Definition of the unique human extraocular muscle allotype by expression profiling

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The extraocular muscles (EOMs) are a group of unique skeletal muscles that are anatomically and physiologically distinct from other skeletal muscles. Perhaps the most striking characteristic of the EOMs is their differential sensitivity to disease. EOMs are spared in Duchenne’s muscular dystrophy (DMD) despite widespread involvement of other skeletal muscles. Conversely, they are early and prominent targets in myasthenia gravis and mitochondrial myopathies. It is unclear how EOMs achieve such specialization or a differential response to diseases; however, this has been attributed to a unique, group-specific pattern of gene expression or “allotype.” To begin to address these issues as well as define the human EOM allotype, we analyzed the human EOM transcriptome using oligonucleotide-based expression profiling. Three hundred thirty-eight genes were found to be differentially expressed in EOM compared with quadriceps femoris limb muscle, using a twofold cutoff. Functional characterization revealed expression patterns corresponding to known metabolic and structural properties of EOMs such as expression of EOM-specific myosin heavy chain (MYH13) and high neural, vascular, and mitochondrial content, suggesting that the profiling was sensitive and specific. Genes related to myogenesis, stem cells, and apoptosis were detected at high levels in normal human EOMs, suggesting that efficient and continuous regeneration and/or myogenesis may be a mechanism by which the EOMs remain clinically and pathologically spared in diseases such as DMD. Taken together, this study provides insight into how human EOMs achieve their unique structural, metabolic, and pathophysiological properties.

regeneration; stem cells; expression profile; Duchenne’s muscular dystrophy; transcriptome

THE EXTRAOCULAR MUSCLES (EOMs) are a group of unique skeletal muscles that are required to locate and accurately track objects by the visual system. The EOMs faithfully, rapidly, and accurately effectuate a variety of reflex and voluntary eye movements. The functional requirements for EOMs are wide ranging and include 1) provision of the relatively slow vestibuloocular and optokinetic eye movement reflexes for baseline ocular stability and 2) effectuating pursuit and vergence eye movements to maintain fixation on slowly moving targets, as well as 3) providing rapid saccadic eye movements to quickly reorient the visual system to new targets. In humans, coordinate functioning of EOMs is critical for provision of binocular vision. Furthermore, the EOMs need to function on demand with high fidelity, for relatively long periods of time (7, 13).

Because the functional demands on EOMs are quite different from those imposed on other skeletal muscles, it is not surprising that EOMs exhibit a number of distinctive properties (50). For example, morphologically, physiologically, or biochemically defined fiber types in EOMs do not correspond to those in other skeletal muscles (34, 47, 54). An array of unusual electromechanical features in EOMs results in fibers that have shorter contraction times but lower tension generation compared with other skeletal muscles (1, 2, 19, 32, 49). Moreover, adult EOMs contain multiply innervated fibers, fibers that are uncommon in other mammalian skeletal muscles. The combination of fast contractile properties, high oxidative capacity, and high fatigue resistance is unusual among skeletal muscles and further emphasizes the complexity of these muscles (7, 50).

From a disease perspective, EOMs are preferentially involved in diseases such as the congenital cranial dysinnervation disorders, myasthenia gravis and mitochondrial myopathies (14, 23). Conversely, EOMs remain clinically and anatomically spared in Duchenne’s muscular dystrophy (DMD), despite the severe and widespread necrosis seen in other skeletal muscles in this disease (22, 26). Indeed, EOMs remain functionally and anatomically spared even until the death of patients with DMD. Unraveling the conundrum of clinical sparing of EOMs could prove of great clinical importance, since the molecular mechanisms utilized by human EOMs can potentially be translated into therapeutic strategies for DMD.

While it is currently unclear how EOMs achieve such specialization or why they have a differential response to disease, it is generally accepted that the EOMs are inherently different in terms of their molecular makeup, and that the complement of genes expressed in EOMs contributes to their unique pathophysiological phenotype. Indeed the term “allotype” was coined to underscore the numerous EOM group-specific properties (31). Expression profiling of rodent EOMs suggests that systemic differences at the transcriptional level exist for this unique muscle group (15, 24, 41, 44). Unfortu-
nately, distinct and often dramatic differences in physiology (e.g., afoveate eyes, lack of binocular vision) and disease phenotype (e.g., the mdx mouse model for DMD does not have muscle wasting, indeed is actually stronger than littermates) make it difficult to transfer inferences drawn from rodent EOM studies to human EOM physiology and disease. Human EOMs differ significantly from other species with respect to fiber type composition as well as expression patterns of myosin heavy chain (8, 28, 35, 48, 52), sarco/endoplasmic reticulum calcium ATPase (4, 5, 27, 30), and laminin (29, 51) genes and/or their isoforms, indicating that significant interspecies differences exist at the molecular level. To identify the molecular components underlying the unique properties of human EOM compared with other skeletal muscles, we performed expression profiling of normal human EOM and quadriceps femoris (QF) limb muscle using Affymetrix (GeneChips) U133A high-density oligonucleotide microarrays (ca. 22,000 human probe sets). Verification of expression changes was undertaken at RNA, protein, and substrate levels using a variety of cellular and molecular methods.

