were similar for both muscles, although this index peaked and dropped sooner for diaphragm (Supplementary Fig. 2A). However, diaphragm DLI plots for other inflammation subcategories, including vascular response (i.e. adhesion, signaling and diapedesis), chemokines and chemokine signaling (i.e. pro-inflammatory chemotaxis), and lymphoid and myeloid cell markers (i.e. attributable to inflammatory cell types), sharply diverged from hindlimb after P23 (Supplementary Fig. 2B–D). Likewise, markers for key components of the inflammatory infiltrate, macrophages, T-cells and mast cells, showed temporal kinetics and muscle group differences similar to the overall inflammation DLI (Supplementary Fig. 2E–G). After P23, hindlimb DLI values were 2- to 7-fold higher than diaphragm for major histocompatibility complex and complement categories.

Fibroblast proliferation and ECM deposition are thought to contribute to the pathogenesis of muscular dystrophy by gradually decrementing vascular perfusion, myoblast/stem cell mobility and, consequently, the regenerative capacity of dystrophic muscle. Although most mdx skeletal muscles are more mildly affected than in DMD patients, fibrosis in the mdx diaphragm approximates DMD (20,21). Thus, the degree of fibrosis may be the underlying reason for muscle group and species phenotypic differences. The overall ECM DLI plots of the diaphragm and hindlimb were very similar (Fig. 3B). However, individual ECM subcategory DLIs behaved very differently from the composite index. While the two muscles exhibited parallel DLI plots for a key component of fibrosis, collagen synthesis, these were oppositely directed (rising for diaphragm and falling for hindlimb) by P112 (Supplementary Fig. 3A). Up-regulation of fibril-forming collagen genes (e.g. Coll1a1, Colla2 and Col3a1) was the principal component of the collagen DLI. The ECM signaling/processing DLI plot
(ECM signal transduction, synthesis, and modification, e.g. $S_{pp1}$, $B_{mp1}$, $F_{ap}$, $L_{ox}$, $M_{mp12}$ and $M_{mp14}$) of diaphragm remained at a steady level after P23, while that of hindlimb first exceeded and then dropped below the diaphragm value by P112 (Supplementary Fig. 3B). ECM glycoprotein and proteoglycan DLIs of diaphragm and hindlimb were similar up to P28, showed divergence at P56 (diaphragm 2-fold $>$ hindlimb, and converged by P112 (Supplementary Fig. 3C). Finally, the DLIs for a heterogeneous group of ECM transcripts, designated ECM-other, showed very different values at P7, but then were similar throughout the time course studied here (Supplementary Fig. 3D).

Dystrophic skeletal muscles recapitulate muscle development as activated satellite and stem cells replace dystrophic muscle fibers. The DLIs for muscle-related transcripts showed parallel increases in both muscles to P23, but then index values diverged, with a decline in hindlimb and a continued rise in diaphragm (Fig. 3C). The DLI plots for several other key contributors to muscular dystrophy, including $C_{a^{2+}}$ homeostasis (Fig. 3D), altered metabolism (Fig. 3E), cytoskeletal adaptation (Fig. 3F), and reactive oxygen species/antioxidant mechanisms (Fig. 3H) were very similar for the two muscle groups. The observed early disease stage muscle group similarities and subsequent divergence of the proteolysis/lysosomal (Fig. 3G) and apoptosis (Fig. 3I) DLIs may be mechanistically linked to the overall inflammation DLIs of the two muscle groups, which showed the same temporal patterns.

DMD skeletal muscles exhibit a general metabolic crisis that has been attributed to mitochondrial dysfunction (29). While the metabolism DLI plots were generally similar for diaphragm and hindlimb, the DLI value for diaphragm at P56 was ~50% higher. The elevated diaphragm metabolism DLI at P56 was attributable to transcript down-regulation (of 54 metabolism transcripts differentially expressed in diaphragm at P56, 95% were down-regulated). Down-regulation of transcripts for glycolysis ($F_{bp2}$, $H_k2$, $P_{ck1}$, $P_{fb1}$, $P_{gam2}$ and $S_{lc2a4}$) and glycogen ($G_{be1}$, $G_{ys1}$, $P_{hkg}$ and $U_{gp2}$) metabolism, particularly $\beta$-oxidation of fatty acids ($A_{cadl}$, $A_{cadm}$, $C_{pt2}$, $D_{ecr1}$, $D_{ch1}$, $E_{ch3}$, $E_{tf8}$ and $S_{lc2a5}$) and energy metabolism ($A_{tp5j}$, $C_{ox7a1}$, $C_{ox8b}$, $C_{ycs}$, $N_{dufa6}$ and $N_{dufs4}$) was prominent. This decrease in metabolism was not as severe at other ages in diaphragm or at any age in hindlimb (26).

