Differences in Gene Expression between Strabismic and Normal Human Extraocular Muscles

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Purpose. Strabismic extraocular muscles (EOMs) differ from normal EOMs in structural and functional properties, but the gene expression profile of these two types of EOM has not been examined. Differences in gene expression may inform about causes and effects of the strabismic condition in humans.

Methods. EOM samples were obtained during corrective surgery from patients with horizontal strabismus and from deceased organ donors with normal EOMs. Microarrays and quantitative PCR identified significantly up- and down-regulated genes in EOM samples. Analysis was performed on probe sets with more than 3-fold differential expression between normal and strabismic samples, with an adjusted P value of $\leq$ 0.05.

Results. Microarray analysis showed that 604 genes in these samples had significantly different expression. Expression predominantly was upregulated in genes involved in extracellular matrix structure, and down-regulated in genes related to contractility. Expression of genes associated with signaling, calcium handling, mitochondria function and biogenesis, and energy homeostasis also was significantly different between normal and strabismic EOM. Skeletal muscle PCR array identified 22 (25%) of 87 muscle-specific genes that were significantly down-regulated in strabismic EOMs; none was significantly upregulated.

Conclusions. Differences in gene expression between strabismic and normal human EOMs point to a relevant contribution of the peripheral oculomotor system to the strabismic condition. Decreases in expression of contractility genes and increases of extracellular matrix-associated genes indicate imbalances in EOM structure. We conclude that gene regulation of proteins fundamental to contractile mechanics and extracellular matrix structure is involved in pathogenesis and/or consequences of strabismus, suggesting potential novel therapeutic targets. (*Invest Ophthalmol Vis Sci.* 2012;53:5168–5177) DOI:10.1167/iovs.12-9785

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A relatively large fraction (approximately 4–6%) of humans has a misaligned visual axis, resulting in strabismus and impaired binocular vision.[1,2] For most cases of childhood strabismus (strabismus not caused by nerve palsy), the reason for this imbalance is unclear. Strabismus may be due to a primary deficit in the central neural pathways controlling extraocular muscles (EOMs),[3–5] but the primary therapeutic intervention is at the level of the EOMs.[6–9] Surgical adjustments of the distal EOMs lead to a rapid (within hours to days) improvement and often long-lasting cure of childhood-onset strabismus.[10,11] This points largely to functional central neural pathways that control a peripheral effector organ that operates "out of range" in the orbit.

Exactly what may cause strabismic EOMs to operate in a dysfunctional manner has remained enigmatic. At the light microscopic and ultrastructural levels, strabismic EOMs show relatively minor alterations.[12–16] Strabismic muscles were reported to have slower contractile properties,[17] but how this may cause strabismus is not clear. There does not seem to be a simple correlation of underacting EOMs being hypotrophic and overacting EOMs being hypertrophic,[18] and differences in the number and activation of satellite cells between strabismic and normal human EOMs are difficult to interpret.[19,20] Strabismus has a genetic component,[21,22] and susceptibility loci for strabismus have been reported based on analysis of families with forms of hereditary strabismus.[23,24] The few gene mutations that have been identified as causing strabismus are related to very rare forms of complex strabismus.[22] We and others have hypothesized that trophic feedback regulates EOM plasticity,[6,9] and imbalance of such feedback may contribute to some forms of strabismus.[9]

Comparison of gene expression between normal and strabismic human EOMs uncovers differences that relate directly to the strabismic condition. Gene expression studies reveal not only compensatory changes that potentially are informative about therapeutic strategies, but also, if investigated early, may inform about causes of strabismus. We compared gene expression by using microarrays and quantitative PCR on EOM samples. Our work showed that strabismic muscles have decreased expression of several genes involved in contractility, calcium handling, and energy balance, but increased expression of genes related to extracellular matrix (ECM) structure.

Methods

Tissue Samples

Human EOM samples were obtained from strabismus correction surgery or from deceased organ-donors. Experimental procedures of human tissue were conducted in compliance with the declaration of Helsinki. The institutional review boards (IRBs) of the University of Nevada and Renown Regional Medical Center approved the research involving human subjects. After explanation of the research study,

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Table 1. List of EOM Samples and Parameters Used in Microarray, PCR Array, and qPCR

	Condition	#	Age	Sex	Length (mm)	Weight (mg)	Deviation Angle	Age at Diagnosis	Previous Treatment	Cause of Death
Microarray										
Pair 1	Normal	1	6	F		56.9	0	–	–	Asthma attack
	Strabismic	1	6	F	5.5	28.4	30	~6 mo		
Pair 2	Normal	2	6	F		38.2	0	–	–	Asthma attack
	Strabismic*	2	9	M	16.0	NA	12–14	~6 mo		
Pair 3	Normal	3	17	M		33	0	–	–	Cerebral edema
	Strabismic	3	12	F	6.5	37	45	Unknown		
Pair 4	Normal	4	38	M		79.7	0	–	–	Acute MI
	Strabismic	4	63	F	8	32	45–55	Childhood	Resection	
PCR array										
Pair 1	Normal	5	6	F		67	0	–	–	Asthma attack
	Strabismic	5	8	M	9.0	42.7	75–80	~6 mo		
Pair 2	Normal	3	17	M		33	0		–	Cerebral edema
	Strabismic	6	8	M	9.0	37.4	75	~6 mo		
Pair 3	Normal	6	32	M		67	0	–	–	Brain trauma
	Strabismic	7	21	M	7.25	47	30–35	16 y		
Pair 4	Normal	7	38	M		41.7	0	–	–	Acute MI
	Strabismic	8	45	M	6.0	37.5	30–35	Childhood		
qPCR										
	Normal	8	17	M		29	0	–		Cerebral edema
	Normal	9	17	M		82	0	–		Cerebral edema
	Normal	10	38	M		68	0	–		Acute MI
	Normal	3,4,6								
	Strabismic	9	34	M	8.0	49.3	45	7 y	–	
	Strabismic	10	77	F	8.0	47.7	50	75 y	–	
	Strabismic	1,3,4,7,8								

All samples are from medial or lateral rectus muscles in exotropic patients, except for one sample, as indicated. Numbering (#) is for each EOM, separate for normal and strabismic. F, female; M, male; NA, information not available; MI, myocardial infarct.