MATERIALS AND METHODS

RNA isolation. Two human EOMs (1478-93, referred to as EOM2, and 1545-93, referred to as EOM3) were obtained at autopsy from 14- and 72-year-old females, respectively, who had no identified neuromuscular disease at the time of death. Both independent samples consisted of the whole superior rectus EOM. Three QF limb muscle biopsies (M5, M6, and M12) were from 5-, 6-, and 12-year-old male patients with as yet unclassified neuromuscular disease at the Children's National Medical Center (Washington, DC). Two independent RNA preparations were made in the case of EOM and three individual preparations of QF for GeneChip screening. Fiber typing of muscle was unremarkable. Total RNA was extracted using the TRIzol reagent (Gibco BRL Life Technologies) and purified using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Briefly, frozen muscle tissue was pulverized in liquid nitrogen using a mortar and pestle and transferred into TRIzol reagent. The solution was homogenized using a Polytron homogenizer (20 s at position 4.5; Kinematica, Kriens/Luzern, Switzerland). RNA was purified by phenol-chloroform extraction, precipitated using isopropyl alcohol, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. This RNA solution was repurified using the RNeasy kit and eluted with 40 μl of DEPC-treated water. An aliquot was used for quantification and verification of quality (260/280 ratio between 1.9 and 2.1), using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Upsalla, Sweden). All human tissue samples used in the study were obtained with appropriate institutional approvals, and protocols were in accordance with the provisions of the Declaration of Helsinki for use of human tissue in research.

Affymetrix GeneChip expression profiling. Affymetrix human U133A series oligonucleotide-based arrays were screened using guidelines provided by the manufacturer (http://www.affymetrix.com/support/technical/manual/expression_manual.affx) and as previously described, optimized for expression profiling of skeletal muscle (3, 12, 15). Briefly, 5 μg of RNA were reverse transcribed, and double-stranded cDNA was synthesized using the SuperScript Choice system (Invitrogen, Carlsbad, CA) with a hybrid oligo(dT) primer containing the T7 RNA polymerase promoter (Genset, San Diego, CA). The double-stranded cDNA was purified using phenol-chloroform extraction and precipitated with ethanol. This cDNA was resuspended in water and served as a template for an in vitro RNA transcription reaction, using the ENZO BioArray High Yield RNA transcript (biotin) labeling kit (Enzo Diagnostics, Farmingdale, NY). An aliquot was quantified by spectrophotometry to calculate RNA yield and to ensure quality of in vitro transcribed RNA yield (≥ 5 × input). Biotin-labeled cRNA transcripts were processed as per the manufacturer’s recommendation, using an Affymetrix GeneChip Instrument System (Affymetrix). Briefly, the samples were purified using the RNeasy kit (Qiagen) and fragmented to ~200 bp in size by incubation in 200 mM Tris-acetate (pH 8.2), 500 mM potassium acetate, and 150 mM magnesium acetate at 94°C for 35 min. Eukaryotic hybridization controls were added to each sample before hybridization to U133A GeneChips on an Affymetrix fluids station 400 (16 h at 45°C and 60 rpm). Subsequently, the GeneChips were washed and stained on the fluids station to detect hybridized cRNA, using phycoerythrin-conjugated streptavidin. The signal was amplified by a second round of staining, and the fluorescence was read using a Hewlett-Packard G2500A Gene Array Scanner. Fluorescence data were analyzed using Microarray Suite 5.0 (Affymetrix). In brief, the intensity of one probe cell containing an oligonucleotide complementary to a specific sequence or perfect match (PM) was compared with the intensity of the adjacent probe cell containing one mismatch (MM) in the center of the oligonucleotide. This analysis was repeated subsequently using multiple (11) probe pairs for each gene. The expression level of each gene was calculated based on a comparison of hybridization, i.e., of the PM vs. MM signal. Iterative comparisons of different GeneChips that had been processed in parallel were conducted using two independent EOM and three QF hybridizations leading to six data sets from five microarrays. Differential expression of genes with consistent fold changes (≥2-fold) in EOM and QF was detected by comparing these six independent data sets, using previously described statistical methods that result in efficient detection of the most significant gene expression change (3, 12, 15). Briefly, the determination of whether a gene was differentially expressed in EOM compared with QF depended on the following parameters: absolute call (abs call), fold change (FC), and difference call (diff call). In turn, the data for these parameters were derived using a decision matrix, which calculated analysis metrics (positive fraction, positive-to-negative ratio, and log average ratio) and was dependent on the probe array’s hybridization intensities. A probe set was defined as increased if it fulfilled all of the following: abs call = present (P), FC ≥ 2, diff call = increased (I) or marginally increased (MI). A probe set was defined as decreased if it fulfilled all of the following: abs call = P, absent (A), or marginal (M) provided baseline ≠ A, FC ≤ −2, and diff call = decreased (D) or marginally decreased (MD). Probe sets that survived these criteria in all six iterative comparisons were considered as having significant expression changes. The average values of these pairwise comparisons are used throughout this study.