Conserved gene expression patterns in diaphragm and hindlimb muscles

Comparison of expression profiles of muscle groups that are differentially responsive to dystrophinopathy may yield insight into core mechanisms that are necessary and sufficient for muscle pathology. Of the transcripts differentially regulated in $mdx$ diaphragm and hindlimb, between 7 and 32% were shared for any single age (Supplementary Fig. 4) and there was ~50% overlap (Fig. 1E) when all ages studied here were combined (diaphragm data served as baseline). We next determined the functional distribution of differentially expressed transcripts that were either shared by or specific to diaphragm and hindlimb muscle (Fig. 4A). Shared transcripts exceeded muscle group-restricted transcripts for all functional categories except metabolism. The DLI analyses shown in Figure 2A for $>700$ transcripts were repeated for the subset of 361 transcripts that was shared by $mdx$ diaphragm and hindlimb. The resulting aggregate and functional class-specific shared DLI plots were very similar to the all-transcript DLI plots (compare Figs 4B and 5 with Figs 2A and 3).

Transcriptional differences between wild-type diaphragm and hindlimb muscle

Baseline transcriptional differences in wild-type diaphragm and hindlimb muscles underlie their novel phenotypes, but also may explain their differential pathogenesis in $mdx$ mice. Pairwise comparisons (consistent increase/decrease call in three of three replicates and absolute value of average fold difference $\geq 2$) of these two muscles in wild-type mice, at
six ages between P7 and P112, identified 726 transcripts (Supplementary Table 2). The number of transcripts was age-dependent, reaching maximum value by P28 to P56 (Fig. 6A). Diaphragm has a higher slow-twitch fiber content than the pooled gastronemius/soleus, so some expression differences were a consequence of fiber type content (slow fiber genes with diaphragm $>$ hindlimb: Atp2a2, Myh7, Myle, Ppp3ca, Tnt1, Tncc, Tnn1 and Tpm3; fast fiber genes with diaphragm $<$ hindlimb: Myom2, Mylpf, and Pva). Fiber type-specific traits represented a very small percentage of total transcripts, so diaphragm and hindlimb muscle differ in ways beyond those attributable to fiber type content. Pairwise comparison of mdx diaphragm with mdx hindlimb muscle showed a similar pattern to that of the wild-type muscles (Fig. 6B).
Given the broad scope of the constitutive diaphragm-hindlimb muscle divergence, we used a data reduction strategy to identify candidate genes linking baseline muscle group traits to the response to dystrophinopathy. Our rationale was that baseline differences that were enhanced or suppressed by disease in only one of the muscle groups might be responsible for muscle group-specific disease patterns in mdx mice. We intersected (Fig. 6C) the 726 transcripts that were differentially expressed in wild-type diaphragm versus wild-type hindlimb with those that were altered in mdx diaphragm (n = 702; Fig. 1C and Supplementary Table 1) and mdx hindlimb (n = 719; Fig. 1D and Porter et al. (26)). We focused upon two groups (Fig. 6C). Group I included those genes that were altered in the mdx diaphragm and also differentially expressed in wild-type diaphragm versus wild-type hindlimb (n = 84). Group II included those genes that were altered in the mdx hindlimb and also differentially expressed in wild-type diaphragm versus wild-type hindlimb (n = 98) (Supplementary Table 3).