* Patient had esotropia and also nystagmus.

informed consent was obtained from patients scheduled for strabismus surgery. Samples consisted of distal segments of horizontal rectus muscles (mostly medial recti from exotropic patients) that were resected during surgery. Samples were placed immediately in vials containing 1.5 mL RNAlater (catalog #AM7022; Ambion, Austin, TX), either during surgery or, in case of normal EOMs, collected within 1 to 2.5 hours of death. All samples were stored at –80°C until processed for RNA. Samples from organ donors with a history of strabismus, eye surgery, or muscle or neurologic disease were excluded from further analysis. Normal and strabismic samples were matched in pairs for microarray and PCR analyses based on EOM type, RNA quality, demographic information, and age at surgery (Table 1); such pairs were selected from a total of 100 strabismic and 28 normal EOM samples. Muscle samples were paired, because it initially was not known whether age, EOM type, or strabismus type may affect the gene expression pattern of EOMs. Specific care was taken to obtain samples from comparable locations along the EOM (part A of Figure). Samples from more distal and more proximal sites (containing more or less tendon) were examined to rule out that gene expression differences could be explained simply by a biased sampling scheme.

RNA Collection

Muscle samples were thawed quickly, weighed, wrapped in foil, and immediately placed into liquid nitrogen, pulverized, transferred into chilled TRIzol (15596-026; Invitrogen, Carlsbad, CA), homogenized, and centrifuged. Supernatant was transferred to Phase Lock Gel 2 mL tubes (2302830; 5 PRIME, Gaithersburg, MD), chloroform was added, and tubes were shaken vigorously and centrifuged. Supernatant was poured into microcentrifuge tubes, ethanol added, vortexed briefly, and loaded onto columns for RNA isolation (RNAeasy Lipid Tissue Mini Kit, 74804; Qiagen, Valencia, CA). The manufacturer's kit protocol was followed, including on-column DNAase digestion. After quantification on nanodrop spectrometer and gel-verification of quality, all samples were stored at –80°C until used for microarray or quantitative PCR (qPCR).

Microarray

Total RNA was collected as described above from eight independent EOM samples (four strabismic and four normal, Table 1), and was processed by the Nevada Genomics Center, as per Affymetrix protocol. In brief, total RNA was amplified to 15 μg using the Ambion MessageAmp Premier RNA Amplification Kit (Ambion). One μL RNA spike (Affymetrix GeneChip Poly-A Control Kit, P/N 900433; Affymetrix, Santa Clara, CA) was added to 4 μL RNA solution. In vitro transcription incubation time was 3 hours, and 20 μg amplified RNA was processed in the fragmentation reaction. Hybridization, washing, staining, and scanning were done as per Affymetrix protocols on Affymetrix HG U133 Plus 2.0 arrays, using a GeneChip Hybridization Oven 640, GeneChip Fluidics Station 450, and GeneChip Scanner 3000 7G. The data acquired for this report were deposited in NCBI's Gene Expression Omnibus[25] and are accessible through GEO Series accession number GSE38780 (from the public domain at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38780).

PCR

Reverse transcription was performed using RT^2 First Strand Kit (C-03; SABiosciences, Frederick, MD) for PCR array experiments or using Superscript III, 200 U/μL (18080-044), oligo (dT) 12–18 0.5 μg/μL (18418-012), dNTP Mix 10 mM (18427-013; all from Invitrogen) for qPCR experiments. Equal amounts of RNA (500–1000 ng) were added to each reverse transcriptase reaction to produce similar amounts of