Semiquantitative RT-PCR. Two hundred nanograms of total RNA from EOM and QF were reverse transcribed into cDNA using the Superscript Choice system (Gibco BRL). For each gene product, 10% (vol/vol) of purified cDNA was used as template for semiquantitative PCR. Primers used for EOM-specific myosin heavy chain (MYH13) fragment (accession no. NM_003802, product size 356 bp) were MYHJ3ex36 F (5'-AAACTGGAGGCTGACATAGCTCAG-3') and MYHJ3ex38 B (5'-ACCTTGGTTCATTGCTGGG-3'); for the γ-subunit of adducin-3 (accession no. NM_019903, product size 356 bp), we used adducin-γ F (5'-CCGATCCCCTGCTCCAAGATT-3') and adducin-γ B (5'-TGGCTGCCACACTGCTGTCAC-3'). The sequences of the other primers were GPM6B F (5'-ACAAGCGTCTCCATTCCAGCAT-3') and GPM6B B (5'-TTAGTGCGACCATCCTCCCGAC-3') for glycoprotein M6B (GPM6B; accession no. AF160004, product size 356 bp), IGFBP6 F (5'-AATTTGCAGGCTTTGCGCA-3') and IGFBP6 B (5'-TTATGGCGACGAATTCCTCCC-3') for insulin-like growth factor-binding protein 6 (IGFBP6; accession no. NM_002178, product size 356 bp), MAOA F (5'-TTTCTTCCCCGAGCAGCAG-3') and MAOA B (5'-AAA-CAAGGGGCGCTATCCCTT-3') for monoamine oxidase A (MAOA; accession no. NM_000240, product size 356 bp), thrombospondin F (5'-TGGAGCAGCACCACAAATTGCAG-3') and thrombo-
plastin B (5′-GAGGGATCCTGCTTTGACACTG-3′) for thromboplastin tissue factor (accession no. NM_001993, product size 356 bp), and thrombopsonidin F (5′-TGTGACACCGATGATGACA-ACG-3′) and thrombopsonidin B (5′-GATTGAAAGGTTGCCACT-GAAAGTC-3′) for thrombopsonidin-1 (accession no. NM_003246, product size 356 bp). All primer pairs were designed to go across an intron-exon boundary and meet following criteria: GC content = 50%, melting temperature = 56 ± 0.6°C, length = 20–25 nucleotides, no secondary structures predicted using MacVector 6.0 (Accelrys, San Diego, CA). As an internal control for efficiency of RT and quantification, we simultaneously amplified part of the human glyceraldehyde-3-phosphate dehydrogenase gene (accession no. BC020308, product size 194 bp) using the primers hGAPDH F (5′-CCATGGAGAACGTGGGG-3′) and hGAPDH B (5′-CAAGATGTCAAGGATGCC-3′).

PCR was performed, and aliquots were removed at 22, 24, 26, 28, and 30 cycles to determine the linear amplification range of each gene, as previously described (15). PCR products were resolved on 1.5% agarose gels and visualized using SYBR Green I (Molecular Probes, Eugene, OR) staining on the Typhoon 8600 PhosphorImager (Amer sham Pharmacia Biotech, Uppsala, Sweden). Quantification was performed on aliquots that lay within the linear amplification range for each reaction and normalized against GAPDH by using ImageQuant 1.2 software (Amer sham) for the Mac OS. The entire experiment was repeated including independent cDNA preparation, PCR, and quantification steps.