Of the 84 transcripts in Group I, 63 were known genes. Several myogenesis-related transcripts (Smad1, Csrp3, Fgf1 and Gdf8) were differentially expressed in both the wild-type muscle group comparison and in the mdx diaphragm. Smad1, Csrp3 and Fgf1 are positive regulators of myogenesis that were more highly expressed in wild-type diaphragm, while Gdf8 is a negative regulator and was expressed at lower levels in wild-type diaphragm. Smad1, Fgf1 and Gdf8 were down-regulated, and Csrp3 up-regulated, in the mdx diaphragm. The patterns in Csrp3 and Gdf8 are consistent with enhanced myogenesis in dystrophinopathy (cf. Fig. 3C). While the Smad1 and Fgf1 patterns in mdx are not consistent with this view, the up-regulation of Fgf1 may have another explanation. Genes related to development and maintenance of vasculature showed the same pattern—Tie1, Vegfb, Vwf and Fgf1 were constitutively higher in wild-type diaphragm and were down-regulated at one or more age in mdx diaphragm. The patterned regulation of these transcripts may support a more substantial microvascular bed in wild-type diaphragm that is compromised by muscular dystrophy.

Of the 98 transcripts in Group II, 81 were known genes. Nearly 20% of these transcripts function in the inflammatory response; 14 of these were both constitutively higher in wild-type diaphragm and up-regulated in mdx hindlimb. This group included several major histocompatibility complex (MHC) and MHC-associated (B2m, H2-D1, H2-K, H2-Q2 and Tapbp) and cytokine-associated (Cebpd, Crip1, Il10rb and Osmr) transcripts. MHC does not appear to be expressed by normal myofibers (30), but is expressed by resident dendritic cells in normal skeletal muscle (31). Dendritic cells are recruited in DMD (29). Our finding of expression of multiple MHC class II transcripts in wild-type diaphragm probably reflects a higher resident population of antigen-presenting cells in this muscle which may condition the severity of its response in dystrophinopathy.

**Verification of differentially regulated transcripts by qPCR**

We tested a subset of transcripts that were differentially expressed in mdx diaphragm using qPCR. The same wild-type and mdx diaphragm samples, between the ages of P7 and P112, used for microarray were used here. These data confirm the temporal trends in the microarray data and are shown in Supplementary Table 4.

**DISCUSSION**

Mutations in dystrophin are the proximate cause of DMD (32), but pathogenic mechanisms linking its absence from the sarcolemma to myofiber necrosis are not fully known. The heterogeneous nature of DMD, including its latency from birth...
to symptom onset, novel sparing of select muscles, and species differences in severity, suggests that dystrophin deficiency alone is insufficient to cause muscular dystrophy (12–19,33). The exceptions to an otherwise stereotypical disease process are invaluable in distinguishing conserved (i.e. those necessary and sufficient for disease) from muscle group-specific mechanisms and, thereby, refining pathogenic models.

Muscular dystrophy invokes coordinated responses from the heterogeneous cell types that comprise skeletal muscle plus cells recruited from other sources. Expression profiling offers the potential to understand the dynamic nature of the disease, including the asynchronous interactions of cell autonomous and non-cell autonomous mechanisms critical to muscle cell fates in dystrophin deficiency. Here, we used DNA microarray

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**Figure 5.** Functional category-specific DLI plots for transcripts shared by dystrophic diaphragm and hindlimb muscles. (A–E) DLI plots were constructed for specific functional categories relevant to the pathogenesis of muscular dystrophy using only those transcripts common (black bars in Fig. 4A) to both diaphragm and hindlimb muscle (‘shared transcript’ DLI). DLI plots illustrate similarities and differences in temporal expression patterns of those genes that were differentially expressed in both diaphragm and hindlimb muscle of mdx mice. Note similarity of temporal profiles to the all-transcript plots in Figure 3.
to functionally dissect temporal expression patterns of dystrophic versus wild-type diaphragm, the mdx skeletal muscle and hindlimb muscle with a phenotype closest to that of DMD patients (20,21). Our data analysis strategy was to characterize the behavior of functional gene categories using DLI. This approach identified fundamental trends, while minimizing the noise introduced by variable regulation of individual transcripts. Comparisons of diaphragm with prior hindlimb muscle data (26) identified patterns common to both muscle groups, those most likely to represent consensus disease mechanisms, and muscle group-specific patterns.

**Basic muscle patterns in the pathogenesis of muscular dystrophy**

The molecular signature of the diaphragm was consistent with reported mdx skeletal muscle histology and mechanisms already implicated in the pathogenesis of muscular dystrophy (34,35). The numbers of differentially regulated transcripts (~700/muscle), general distribution among functional categories, and aggregate DLI plots were similar for diaphragm and hindlimb. However, there was only modest overlap (50%, when all ages were combined) of differentially regulated transcript identities in the two muscle groups, consistent with the hypothesis of their divergence in mechanisms of and/or downstream responses to muscular dystrophy.