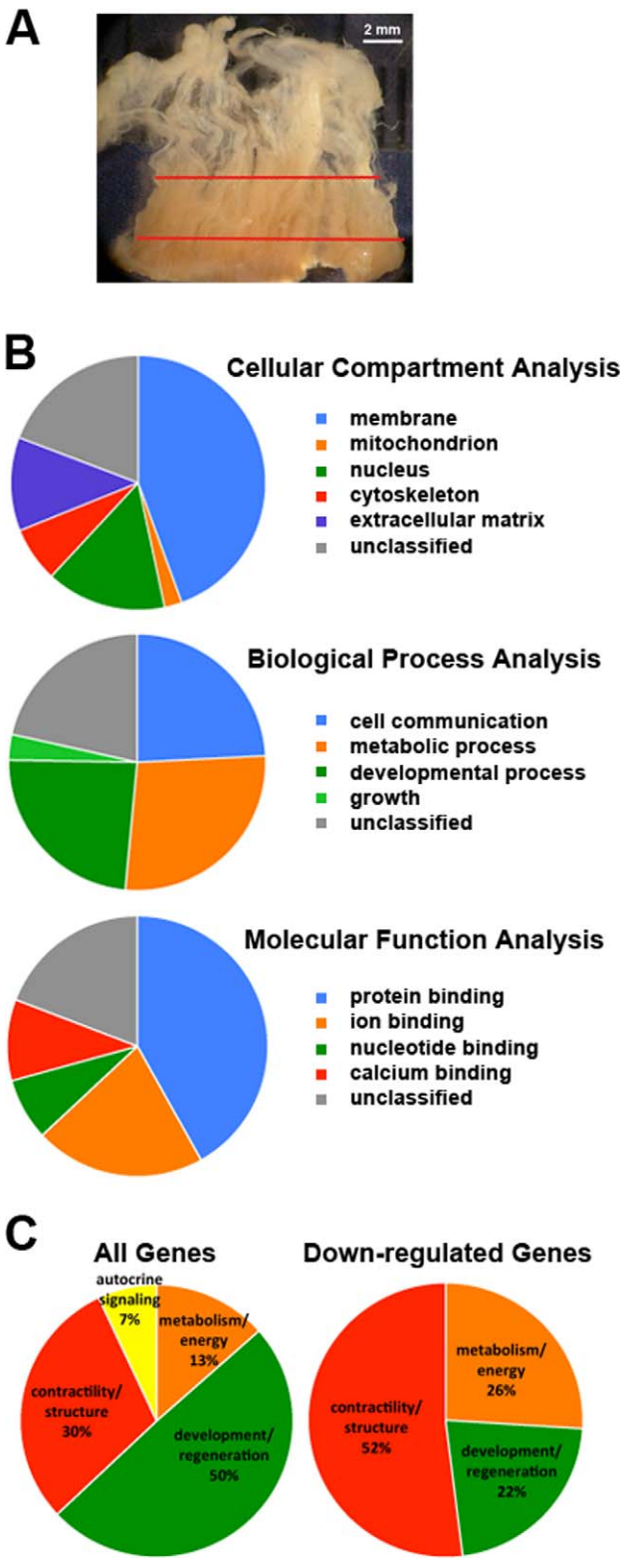

Figure. Location of resected tissue samples in EOMs, and classification of gene expression differences between normal and strabismic human EOMs. (**A**) The typical position of resected tissue samples (*between the two red bars*) is indicated in the distal segment of a lateral rectus muscle. (**B**) Analysis of significantly different gene expression was performed for cellular component, biologic process, and molecular function. Category names are listed to the right of each chart. Some genes are assigned to more than one category, only a selection of categories is shown. (**C**) Comparison of functional groups on the PCR array based on relative number of all 89 genes on array (*left*) and the 22 down-regulated genes in strabismic EOM (*right*). Functional groups as per PCR array design (PAHS099; SABiosciences). The transcripts associated with contractility and energy/metabolism are over-represented among the down-regulated genes. Note that some genes belong to more than one group.

cDNA. Starting amounts of RNA ranged from 390 to 860 ng. Resulting cDNA was tested on a gel to confirm quality and to check for genomic contamination. All experiments were conducted as pairs (Table 1), one normal and one strabismic cDNA sample with one master mix of reference dye, labeling dye, nucleotides, and enzymes. We processed samples on a human skeletal muscle PCR array for 89 muscle-specific genes (PAHS-099C; SABiosciences) according to the manufacturer's protocol with SYBR Green/ROX qPCR Master Mix (PA-012; SABiosciences). Normal and strabismic cDNAs were processed on the same day and on the same Applied Biosystems 7900HT real-time PCR System (Applied Biosystems, Carlsbad, CA). For qPCR, 96-well plates were set up with housekeeping primers and gene of interest (GOI) primers, RT^2 Fast SYBR Green qPCR Mastermix (4385612 ABI; SABiosciences) using normal or strabismic cDNA template. Primers were added to final concentrations of 10.0 μM: IGFR – f:GCTGCCCGTGCGCTGGATGT, r:GCTGCTCGGCCAGTGTGGCG; GDNF – f:CCCAGCCATCCAGTCATTCG, r:ATCGCACTGCCAAGGTTCTCT; GFRa1 – f:GAAGGACAGAGCC CAAAACG, r:ATCGGGGGAGTGGTCTACCT; MYH13 – f: GCAGAGGAACTTCGACAAGG, r:ACCTGGACTCCTTCTGAGCA (Operon, Huntsville, AL). Primer sets for *IGF1* and housekeeping genes, *CAPN2*, and *UTRN* were purchased from SABiosciences. These primer sets are identical to those on the skeletal muscle PCR Array. All experiments were run on an Applied Biosystems 7900HT real-time PCR System. Data were collected using ABI software and analyzed as described below.

Data Analysis

Microarray data first were assessed using Expression Console (Affymetrix, Inc) provided by Affymetrix to confirm quality control performance and establish the presence or absence of each probe set. All eight arrays underwent strict quality control standards as reported previously.[26,27] All were of good quality. We focused on the 31,468 (57%) non-control probe sets that were detected on at least two of the eight arrays. Principal component analysis (PCA) showed a clear separation between normal and strabismic cohorts, with 96% of overall variation explained by the first component and 2% of overall variation by the second component. Data were cleansed to exclude highly outlying replicate expression values. Specifically, any set of replicates having a coefficient of variation greater than 0.5 and a replicate value with a SD (across the four replicate expression values) of greater than 1 was excluded. (The maximum possible SD for four measures was 1.5; thus, if one measure was near 1.5, this indicated that the fourth replicate was at its maximum outlying capacity and was removed.) Only 2530 (1%) of the data values were excluded during this step. We found that this cleansing process allows us to identify notable outliers within a replicate set.[26,28] Expression values were averaged across replicates. Table 2 presents expression ratios of strabismic/normal and the log-transformed expression ratios, using the log base 2 transform. *P* values were calculated by unpaired Student's *t*-test, assuming equal variance for each probe set. *P* values were adjusted using a multiple testing correction (false discovery rate),[29] and significance thresholds were set at $\geq$3-fold change, either up or down, with an adjusted *P* value of ≤ 0.05. WebGestalt Gene Kit Analysis ToolKit (from the public domain at http://bioinfo.vanderbilt.edu/webgestalt, Vanderbilt University),[30] and Database for Annotation, Visualization and Integrated Discovery analysis (DAVID, version 6.7, from the public domain at http://david.abcc.ncifcrf.gov/home.jsp)[31] were used to annotate and characterize genes that showed a $\geq$3-fold change (either up or down)

Table 2. Differential Gene Expression in Human Strabismic EOM Compared to Normal EOM as Measured on Microarray