Immunocytochemistry and histochemistry. In addition to the samples used for profiling, autopsy samples from EOMs and biopsy samples for limb muscles were obtained from individuals (ages 17–34 yr) and used for independent biological validation by immunocytochemistry and histochemistry. The samples were mounted on cardboard, rapidly frozen in liquid nitrogen-chilled isopentane, and stored at −80°C. Serial cross sections (5 μm) were cut using a Reichert-Jung cryostat and processed for immunocytochemistry, with previously characterized monoclonal antibodies (mAb) recognizing the laminin alpha 4-chain, the laminin alpha 5-chain, the Na+K+-ATPase alpha subunit, and MYH13. The tissue sections were processed as previously described (15). Control sections were processed as above, except that the primary antibody was omitted. No staining was observed in the control sections. Glycogen content was revealed using periodic acid-Schiff (PAS) reaction. Serial sections were pretreated with α-amylase enzyme to control for specificity of the glycogen-staining procedure. Mitochondria content was monitored using nico tinamide dehydrogenase tetrazolium reductase diaphorase (NADH-TR) staining. The sections were photographed under a Nikon microscope equipped with a CCD camera. The number of capillaries was noted in randomly chosen areas (500 μm²) from sections stained with the antibody recognizing laminin alpha 5-chain. All vessels with an outer diameter <15 μm were scored as capillaries according to the criteria defined by Jerusalem et al. (21).

RESULTS

Expression profiling of EOM and QF muscle. To define the expression profile of human EOM compared with limb muscle, we screened the Affymetrix U133A GeneChips with RNA extracted from human EOM and QF. We chose rectus EOM and QF, since these muscles have been studied extensively, and profiling data are already available for QF from normal and DMD patients (12, 17). To verify lack of intrasample variability, we determined the overall variability of expression profiles between two EOM and two QF samples. Scatter graphs of expression levels of all probe sets represented on individual microarrays compared either between the two EOM samples (Fig. 1A) or the two QF samples (Fig. 1B) show much less scatter (R² = 0.88 for EOMs, and R² = 0.89 for QFs, respectively) compared with the scatter between individual EOM and QF samples (Fig. 1C; R² = 0.73). Differential expression of MYH13, which has previously been shown to be highly upregulated in EOM compared with limb muscles at mRNA and protein levels in a variety of species (8, 15, 28, 35, 48, 52), demonstrates the specificity and sensitivity of the approach (Fig. 1C).

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Fig. 1. Expression profiling data sets of extraocular muscle (EOM) and limb muscle (quadriceps femoris; QF). The 3 scatter graphs represent probe sets, with axes showing relative expression levels of each gene on a log scale. Graphs compare the expression profiling data sets for the following independent samples: EOM3 vs. EOM2 (A), limb m12b vs. limb m5b (B), and EOM2 vs. limb m12b (C). The high concordance (or lack of scatter) represents low experimental variability, which is minimal in A and B. Panel C, which compares the expression profile of limb and EOM, demonstrates considerably greater scatter. Solid lines indicate 2-fold difference cutoff. Genes lying off the diagonal show greatest differential expression, exemplified by the probe set for MYH13, which is upregulated in EOM. Slopes and correlation coefficients (R²) for the scatter graphs are as follows: y = 0.952x + 58.261, R² = 0.893 (B), and y = 1.069x + 2.144, R² = 0.725 (C).
To analyze and interpret the data, we performed iterative comparisons of the data sets as previously described (3, 12, 15). Multiple (6) iterative comparisons using a twofold difference cutoff were performed to control for intrasample variability (e.g., in hybridization of cRNA) and to perform a stringent determination of significant and reproducible expression changes. Figure 2A shows six iterative comparisons of “differ-ence calls” from the five independent RNA preparations and hybridizations (2 EOM preparations and 3 QF preparations). The number of surviving increase and decrease calls (or difference calls) is stabilized by the 6th iterative comparison. The nos. of increase and decrease calls in the 1st to 6th iterative comparisons are as follows. First iterative comparison: 1,199 increase and 858 decrease calls. Second iterative comparison: 742 increase and 465 decrease calls. Third iterative comparison: 525 increase and 382 decrease calls. Fourth iterative comparison: 344 increase and 209 decrease calls. Fifth iterative comparison: 245 increase and 158 decrease calls. Sixth iterative comparison: 222 increase and 144 decrease calls. Individual points on the graph represent genes that met the 2-fold difference cutoff (i.e., differentially expressed genes). Genes lying off the diagonal show the greatest differences in expression between EOM and limb muscle.