Although the up-regulation of transcripts was the predominant pattern in mdx diaphragm and hindlimb, and is a general feature of dystrophinopathy (26,28,36), both groups had an unusual, transient phase when most differentially regulated transcripts were down-regulated (at P7, 37/38 in diaphragm and 19/28 in hindlimb were down-regulated). Of the repressed transcripts in P7 diaphragm, 27% were muscle-specific/enriched genes (Acta3, Actc1, Cacng1, Car3, Csrp3, Gamt, Kcnel1, Mybph, Pdlm3 and Pva). We speculate that this pattern might reflect neonatally compromised dystrophin or dystrophin-glycoprotein complex signaling functions (11,37,38). Whether this also occurs in pre-necrotic DMD muscle, and what role, if any, this pattern plays in pre-conditioning the pathogenesis of dystrophin-deficient skeletal muscles, are unknown.

Phenotypes are not identical for all mdx skeletal muscles; while pathology is life-long in most muscles, diaphragm exhibits a more severe phenotype at all ages (20–24). A data reduction strategy, DLI analysis, was employed here to compare DNA microarray profiles of the two muscle groups by functional transcript category. We observed a remarkable concordance in functional gene category patterns for diaphragm and hindlimb up to P23, supporting the concept that pre-necrotic and early necrotic mechanisms triggering muscular dystrophy are highly conserved. This finding is entirely consistent with morphopathologic evidence that the initial lesions in diaphragm do not appear earlier nor are they more severe than those of other mdx skeletal muscles (20). However, DLIs for some key functional categories diverged after P23, suggesting that the mechanisms that help shape differential muscle group fates appear after the early stages of muscular dystrophy.

**Conserved and muscle group-specific patterns in muscular dystrophy**

While regeneration is a conserved feature of dystrophic skeletal muscle, diaphragm and hindlimb differed in the magnitude and dynamics of their response. The muscle-specific category DLI of diaphragm exceeded that of hindlimb muscle after P23 and was at peak value and on an upward trajectory at P112, as the hindlimb DLI is declining. These data probably reflect the partial stabilization of dystrophy in hindlimb, but not diaphragm (20,21). The shared transcript DLI plots (Fig. 5C) for the muscle-specific gene category were nearly identical to the all-transcript DLI plots (Fig. 3C), suggesting that genes altered in only one of the two muscles do not define fundamentally different mechanisms. Indeed, up-regulation of muscle development/regeneration transcripts (e.g. Chrna1, Crap, Mybph, Myh3, Myh8, Myla, Myod1, Myog and Tnnt2) was a conserved feature, but fold change values were generally higher for diaphragm. Some muscle development-related
transcripts were differentially expressed only in diaphragm (e.g. Chrnb1, Ncam1, Myf5 and Pdlim3) or hindlimb (e.g. H19, Siat8d and Tgfb1). The comparatively high myogenic activity in mdx diaphragm for the ages studied here may, in part, explain the subsequent depletion of regenerative capacity in this muscle in mice ≥16 months old (20,21). For this transcript category, the mdx mouse appears to be a good model of DMD, as several muscle regeneration-related transcripts seen here also were differentially expressed in DMD patient biopsies (29,36,39–41).

An energy crisis in DMD skeletal muscle is thought to result from Ca\textsuperscript{2+} overload and consequent mitochondrial dysfunction (29). Although metabolic alterations are considered a less significant feature of mdx skeletal muscle (26,42–44), decreased glucose and fatty acid breakdown, and alterations in gene transcription for these pathways, have been reported (45,46). Based upon our DLI analyses, it is likely that the inconsistent reports of metabolic alterations in DMD and mdx skeletal muscles (29,36,39,41–44) are a consequence of disease stage sampled. Here, we observed transcriptional evidence of a severe metabolic crisis in mdx diaphragm. Hindlimb did not exhibit as severe a response at any age (Fig. 3E and Porter et al., 26). In addition to the down-regulation of numerous mitochondrial transcriptional genes, gene transcription data suggest that glycogen synthesis, glycolysis and fatty acid β-oxidation are severely impaired in mdx diaphragm. The substantial reduction of the metabolic changes in diaphragm by P112 is consistent with a prior report (46). We suggest that the energy crisis is a conserved feature of DMD and mdx skeletal muscle, but there is a defined temporal window, late in the dystrophic process, when metabolism transcripts are affected.