Gene Symbol	Description	Fold-Change	Fold-Change (Log Transformed)*
Extracellular matrix			
COL1A1†	Collagen, type I, alpha-1	20.3	4.3
COL1A2†	Collagen, type I, alpha-2	12.1	3.6
COL8A2†	Collagen, type VIII, alpha-2	11.5	3.5
COL11A1†	Collagen, type XI, alpha-1	14.1	3.8
COL11A2	Collagen, type XI, alpha-2	10.6	3.4
CTGF	Connective tissue growth factor	7.0	2.8
CYR61†	Cysteine-rich, angiogenic inducer, 61	8.4	3.1
LOX	Lysyl oxidase	5.0	2.3
LOXL1	Lysyl oxidase-like-1	7.5	2.9
MMP16	Matrix metallopeptidase-16	8.3	3.1
MMP3‡	Matrix metallopeptidase-3	0.06	−4.2
TIMP4	TIMP metallopeptidase inhibitor-4	0.26	−1.9
TNC	Tenascin C	6.0	2.6
VCAN†	Versican	4.6	2.2
Muscle			
ANK1	Ankyrin-1, erythrocytic	0.24	−2.1
ANKRD1	Ankyrin repeat domain-1 (cardiac muscle)	0.03	−4.9
DES	Desmin	0.56	−0.8
MYH1	Myosin, heavy chain-1, skeletal muscle, adult	0.03	−5.2
MYH13	Myosin, heavy chain-13, skeletal muscle	0.02	−5.8
MYH3	Myosin, heavy chain-3, skeletal muscle, embryonic	7.3	2.9
MYO1D	Myosin ID	4.3	2.1
MYOG	Myogenin (myogenic factor 4)	0.32	−1.7
TNNT3‡	Troponin T type 3 (skeletal, fast)	0.53	−0.9
TRIM63	Tripartite motif-containing-63	0.39	−1.4
Metabolism/energy			
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator-1-alpha	0.73	−0.5
Growth factors			
GDNF	Glial cell line-derived neurotrophic factor	0.40	−1.3
GFRA1	GDNF family receptor alpha-1	NS	
IGF1	Insulin-like growth factor-1 (somatomedin C)	NS	
IGF1R	Insulin-like growth factor-1 receptor	0.63	−0.7
IGFBP5	Insulin-like growth factor binding protein-5	3.6	1.8
IGFBP6	Insulin-like growth factor binding protein-6	2.9	1.5

Genes are grouped into categories based on function or location. The significance was $P \leq 0.05$ for all values. This is a selection of genes with major expression differences as discussed in the text. (For a complete list, see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9785/-/DCSupplemental.) NS, not significant.

* Log-transformed values of ratios <1 are negative.

† Value shown is average of multiple probe sets.

‡ Genes that encode proteins that are calcium-responsive.

between strabismic and normal groups. PCR array data were analyzed using web-based analysis and a downloadable template provided by SABiosciences (available in the public domain at www.sabiosciences.com/pcrarraydataanalysis.php). We chose housekeeping genes for PCR array by analyzing all 89 genes on the array using geNorm analysis (from the public domain at http://medgen.ugent.be/~jvdesomp/genorm/). Genes deemed most stable over three normal and three strabismic groups were Calpain2 (*CAPN2*) and Utrophin (*UTRN*). Expression data for 87 genes (89 on array minus two designated housekeeping genes) obtained from each PCR array first were normalized to the expression of the two housekeeping genes (the geometric average Ct value of *CAPN2* and *UTRN* combined), and then compared to other arrays. We combined the results from all arrays creating one normal ($n = 4$) and one strabismic ($n = 4$) group, which were compared for gene expression. The qPCR data were analyzed similarly. Gene expression was first normalized to housekeeping gene expression, then the difference of expression between strabismic and normal was calculated using the same equations as on the SABiosciences template. To validate the accuracy of the expression measures determined by the microarrays, expression values were compared to those obtained by qPCR for 10 genes. The qPCR and microarray data ratios for these 10 pairs showed a statistically significant correlation of 0.833, $P < 0.005$.

Results

Sample Selection and Description

The large majority of our strabismic samples were medial rectus EOMs that had been resected to correct medium-to-large angle exotropia (deviation of 30–80 prism diopters). To rule out the possibility that gene expression differences between samples simply were due to strabismic samples being derived from the more tendinous region of the muscle, we checked expression values of standard markers for tendon and muscle, and found no significant differences. Furthermore, we compared gene expression of prospective "strabismic" genes by analysis of samples obtained from more proximal and more

distal locations to assess potential gradients along normal EOMs. No consistent differences or even trends were seen. We also confirmed that genes reported previously as EOM profile genes[32] were expressed in both muscle samples, specifically laminin alpha-4, laminin alpha-5, insulin-like growth factor binding protein-6, ATPaseA1, adducins gamma, neuronal membrane glycoprotein M6-b, thrombospondin, thromboplastin, and monoamine oxidase A. We did not find expression differences in genes linked to congenital fibrosis of EOMs[22] between normal and strabismic EOM on the microarray.

Transcriptome Differences between Strabismic and Normal Muscle by Microarray

Our microarray analysis of 54,675 probe sets represented 39,500 genes. Of these probe sets 57% were detectable in strabismic and normal EOM samples. From this subset of probe sets, representing the EOM transcriptome, 2.8% (897) had $\geq$3.0-fold differential expression between normal and strabismic EOM with an adjusted P value of ≤ 0.05 (see Supplementary Material and Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9785/-/DCSupplemental). These 897 probe sets represented 604–666 known genes/gene loci, depending on the analysis program used (see below). The majority (90.5%, 812 probe sets) were upregulated, while 9.5% (85 probe sets) were down-regulated. Transcripts of interest that were upregulated in significantly strabismic EOMs included genes necessary for ECM structure and function, while transcripts regulating individual sarcomeres as well as final composition and arrangement of the EOMs tended to be down-regulated in strabismic EOMs.