Characterization of differentially expressed genes in EOM. Further analysis of the 366 probe sets revealed the presence of some transcripts that could be readily ascertained as emanating from the same genetic loci (e.g., splice variants), reducing the actual number of differentially expressed genes to 338. Of the 204 upregulated genes in EOM, 149 were well-characterized genes (representing 73.0% of upregulated genes; 0.7% of all probe sets screened) and 55 encoded genes of unknown function (representing 27.0% of upregulated genes; 0.2% of all probe sets screened). In the case of the 134 genes that were found to be downregulated in EOM, 106 were previously described genes (representing 79.1% of downregulated genes; 0.5% of all probe sets screened) while 28 encoded genes of unknown function (representing 20.9% of downregulated genes; 0.1% of all probe sets screened). The set of genes of unknown function is not discussed in detail in this study, since their functions have currently not been elucidated. Nonetheless, because this set of genes helps to accurately and comprehensively define the EOM expression profile, this set is also listed as Supplementary Data (see the Physiological Genomics web site). Additionally, profiling data was reevaluated using different statistical methods, and the (concordant) results are provided as Supplemental Tables S1–S7 and Supplemental Figs. S1 and S2.

We tabulated and sorted the known differentially expressed genes into various functional groups (see Supplemental Table S8). Genes related to protein biosynthesis (10.7%) and genes involved in intracellular signaling (8.3%) are the largest functional groups of all differentially expressed genes. Other groups include genes encoding neuronal proteins (7.7%) and sarcomeric elements (3.6%); genes involved in maintenance of intracellular homeostasis (2.1%), energy metabolism (4.4%), and protein metabolism (5.0%); and genes related to growth, development, and regeneration (3.0%). Interestingly, genes encoding development-related proteins within the vascular and neuronal groups make up 5.6% of all differentially expressed genes. Taken together, these data are consistent with the hypothesis that EOM has a unique molecular makeup, different from that of limb muscle.

Validation of expression profiling: structural and metabolic elements. We used a number of independent methods to validate important functional components and predictions revealed from expression profiling of human EOMs. Expression levels of seven genes (MYH13, adducin-γ, GPM6B, IGFBP6, MAOA, thromboplastin, and thrombospondin) representing different functional groups were verified at the mRNA level by RT-PCR (Fig. 3). Furthermore, a number of features of the
expression profile were also verified at the protein and/or substrate level (Fig. 4) using immunohistochemistry. The increased expression of the previously well-characterized EOM marker MYH13 (Figs. 1 and 3) was also validated at the protein level using immunohistochemistry (Fig. 4, A and B). Increased expression of laminin-α4 (Fig. 4, C and D) and laminin-α5 (Fig. 4, E and F) offers additional internal controls of the expression profiling, as it reflects the well-characterized increased vascularity of EOM (7, 10). Morphometric analysis, based on counting six areas from the medial rectus EOM and three from QF (total of 1,823 capillaries), revealed that EOM had an approximately five times higher abundance of capillaries compared with limb muscle. The increased expression of membrane channel Na+/K+-ATPase was validated using immunohistochemistry (Fig. 4, G and H). Histochemical analysis of metabolic pathways using PAS (Fig. 4, I and J) and NADH-TR diaphorase activity (Fig. 4, K and L) validated the well-characterized low glycogen content coupled with increased mitochondrial oxidative pathway utilization (7, 9–11) by EOM (Supplemental Table S8). Overall, the expression profile of EOM defined in this study correlates well with what is known about EOM structure and function, suggesting that our expression profiling was sensitive and comprehensive.

**DISCUSSION**

In this study, we used Affymetrix GeneChip-based transcriptome analysis to define the expression profile of human EOM compared with limb muscle. Three hundred thirty-eight genes were found to be differentially expressed in EOM compared with QF muscle, defining the EOM profile. Verification of expression changes was undertaken at RNA, protein, and metabolic levels using a variety of cellular, molecular, and histochemical methods. The use of multiple independent assays to validate major components of the EOM profile should
afford a high degree of confidence for this study. Taken together, our results support the hypothesis that the overall pattern of gene expression or allotype of human EOM is fundamentally different from that of limb muscle.

The last three years have seen a number of comparative analyses being performed on the rodent EOM due to the advent and advances in genetic technologies related to transcriptome analysis. Using a screening-cum-cloning technique called differential display, we had previously demonstrated that the rat EOM transcriptome was indeed different compared with limb muscle (41). More recently, oligonucleotide microarrays (GeneChips) have been used to greatly extend these findings and compare expression levels of genes encoded by the \( \sim 10,000 \) probe sets present on rat and mouse GeneChips, thus allowing interrogation of about one-fourth of the rodent transcriptome (15, 24, 44).