Fibrosis compromises muscle regeneration and the more severe phenotypes shared by the mdx diaphragm and DMD muscles are thought to be a consequence of the level of connective tissue deposition (20,21). Consistent with this view, many ECM transcripts reported here for diaphragm were also identified by expression profiling of DMD muscle biopsies (29,36,39–41). By contrast, ECM DLI plots were very similar for diaphragm and hindlimb muscle groups. Taken together with the observed similarities of the ECM all-transcript and shared DLI plots (compare Fig. 3B with Fig. 5B), our microarray data suggest that, at least at the transcriptional level, fibrosis is very similar in these two muscle groups. These data are consistent with the view that disease processes in mdx hindlimb and diaphragm both may more closely resemble those in DMD patients. One ECM transcript pattern does, however, support a partial divergence in hindlimb and diaphragm: while differentially regulated collagen transcript identities were highly conserved among the two muscle groups, there was a trend of collagen transcription increasing in diaphragm and decreasing in hindlimb by P112.

Inflammation is a recognized feature of muscular dystrophy and the inflammatory response reported here was consistent with prior studies (26,28,42–44,46,47). The overall inflammation DLI, and its subcategory DLIs, were tightly correlated for diaphragm and hindlimb between P7 and P23, but then diverged as diaphragm values leveled off while hindlimb continued to increase. Yet, the all-transcript DLI plots were very similar to the shared transcript DLIs (compare Figs 3A and 5A), suggestive of conserved mechanisms in the two muscles that primarily differ in degree.

The chemokine category is representative of the muscle group differences in the magnitude, but not particularly the nature, of inflammation. Chemokines are pro-inflammatory chemoattractants, initially produced in dystrophic muscle by resident inflammatory cells and muscle fibers proper (47), that recruit circulating mononuclear cells. At peak, the hindlimb chemokine index was ~7-fold higher than diaphragm. Although 55% of chemokines induced in diaphragm were conserved (Ccl2, Ccl7, Ccl9, Ccr2, Ccr5 and Cmkr4), they differed in level (e.g. Ccl7 induced by 18- and 5-fold in hindlimb and diaphragm, respectively). These data are consistent with prior reports that CC class chemokines, which generally have specificity for monocytes and T-cells, predominate in dystrophic muscle (47,48); three CXC class chemokines (Cxcl9 and Cxcl13) were induced in dystrophic diaphragm only, while two CC class (Ccl6 and Ccl8) and one CXC class (Cxcl14) chemokine were induced in hindlimb only. Lower chemokine expression translates into a modest inflammatory infiltrate, a prediction consistent with histologic and microarray (i.e. lower myeloid/lymphoid marker DLI in diaphragm; Supplementary Fig. 2D) data for the two muscles.

Taken together, the level of inflammatory response was very different in diaphragm and hindlimb. Interpretation of these differences is complex, since inflammation plays both positive and negative roles in dystrophic skeletal muscle (49–53). Moreover, pro-inflammatory cytokines may even have distinct functions in hindlimb and diaphragm: tumor necrosis factor deficiency on an mdx background decreased pathology in quadriceps, but had the opposite effect on diaphragm (54). By transcript level, macrophages are the most abundant inflammatory cell in dystrophic muscle (present data and 26,28) and are essential for muscle regeneration (50,55,56). This may explain why the greater inflammatory load in mdx hindlimb (26,28) does not translate into more severe pathology than in the diaphragm. DMD biopsies and mdx diaphragm share a more modest level of pro-inflammatory transcript induction (present data and 29,36,39). Thus, inflammation transcript induction may have a positive effect upon regeneration, and, in its absence, muscles may become severely dystrophic. Understanding the dynamic balance between the recruited cell types and mechanisms that are positive regulators of damage/repair will be essential to modeling the role of inflammation in muscular dystrophy.