Many collagen genes were >3-fold upregulated (Table 2). Collagens *1A1*, *1A2*, *8A2*, *11A1*, and *11A2* were >10-fold upregulated, with collagen *1A1* upregulated 20-fold. Similarly, several additional genes associated with and necessary for ECM composition and function were significantly upregulated $\geq$5-fold. In this group were connective tissue growth factor (*CTGF/CCN2*), cysteine-rich angiogenic inducer 61 (*CYR61*, also known as IGF binding protein-10 and *CCN1*), lysyl oxidase (*LOX*), lysyl oxidase-like-1 (*LOXL1*), matrix metallopeptidase-16 (*MMP16*), and tenascin C. Metallopeptidase-3 (*MMP3*) was down-regulated 16-fold (expression ratio is 0.06) and metallopeptidase inhibitor-4 (*TIMP4*) was down-regulated 4-fold (expression ratio is 0.26) in strabismic compared to normal EOM (Table 2). Other ECM genes, such as elastin and agrin, were expressed, but not significantly different between the two muscle samples.

In contrast to ECM transcripts, which largely were upregulated, transcripts associated with the contractile mechanics and sarcomere structure were considerably down-regulated. Myosin heavy chain-13 (EOM specific), myosin heavy chain-1 (fast 2x), and ankyrin repeat domain-1 (possibly involved in the myofibrillar stretch-sensor system) have differential expression of 0.02, 0.03, and 0.03, respectively, between strabismic and normal EOM. Also significantly down-regulated were ankyrin-1 and myogenin, both essential for proper development and function of muscle. Upregulated muscle biology genes included myosin heavy chain-3 (embryonic), at >7-fold, and myosin ID (unconventional myosin I), upregulated at >4-fold (Table 2). We concluded that the myosin composition of strabismic muscles is altered, favoring embryonic forms of myosin over EOM-specific and typical fast myosin. Sarcomere markers vimentin and *TP63* had comparable expression levels between the two EOM samples. Overall, we found that expression of ECM-related genes was increased, suggesting a change in muscle length and/or stiffness,[33] while expression of genes related to muscle contraction was decreased, suggesting altered mechanics and function of sarcomeres.

Functional analysis of the differentially expressed genes can give insights into the cellular processes that are affected in strabismic muscle. The WebGestalt Gene Analysis Tool Kit [30] and DAVID Bioinformatics Resource 6.7[31] were used to describe functionally, categorize, and cluster the genes identified by microarray as significantly different in strabismic muscle. WebGestalt identified 604 genes from the 897 probe set IDs submitted, while DAVID analysis found 666 genes. All genes were analyzed together, rather than separated into up- and down-regulated groups. Analysis of our gene list took into account cellular compartments, biological process, and molecular function (part B of Figure). Genes were grouped by cellular component terms (Gene Ontology "GOTERM_SLIM" terms) and analyzed by WebGestalt. Of the 604 differentially expressed genes 58% (483) were designated as "membrane," 20% as "nucleus," 16% as "ECM," 9% as "cytoskeleton," and 3% as "mitochondrion" (14 of 483 genes). Categorizing by annotation for biologic process placed 46% in "metabolic process," and 40% in "cell communication" and "developmental process," suggesting altered muscle tissue metabolism and signaling. By molecular function analysis, the largest group (64%) are "protein binding," while 32% (151 of 467 classified) were tagged as "ion binding," and 15% specifically as "calcium-ion binding," suggesting alterations of calcium signaling in strabismic EOMs. Altogether, GO analysis points to an imbalance of cellular components, with membrane proteins grossly changed in strabismic EOM. Additionally, metabolic processes and cell communication of strabismic EOM potentially were compromised as reflected by the 40 to 46% of differentially expressed genes.

Functional annotation clustering (DAVID analysis of 666 identified genes) used multiple annotation databases to describe each gene functionally, and then clustered the descriptions by common terms, resulting in gene lists organized by related functional terms. The most enriched cluster was defined by the terms "signal," "secreted," and "extracellular region," while the second-most enriched cluster was defined by "ECM," "extracellular region part," and "proteinaceous ECM." The third-most enriched cluster was grouped by annotations of "cell adhesion" and "biologic adhesion." The most common annotations after "sequence variation" and "polymorphism" were "glycoprotein" (49%) and "signal" (48%, $P < 0.0001$). This analysis revealed a heavy bias of gene expression changes towards those genes involved in signaling, particularly regarding ECM interactions.

Pathway analysis of the 666 genes grouped the genes by annotation to specific known biologic pathways, based on two criteria: (1) at least two genes from the differentially expressed group needed to be in the same pathway, and (2) a P value $<$ 0.01 (signifying confidence for being in pathway). A total of 120 genes (18%) passed these stringent criteria and, thus, were assigned to a pathway. Pathways with the most gene members and highest significance were described as "focal adhesion" and "ECM-receptor interaction" with 3.9% (26 genes, $P <$ 0.0001) and 3.3% (22 genes, $P < 0.0001$), respectively. Other pathways of interest were "cell adhesion molecules" with 2.3% (15 genes, $P = 0.0004$), and "inhibition of matrix metallopeptidases" with 0.6% (4 genes, $P = 0.01$). Altogether, DAVID analysis points to a significant difference in strabismic muscle structure in regards to sarcomere and ECM interaction.