The present study used GeneChip-based expression profiling to analyze and define the human EOM allotype. Because our knowledge of functional genomics and genetic disorders is greater in human compared with rodents, analysis of EOM and limb muscle from humans allowed a more comprehensive interrogation of the transcriptome. Indeed, the U133A series of Affymetrix GeneChips used in this study is about twice as informative as rodent-based GeneChips, since it allows interrogation of \( \sim 22,000 \) probe sets covering all well-characterized expressed genes or approximately one-half of the human transcriptome. Because rodents lack a fovea or binocular vision, in contrast to humans, definition of the human EOM allotype has greater relevance for understanding pathophysiological mechanisms of human EOM.

The expression profile obtained in this study was concordant with many known structural and metabolic features of EOM. Detection of MYH13 (Supplemental Table S8; Figs. 1, 3, and 4) demonstrates the sensitivity of the approach, since this well-characterized marker of EOM, while highly differentially regulated, is expressed at low abundance. Validation of the differential expression of a number of genes (MYH13, adducin, GPM6B, IGFBP6, MAOA, thromboplastin, and thrombospondin) at the mRNA level (Fig. 3) demonstrates the specificity of the approach, since the genes occur in separate functional groups (Supplemental Table S8). Differential expression of genes encoding vessels, mitochondria, and sets of metabolic enzymes provides transcriptome-based suggestions that human EOMs preferentially utilize glucose for energetically efficient oxidative phosphorylation (Supplemental Table S8). A number of genes related to growth/development and apoptosis were differentially expressed in EOM (Fig. 3; Supplemental Table S8). This is interesting, since continual myogenesis in uninjured adult EOM has been suggested based on expression profiling, metabolic and/or satellite cell-labeling studies in a variety of species including humans (15, 37–39). Taken together, these studies suggest that superior regeneration/myogenic potential may contribute to EOM sparing in DMD. The high degree of concordance with previous studies in other species, as well as validation by structural, molecular, and biochemical means, suggests that our molecular definition of the human EOM allotype should provide mechanistic insights toward the functioning of this unique muscle.

Expression profiling: structural elements. The expression profile obtained in this study was concordant with the known structural features of EOM (7, 13). The increased expression levels of MYH13 mRNA in human EOM is consistent with previous reports analyzing the distribution of the protein product (28). The differential expression of genes that are known to modulate contractile properties of muscle such as tropomyosin-4, the essential light chain, myosin regulatory light chain-interacting protein, and caldesmon-1 was noted. A number of genes associated with calcium/calmodulin-dependent signal transduction pathways involved with muscle contractility were differentially regulated in EOM as well. This is exemplified by differential regulation of genes such as calciuin A2, calcrepressin, adducin-3, and calcsarcin-1. Interestingly, calcsarcin-1 binds the Z-disc protein α-actinin in developing, cardiac and/or slow-twitch muscle as well as calcineurin and thus could play an important role in tethering it to the sarcomere in EOM (16). Genes related to neuronal outgrowth and synapse formation were upregulated, providing clues for the development of multiply innervated fibers that are seen in EOMs (Supplemental Table S8). The specific combinatorial complement of structural proteins, calcium/calmodulin-dependent signaling pathways, and specialized innervation would be predicted to provide the EOM with unique contractile properties.

Expression profiling: conserved features of energy metabolism. Expression profiling of human EOM revealed features strikingly similar to those previously noted with respect to metabolic pathways in rodents (15, 24, 44, 44). Consistent with the model of unidirectional glucose-based energy metabolism, we noted excessive downregulation of the gene encoding fructose-1,6-biphosphatase (F-1,6-BP), the key antagonist to the phosphofructokinase enzyme. Low levels of F-1,6-BP would be predicted to promote unidirectional glucose breakdown. The increased expression of vascular genes provides the mechanistic basis for increased vascularity, a structural feature of EOMs that would facilitate adequate availability of bloodborne glucose for metabolic needs. The EOMs are extremely vascular (7, 42) and possess the highest capillary density measured among human muscles (29). Interestingly, levels of laminin mRNA were not found to be differentially expressed, which may be related to a wider subcellular distribution of the mRNA than protein, transcriptional/translational stability or the sensitivity of profiling for this gene. An important feature noted in our rat EOM profiling study was decreased glycogen content, coupled with decreased expression of key regulatory enzymes for glycogen synthesis and glycogen breakdown. This mechanism seems conserved in humans; the glycogen content of human EOM was also reduced in a manner comparable to what we had noted previously in rat EOM (15). Expression levels of key regulatory enzymes of glycogen synthesis protein phosphatase-1, regulatory inhibitor subunit-2 (PPP1R2) and protein phosphatase-1, and regulatory inhibitor subunit-1A (PPP1R1A) as well as phospholipase kinase, the key regulator of glycogen breakdown, were downregulated. Additionally, differential expression of the brain isoform of creatine kinase (CK-BB) as well as adenosine monophosphate deaminase-1 (AMPD1) was noted in EOM. Both of these enzymes provide alternative pathways for energy metabolism and may play an important role for the unique contraction dynamics and properties of EOM that allow rapid contractions for extended periods of time.