Baseline transcriptional differences between wild-type muscle groups may be a determinant of muscular dystrophy phenotypes

Skeletal muscle groups may be more different from one another than previously appreciated (3,5,7). The emergence of group-specific transcriptomes during wild-type muscle development may interact with developmental-based disease processes to affect muscular dystrophy phenotypes. Here, we identified 726 differentially expressed transcripts in age-matched comparisons of wild-type diaphragm and hindlimb muscle, only a modest number of which could be attributed to known fiber type differences between these two muscles. The diaphragm versus hindlimb difference is much more significant than that between
predominately white (quadriceps) and predominately red (soleus) skeletal muscles (8). A similar transcriptional divergence of adult extraocular and hindlimb muscles may underlie their differences in energy metabolism and disease susceptibility (4,5,7,25,57–59). Thus, the constitutive gene expression profiles of adult diaphragm and hindlimb muscle differ broadly and, during development, diverge concurrent with the appearance of the morphopathologic signs of muscular dystrophy (Fig. 6). These data suggest that normative muscle group differences emerging during development are additive with the progression of a developmental disease in producing the diverse mdx phenotypes.

Among the constitutive muscle group differences, we identified differential expression of several myogenesis-related transcripts in adult wild-type diaphragm versus hindlimb muscle (diaphragm > hindlimb: Smad1, Csp3 and Fgf1; hindlimb > diaphragm: Gdf8). Diaphragm is also known to have a higher satellite cell content than fast-twitch muscles (60,61). Together, these data support the concept that ongoing repair processes in wild-type diaphragm offset any deleterious consequences of its continuous activity. A similar process may function in the highly active extraocular muscles (62). Inhibition of the negative regulator of myogenesis, Gdf8 (myostatin), functionally improves dystrophic muscle (63), including reduction of fibrosis and fat accumulation in diaphragm (64). The constitutively low Gdf8 in wild-type diaphragm, and its further reduction in dystrophy, may augment regeneration in diaphragm until later ages when it becomes highly fibrotic and dystrophic (20,21). The muscle-specific and ECM gene expression patterns seen here are consistent with this interpretation.

Toward a consensus model of the pathogenesis of muscular dystrophy

The heterogeneity of muscle group and species responses to dystrophinopathy offers a window into the core mechanisms of DMD. Some processes common to mdx mice and DMD patients represent stereotypical muscle degeneration/regeneration, and thus are similar to those from traumatic/cytotoxic insult models (51,52,65). Through the use of DNA microarray and a novel data analysis strategy, we have shown that the temporal patterns of many functional gene categories relevant to muscular dystrophy are highly conserved in mdx diaphragm and hindlimb. Our data confirm the operation of several mechanisms previously implicated in muscular dystrophy, but also highlight unexpected areas of muscle group divergence (Fig. 7). The comparisons of all-transcript and shared transcript DLIs suggest that the diverse responses of diaphragm and hindlimb muscles to muscular dystrophy are primarily the result of the level, rather than the nature, of differentially expressed transcripts. These data support the hypothesis that muscular dystrophy is mediated by spatially and temporally conserved mechanisms. Future studies can better resolve these interactions by experimentally blocking one or more processes and globally assessing the consequences for the pathogenesis of muscular dystrophy.

DMD biopsies and the mdx diaphragm appear to share the properties of low inflammation, moderate metabolic arrest, and the ultimate failure of muscle regeneration, traits not found in mdx hindlimb muscle. A recent study in mdx mice supports the notion that the level of inflammation may shift the balance toward either myofiber damage or repair (38). Both mdx diaphragm and DMD patient muscles exhibit a lower level of pro-inflammatory transcript induction than that of mdx hindlimb (present data and 26,28,29,36,39). Thus, inflammation-mediated repair may be low in the most severely affected muscles, and ultimately this may compromise regeneration.

Finally, we propose that the interaction of conserved disease mechanisms, initiated by the absence of dystrophin, with the diverse gene expression profiles of developing muscle groups is an essential determinant of the response to dystrophinopathy. Put another way, skeletal muscle is not stereotyped, but rather exhibits a broad range of normative expression profiles that will condition all muscle group responses to disease. Skeletal muscle transcriptomes may function to either elevate (diaphragm) or suppress (extraocular) muscle group sensitivity to dystrophinopathy. Knowledge of the precise nature of the differences between normal skeletal muscles is essential to understanding of muscle group/species specificity in DMD and a variety of other neuromuscular diseases.

MATERIALS AND METHODS

Animals

Male wild-type (C57BL/10SnJ) and mdx (C57BL/10ScSn-DmdmdxJ) mice (breeding pairs originally from Jackson Laboratory, Bar Harbor, ME, USA) were killed by asphyxiation with carbon dioxide at postnatal day (P) 7, P14, P23, P28, P56 and P112. Costal portions of diaphragms (excluding central tendon) were dissected bilaterally (five mice/replicate) and pooled for each of three independent replicate DNA microarray analyses per time point and strain. Samples were flash-frozen and stored at −80°C.