Gene Expression Differences by PCR Array

To validate data from the microarray analysis and to explore further the implications, particularly the finding that genes involved in muscle contraction were down-regulated in

strabismic EOMs, we processed samples on a human PCR array for 87 skeletal muscle-specific genes, with primer sets for genes involved in mechanics of muscle contraction, muscle metabolism, myogenesis, and autocrine signaling, as well as markers for disease and muscle atrophy. Data were collected for four medial rectus pairs (Table 1), each composed of one strabismic and one normal EOM. All of the genes on the skeletal muscle array were expressed in EOMs. We first analyzed the individual gene expression differences using the same significance thresholds of greater than 3-fold differential expression, with P values of $\leq$ 0.05. Of the genes 22 (25%) had significantly different expression levels (see Supplementary Material and Supplementary Table S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9785/-/DCSupplemental); they all were down-regulated in strabismic muscle.

The most-down-regulated genes were genes involved directly in contraction mechanics, including genes for proteins that bind or are part of the titin complex, and proteins that bind or respond to calcium. Titin complex genes that were significantly different in strabismic muscle included titin itself (0.20-fold change), tripartite motif containing 63 (*TRIM63*, 0.11-fold change); troponin T type 3 and troponin I type 2 (0.15-fold change), and crystalline alpha B (0.24-fold change, Table 3). *TRIM63*, the most down-regulated gene, is a titin-binding protein, localized to the Z-line in skeletal muscle. The next most down-regulated gene from these PCR array experiments was *ATP2A1*, a calcium pump. *ATP2A1* was expressed differentially with ratio equal to 0.12 (more than 8-fold down-regulated) in strabismic EOM. In fact, seven of the 22 down-regulated transcripts (32%) were from genes encoding proteins that bind, are responsive to, or are activated by calcium, indicating that strabismic muscles may have a reduced calcium handling capacity (Table 3).

The PCR arrays contain genes that are markers for specific functional groups (part C of Figure). We found that among the functional groups designated on this array, 14 (42%) of 33 genes involved in skeletal muscle contractility were significantly down-regulated and five (50%) of 10 metabolic syndrome markers were down-regulated, plus two genes, normally designated housekeeping genes, *GAPDH* and *HPRT1*. Of key interest among the metabolic marker genes were two peroxisome proliferator-activated receptors (coactivator 1 alpha and beta), and two protein kinase AMP-activated genes (beta 2 and gamma 3). All four of these marker genes encode proteins that are important for regulation of energy metabolism. The other functional groups on the PCR array, myogenesis, hypertrophy, and atrophy/wasting, represented 22% of the down-regulated genes (part C of Figure). No genes related to autocrine signaling were different between normal and strabismic EOM on this array set. *TRIM63*, the most down-regulated transcript on the PCR array, binds titin, but also is known as *MURF1*, an E3 ubiquitin ligase strongly linked to muscle atrophy.[34] The findings from the PCR array, when analyzed as individual genes or by functional grouping, point to a dysregulation of muscle contractile mechanics and perhaps a related deficiency in energy production.

Gene Expression Differences by qPCR

Some GOI in regards to trophic signaling and EOM structure were not present on the muscle PCR array. We examined these genes by focused, specific qPCR. We compared the expression level of glial cell line-derived neurotrophic factor (*GDNF*), GDNF receptor alpha 1 (*GFRa1*), insulin-like growth factor-1 (*IGF1*), IGF receptor (*IGFR*, $n = 4$, Table 1). We also measured the expression level of EOM specific myosin (*MYH13*) by qPCR ($n = 6$). *IGF1* was significantly upregulated, being expressed at 5.3-fold, $P = 0.0007$ (Table 3) in strabismic samples when compared to normal samples. *GDNF* and *IGFR* had reduced expression level in strabismic samples, but the P value did not meet our significance threshold. *MYH13* was down-regulated significantly, expression ratio is 0.02 (50-fold down-regulation), $P = 0.0016$, in strabismic EOM. These findings indicate some alterations in trophic signaling in the strabismic EOM.

To assess the validity of our microarray, we compared those genes to a significant differential expression ranging from 0.11 and 5.6 between normal and strabismic EOM on qPCR with fold-change as measured on microarray. Nine of 10 genes sampled showed the same direction of change, thus providing confidence in the microarray results. Expression of myogenin and tripartite motif containing-63 showed 0.32-fold and 0.39-fold-change, respectively, in strabismic when compared to normal EOM on microarray. Desmin, peroxisome proliferator-activated receptor gamma, coactivator-1-alpha, and troponin T type 3 expression ratio ranged from 0.63 to 0.56 on the microarray, consistent with the results from the PCR array (Tables 2, 3). The other 65 genes on the PCR array had similar expression in normal and strabismic EOM, except *IGFBP5*, which was upregulated, expression at 3.6-fold ($P = 0.03$), on microarray. *MYH13* differential expression measured at 0.02 (50-fold down-regulation) in strabismic EOM with both approaches. *IGF1* was upregulated on qPCR and also on microarray, although it did not meet our significance criteria on the microarray. Taken together, the results from both approaches point to a dysregulation of genes involved in sarcomere contraction, energy processing, and EOM structure, specifically affecting the interaction and signaling between ECM and sarcomeres.

Discussion

To our knowledge, our study has defined for the first time the gene expression values of human strabismic EOMs compared to normal human EOMs. There are two important caveats of our study. First, most of our EOM samples were obtained from strabismus surgery to correct childhood-onset strabismus. Exotropia comprises approximately 40% of childhood-onset strabismus cases.[35,36] Accordingly, our data pertained primarily to gene expression changes in this type of strabismus, and did not necessarily apply to other types. Second, as noted in previous reports,[14,37] samples obtained during strabismus surgery typically were restricted to the distal segment of the EOM (myotendinous junction). Therefore, our analysis of gene expression was limited to this segment. The molecular and structural composition of EOMs, and even of individual EOM myofibers, differs along the longitudinal axis.[38–40] Therefore, although we did not find significant gradients in gene expression within the distal segment, it is possible that other segments of the EOM contained genes that are regulated differently. Fortunately, the distal segment of the human strabismic EOM, specifically the myotendinous junction, is of prime interest and presumably highly relevant in the context of strabismus, since it displays the most pronounced morphologic changes compared to normal human EOMs.[16]