Expression profiling: mitochondria and reactive oxygen species metabolism. Additional support for the hypothesis that EOMs utilize oxidative rather than glycolytic pathways comes
from the differential expression of genes encoding mitochondrial proteins. High levels of genes such as 3-oxoacid-CoA transferase (OXCT), a mitochondrial matrix protein, as well as MAOA, an outer mitochondrial membrane protein, were detected in the EOMs. Interestingly, we also noted downregulation of the mitochondrial gene uncoupling protein (UCP3), a feature noted previously in rat EOMs as well. Because UCP3 catalyzes thermogenesis rather than ATP production, downregulation of UCP3 in EOMs suggests a mechanistic adaptation for efficient oxidative metabolism. The differential expression of genes related to energy metabolism suggests that energy utilization in human EOMs is similar to that proposed for rat EOMs, i.e., preferential, unidirectional, glucose-based oxidative metabolism dependent on blood-borne energy fuels rather than intracellular storages of glycogen.

Dependence of EOMs on oxidative metabolism is predicted to generate high levels of reactive oxygen species (ROS), which have the potential of causing significant cellular damage. The profiling data suggest that human EOMs have increased expression of genes encoding chemoprotective proteins such as P450 cytochrome oxidoreductase (POR), CYP1B1, aldehyde dehydrogenase A3 (ALDH1A3), and aldehyde oxidase. Because microsomal NADPH-dependent lipid peroxidation is triggered by the passage of electrons from NADPH to ferric ions, the high expression level of POR mRNA would be predicted to attenuate the deleterious effects of lipid peroxidation in EOM by competition with the NADPH oxidase. The high levels of ALDH1A3 expression indicate a greater ability to protect against cellular damage downstream of lipid peroxidation, since ALDH is known to play a major role in the detoxification of aldehydes generated by ethanol metabolism and/or lipid peroxidation. Interestingly, the downregulation of the gene encoding Kelch-like ECH-associated protein-1 in human EOMs is consistent with increased expression of antioxidants such as ALDH1A3, since the protein encoded by this gene forms part of a negative feedback loop and is known to repress antioxidant response element-mediated upregulation of detoxifying enzymes.

Expression profiling: growth, development, and regeneration. Profiling demonstrated differential expression of a number of genes related to growth/development and apoptosis, suggesting 1) superior regeneration/myogenic potential and 2) continual remodeling of human adult EOM. This is an unusual feature given that skeletal muscle like neuronal tissue is considered to possess limited regenerative capacity, and the majority of myofiber nuclei are thought to be postmitotic. Our expression profiling detected differential expression of numerous genes in human EOM, usually associated with growth, development, and regeneration of muscle. This is exemplified by increased expression of brain-specific isoform of fatty acid-binding protein-7 (FABP), a marker for multipotent stem cells of neuroepithelial origin, and increased expression of the satellite cell marker vascular cell adhesion molecule-1 (VCAM1) (18). Increased expression of growth factors, binding proteins, and receptors [e.g., fibroblast growth factor-2 (FGF2), frizzled-related protein (FRZB), secreted frizzled-related protein-4 (sFRZB4), IGF6, and phospholipid scramblase-1 (PLSCR1)] was noted, as was increased expression of apoptotic markers. FGF is a very well-characterized growth/developmental factor that has profound effects on muscle development and regeneration as well as angiogenesis, embryogenesis, differentiation, and proliferation. The FGF2 isoform is considered an important mitogenic isoform of FGF and has recently been shown to enhance proliferation of neural stem cells (20). Hence, its upregulation in EOM suggests conditions that would greatly facilitate remodeling. Also upregulated was PLSCR1, which is known to colocalize with the EGFR receptor and plays a role in EGF-induced metabolic and mitogenic responses. Transcripts encoding clusterin were upregulated in EOM compared with limb muscle. High expression levels of clusterin have previously been noted in tissue undergoing remodeling after injury. The downregulation of four and a half LIM domains-3 (FHL3) further suggests that EOMs are arrested in a state of continuous muscle remodeling, since reduced expression has previously been noted in C2C12 cells during stimulation with IGF2 and myogenin (40). Additionally, we detected differential expression of numerous genes in human EOM related to angiogenesis [e.g., thrombospondin-1, fibrin-1, fibrin-2, and fibrin-3, connective tissue growth factor (CTGF), and hypoxia-inducible factor-1 (HIF1α)] and neurogenesis [e.g., GM6B, ephrin receptor (EPHA3), actin-binding LIM protein-1, pleiotrophin, receptor protein tyrosine kinase type-2, drebrin-1, Down syndrome critical region gene-1-like-1 (DSCR1L1), epithelial membrane protein-1 (EMP1), and N-myc downstream regulated (NDRG1)]. This is consistent with the notion that continuous EOM remodeling would be predicted to require remodeling of neural and vascular elements along with myofibers to retain full functionality. In general, these features are similar to those noted previously while profiling rodent EOM (15). Continual myogenesis in uninjured adult EOM has also been independently suggested and validated directly, using satellite cell- and apoptotic cell-labeling studies, in a variety of species including humans (36–39, 53). Conservation of this important property across species signifies the inherent importance that continual myogenesis/remodeling plays in these unique muscles.