DNA microarray

DNA microarray methods were described previously (5,7,26,28). Briefly, total RNA was extracted from diaphragms using TRIzol reagent (GibcoBRL, Rockville, MD, USA). RNA pellets were resuspended at 1 µg RNA/µl DEPC-treated water and 8 µg was used in a reverse transcription reaction (SuperScript II; Life Technologies, Rockville, MD, USA) to generate first strand cDNA. Double strand cDNA was synthesized and used in an in vitro transcription (IVT) reaction to generate biotinylated cRNA. Fragmented cRNA (15 µg) was used in a 300 µl hybridization cocktail containing herring sperm DNA and BSA as carrier molecules, spiked IVT controls, and buffering agents. A 200 µl aliquot of this cocktail was used for hybridization to Affymetrix (Santa Clara, CA, USA) MG-U74Av2 microarrays for 16 h at 45°C. The manufacturer’s standard post-hybridization wash, double-stain, and scanning protocols used an Affymetrix GeneChip Fluidics Station 400 and a Hewlett Packard Gene Array scanner.

Data analysis

The 36 microarrays used here were scaled to the same target intensity and analyzed using Affymetrix Microarray Suite
Pairwise comparisons were made between age-matched wild-type and mdx samples that were processed concurrently. Transcripts were defined as differentially regulated if they met the criteria of: (a) consistent increase/decrease call in mdx versus wild type in all replicates (three of three) at one or more ages, based upon Wilcoxon's signed rank test (the algorithm assesses probe pair saturation, calculates a P-value and determines increase, decrease, or no change calls) and (b) absolute value of the average fold difference $\geq 1.5$. Average fold difference is the arithmetic mean of the three independent replicate pairwise comparisons and reported $P$ values represent the worst-case value from the three comparisons. Hierarchical clustering analysis used Silicon Genetics GeneSpring software (version 5.0; Redwood City, CA, USA).

As a means of weighting the relative impact of differentially regulated transcripts across a temporal expression profiling series, we previously developed a measure defined as the 'disease load index' (DLI) (26). The aggregate DLI is defined as the sum of fold change absolute values for all differentially regulated transcripts at a given age (i.e. if five transcripts were induced or repressed in mdx versus wild-type by exactly 2-fold each, the aggregate DLI value would be 10). Overall, 85% (individual age values ranged between 77 and 92%) of all differentially regulated transcripts had fold change values $\leq 4.5$-fold; thus, the few transcripts with high fold change values did not disproportionately influence the DLI. Differentially expressed transcripts were assigned to one or two of 11 major functional classes and to as many as five of 28 functional subclasses using NCBI LocusLink and gene ontology databases. This approach took the multiple functions of some genes into account in the construction of DLI plots for disease-relevant disease processes (e.g. inflammation) and molecular classifications (e.g. glycoprotein/proteoglycan).

To identify molecular mechanisms underlying the recognized muscle group-specific responses to muscular dystrophy (14,19,20), the diaphragm DNA microarray data obtained here were compared with those from our prior study of hindlimb (combined gastrocnemius/soleus) muscles (26). Experimental and analytic procedures were identical in both studies.

**Quantitative real-time PCR (qPCR)**

Some transcripts were verified by qPCR, using the same samples as in the microarray studies. Briefly, transcript-specific primers (Supplementary Table 4) were designed using Primer Express software (Applied Biosystems Inc. (ABI), Foster City, CA, USA) and their specificity confirmed by NCBI BLAST. Reverse transcription was carried out on 2 $\mu$g total RNA with ABI TaqMan reverse transcription reagent. qPCR used SYBR green PCR core reagent in 25 $\mu$l volume, with an ABI PRISM 7000 Sequence Detection System. Mouse GAPDH (forward primer: 5-AACGACCCCTTCATTGAC-3; reverse: 5-TCCACGACATACTCAGCAC-3) was used as an internal positive control to ensure that equivalent amounts of RNA were included in each assay. Fold change values between mdx and wild-type represent averages from triplicate measurements, using the $2^{-\Delta \Delta CT}$ method (66).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.
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