Changes in ECM, Contractile Proteins, and Energy Metabolism

The microarray and PCR experiments supported three major conclusions. First, a large cohort of collagen and collagen-related genes were upregulated substantially. Collagens-1A1 and –1A2 are abundant fibril-forming collagens found in most connective tissue, including tendon. Over-expression of collagen-1 increases age-related fibrosis,[41] Specific collagens have not been measured in EOM; however, morphologic

Table 3. Differential Gene Expression in Human Strabismic EOMs Compared to Normal EOMs as Measured on Human Skeletal Muscle PCR Array or qPCR for Specific Genes

Gene Symbol	Description	Fold-Change	Fold-Change (Log Transformed)*
Muscle			
ATP2A1†	Atpase, Ca++ transporting, cardiac muscle, fast twitch-1	0.12	−3.1
CRYAB†	Crystallin, alpha B	0.24	−2.1
DES	Desmin	0.19	−2.4
MYH2†	Myosin, heavy chain-2, skeletal muscle, adult	0.28	−1.8
MYH13	Myosin, heavy chain-13, skeletal muscle	0.02	−5.6
MYOG	Myogenin	0.23	−2.1
MYOT	Myotilin	0.25	−2.0
NEB	Nebulin	0.23	−2.1
TNNI2†	Troponin I type 2	0.15	−2.7
TNNT3†	Troponin T type 3	0.15	−2.7
TRIM63	Tripartite motif-containing-63	0.11	−3.2
TTN	Titin	0.20	−2.3
Metabolic/energy			
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.23	−2.1
HPRT1	Hypoxanthine phosphoribosyltransferase-1	0.30	−1.7
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator-1-alpha	0.20	−2.3
PPARGC1B	Peroxisome proliferator-activated receptor gamma, coactivator-1-beta	0.24	−2.1
PRKAB2	Protein kinase, AMP-activated, beta-2-non-catalytic subunit	0.24	−2.1
PRKAG3	Protein kinase, AMP-activated, gamma-3-non-catalytic subunit	0.26	−1.9
Growth-factor			
GDNF	Glial cell line-derived neurotrophic factor	NS	
GFRA1	GDNF family receptor alpha-1	NS	
IGF1	Insulin-like growth factor-1	5.33	2.4
IGF1R	Insulin-like growth factor-1-receptor	NS	

$P \leq 0.05$ for all values unless noted. NS, not significant.

* Log-transformed values of ratios <1 are negative.

† Genes that encode proteins that are calcium-responsive.

studies of normal human EOM compared to strabismic EOM described a consistent increase in perimysial and endomysial collagenous tissue.[14] Collagen density in strabismic EOMs is increased abnormally and the amount of increase correlates with the duration of sensory exotropia.[42] Genes necessary for collagen formation and function, such as the CCN gene family, also showed increased expression in strabismus. Both *CTGF* and *CYR61* are members of the CCN family, a group of secreted proteins that associate with the ECM.[43] In normal conditions, CCN family genes principally modify signaling by other molecules and, therefore, are associated with a large repertoire of functions, including cell adhesion, ECM production, wound repair, and apoptosis. In pathologic situations, CCN family members are associated with development of fibrosis.[43] Besides ECM-associated proteins, enzymes necessary for building and maintaining collagen structure were dysregulated. *LOX* and *LOXL1*, both necessary for enzymatic cross-linking of collagen to elastin,[44] were upregulated on our microarrays. Several matrix metallopeptidases (*MMPs*) and their inhibitors, which are crucial for ECM homeostasis, also were expressed differentially. Thus, not only the structural components of the ECM are altered, but also the processes that use these components to build functional ECM are dysregulated in strabismic EOM.

The accessory proteins versican and tenascin C are another type of ECM-associated proteins that were expressed differentially in our microarray experiments. Versican is a major component of the ECM and involved in cell-adhesion. Tenascin C protein also is involved in cell-adhesion, as well as a marker for collagen-containing tendon of skeletal muscle. Tenascin C protein is expressed robustly in strabismic EOMs, but was not detected initially in normal EOMs (Strominger MB, Laver NV. *IOVS* 2008;49:ARVO E-Abstract 4121); subsequent work showed it also is expressed there (Strominger M, Laver N, personal communication). We found the tenascin C gene significantly upregulated in strabismic EOMs, suggesting that the distal segment of the strabismic EOM has more structural components of tendon than that of normal EOM. Altogether, it appears that the imbalance of matrix MMPs, inhibitors of MMPs, CCN family genes, and structural enzymes of the ECM results in a net increase in collagen (as has been noted in morphologic studies); this does not necessarily imply that this increase in structural components results in enhanced or beneficial function.

The second interesting characteristic of strabismic EOM as revealed on microarray and PCR array is the down-regulation of specific myosins, notably EOM-specific myosin (*MYH13*) and myosin heavy chain-1 (*MYH1*), and related contractile genes. An imbalance within an agonist-antagonist EOM pair can result in a strabismic phenotype. One explanation for the asymmetrical positioning of the globe by the EOM pair is a lack of muscle tone and/or contraction tension in one of the muscles of the pair. In this scenario, strabismus may be the result of ineffective contraction mechanisms. Consistent with this notion, the following has been described in strabismic sarcomeres: myofilament disorganization,[13,16] Z-disk disorganization, "double" Z-disk,[13,45] increase in rods,[13,16] and increased, enlarged, small, and internally disorganized mitochondria.[16] By affecting the structure of the sarcomere, the contraction parameters could be modified consequentially.