Expression profiling: disease perspective. Because the functional demands on EOMs are distinct from those on other skeletal muscles, it is not surprising that the EOMs possess a unique set of structural and metabolic adaptations as reflected by their expression profile. What is surprising is the differential sensitivity to certain diseases, which seems in part a functional consequence of their unique expression profile. Differential expression of genes related to energy metabolism suggests a model in which human EOM preferentially utilize glucose-based oxidative metabolism dependent on blood-borne energy fuels rather than intracellular storages of glycogen. In humans, the inability of muscle to metabolize glycogen causes McArthur’s disease. This disease presents with painful exercise-induced cramps in limb muscles; however, the EOMs remain unaffected. The relative independence from glycogen as an energy source (as opposed to limb muscle) offers an explanation as to why EOMs are spared in this disease. Ironically, these adaptations are predicted to render the EOMs vulnerable to disorders of oxidative metabolism. Indeed, EOMs are involved early, frequently, and prominently in the mitochondrial myopathies, where oxidative metabolism is compromised due to genetic mutations of mitochondrial genes (7, 13).

EOMs are functionally (22) and anatomically spared in DMD (26). This sparing presents an intriguing conundrum, since we have previously shown that both normal human limb muscle and EOM produce full-length dystrophin, and that
dystrophin is completely absent in both limb and EOM of DMD patients (26). This phenomenon extends across species, as EOMs are spared in the canine (26) and murine (mdx) models of DMD as well (26, 33, 45). A number of hypotheses have been proposed to explain EOM sparing in DMD and seem to converge on the notion that EOM group-specific properties prevent, adapt, or compensate against dystrophin deficiency (25, 43). Unfortunately, independent tests of many of these hypotheses have yielded equivocal results. EOM-specific utrophin upregulation was suggested to be protective based on mouse studies (33, 45). However, studies from our laboratory have demonstrated that utrophin is not upregulated in EOMs of normal or dystrophic mice, dogs, or humans at the protein level (26). Additionally, no evidence has been found for utrophin mRNA upregulation at the transcriptome level in studies performed either at our laboratory (15) or other laboratories (24, 44) in rats or mice. The current profiling study also failed to find evidence for utrophin upregulation in human EOMs. Taken together, it is unlikely that utrophin upregulation plays a role in sparing of EOMs in DMD. Higher antioxidant capacity (46) and/or enhanced capacity for calcium homeostasis (26) has been suggested to play a protective role, and the current profiling data support this hypothesis. The current profiling study supports the hypothesis that human EOMs have superior regeneration/myogenesis and constant remodeling (37). Indeed, increasing myogenesis/regeneration using pharmacological myostatin blockade has been shown to ameliorate the dystrophic phenotype in the mouse model of muscular dystrophy (6).

In conclusion, we used a variety of molecular and structural methods to identify, validate, and help define the molecular determinants of the unique EOM allotype in humans. Our current study showed a high degree of concordance with profiling studies that defined the rodent EOM allotype. The concordance was somewhat surprising, since interspecies differences of the visual system between rodents and humans are quite pronounced; rodents have a simpler visual system lacking a fovea and do not use binocular vision. Comparative studies in nonmammalian species may help clarify this issue. The expression profile and biochemical data presented in this study suggest the preferential use of glucose-based aerobic metabolic pathways in EOM. The study supported the hypothesis that human EOMs have superior regeneration/myogenesis and constant remodeling, presumably due to an increased complement of satellite and/or stem cells. We believe that our definition of the EOM-specific expression profile will help provide a more mechanistic understanding of the distinct metabolic and pathophysiological properties of EOM.

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