In addition to our findings of down-regulation of specific myosins, several genes that affect cytoarchitecture were

expressed differentially between normal EOM and strabismic EOM (according to PCR array). Desmin (DES) is a scaffolding protein essential for maintaining muscle cytoarchitecture. It also connects successive Z-disks longitudinally and is expressed highly at the myotendinous junction of striated muscle.[46] By PCR array, the expression ratio of *DES* was measured at 0.19 (5.2-fold down-regulation) in strabismic compared to normal EOM. Myosin heavy chain-2 (MYH2) is a conventional myosin heavy chain protein that functions in muscle contraction. Recessive mutations in *MYH2* have been identified in a family study of early onset muscle weakness, and some of the affected individuals presented with ophthalmoplegia.[47] A missense mutation in *MYH2* can be associated with muscle weakness, progressive muscle dystrophy, and an aberrant mix of myosin fiber types, with paresis of the EOMs.[48] EOM has a unique expression pattern of myosin heavy chains within individual fibers as well as across the muscle.[49,50] We found *MYH2* to be expressed at 0.28 (3.8-fold down-regulation) in strabismic compared to normal EOM as measured on PCR array. This finding in conjunction with the evidence from microarray showing down-regulation of *MYH13* and *MYH1* points to the possibility that strabismic EOMs have an irregular pattern of myosin heavy chain fibers.

Myotillin (MYOT) is a core structural molecule of nemaline rods and central core lesions in diseased skeletal muscle.[51] Normally, MYOT functions to cross-link actin filaments and also to prevent disassembly of actin filaments. MYOT is considered essential for thin filament stabilization, which leads to organized Z-disk formation.[52] *MYOT*'s expression ratio is 0.25 in strabismic EOM as measured on PCR array. Nebulin (*NEB*) is a large protein implicated in many pathologies affecting muscle strength.[53] NEB regulates the length of actin filaments[54] and contraction strength.[55,56] NEB interacts with many other proteins, such as actin, DES, and titin; the latter two were down-regulated in strabismic EOMs on PCR array. NEB had a decreased expression at 0.23 or more than 4-fold down-regulated in strabismic compared to normal as measured on PCR array. Troponin T type 3 (TNNT3) is part of the troponin complex that regulates calcium-dependent contraction. Without troponin, the sarcomeric structure is disrupted completely, leading to disintegration of the muscle fibers. When troponin function is disrupted partially, thin filament composition is compromised. Troponin family members *TNNT3* and troponin I, type 2 (*TNNI2*) showed a down-regulation in strabismic EOM, having an expression ratio of 0.15 in strabismic compared to normal EOM, more than 6-fold down-regulated. Altogether, the finding that this group of sarcomeric structural components is regulated differentially in strabismic EOM suggests a defect in molecular composition of myofibers.

The third interesting set of dysregulated genes revealed by comparing strabismic EOM with normal EOM are those involved in mitochondrion homeostasis and function, regulation of energy metabolism, or regulation of energy expenditure. The PCR array data showed significant down-regulation of peroxisome proliferator-activated receptor coactivator-1-alpha (*PPARGC1A*), which has a role in mitochondrial biogenesis, muscle fiber-type determination, and is believed to be a direct link between external stimuli and energy production[57]; coactivator-1-beta (*PPARGC1B*), also involved in energy production and in non-oxidative glucose metabolism,[58] and a regulator of mitochondrial homeostasis;[59] protein kinase AMP activated, beta-2 (*PRKAB2*), which is highly expressed in muscle tissue, suggesting a specific role for muscle,[60] and protein kinase AMP activated, gamma-3 (*PRKAG3*), which is thought to have a pivotal role in regulation of energy metabolism for skeletal muscle.[61] This group of down-regulated transcripts, essential for cellular energy processing and mitochondrial function, complements the morphologic changes of strabismic muscle. The presence of abnormal mitochondria at the ultrastructural level[16] and the dysregulation of mitochondrial genes found in our study suggest a defect in energy homeostasis and possibly indicate cellular stress[62] in the strabismic muscle.

Causes of Strabismus

Strabismus is a condition that can have numerous etiologies,[9,63] including a genetic component, although the exact type of inheritance is yet to be established.[23,64] As mentioned in the introduction, the primary cause of the visual misalignment in typical childhood-onset strabismus is not known. Our finding of a considerable number of significantly up- and down-regulated genes points to a major contribution of the EOM itself in common forms of strabismus. This hypothesis is consistent with the rapid onset of therapeutic effects after manipulations of the EOM by surgical correction[10] or after EOM rebuilding.[33] However, this does not exclude a contribution of the upstream neural networks controlling the EOM.

Growth factors, such as insulin-like growth factor 1 (IGF1), regulate skeletal muscle, including mass and contraction kinetics of EOMs,[65–67] and IGF1 is expressed prominently in adult mammalian EOM.[68] In our study we found an upregulation of expression of *IGF1* via qPCR, but only marginal upregulation via microarray. Differences between these techniques are not unexpected, because microarray measures expression via multiple probe sets and does not distinguish between *IGF1* splice forms, while PCR-based techniques target one specific sequence within the gene. Alteration of the IGF system in strabismic EOM is confirmed further by upregulation of IGF binding proteins-5 and -6 (*IGFBP5*, *IGFBP6*) via microarray. ECM composition can stimulate the synthesis of IGF1[69] and modulate the amount of growth factors available to tissues.[70,71] Accordingly, the dysregulation of the ECM and associated proteins may affect the efficacy of IGF signaling to EOM. Additionally, many of the key ECM proteins (collagens, tenascin C) are regulated by IGFs.[69] IGF1 signals from the EOM to innervating motoneurons[72] and possibly also in the opposite direction, from nerve to EOM.[73] On a larger scale, IGF1 has been proposed to be the key growth factor system that interfaces the brain with the rest of the body.[74] Therefore, both the periphery (EOM) and the innervating neural networks are interdependent functionally and are part of a larger framework – possibly linked through trophic feedback systems, such as IGFs.

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