Eye Movements, Strabismus, Amblyopia and Neuro-Ophthalmology

Altered Protein Composition and Gene Expression in Strabismic Human Extraocular Muscles and Tendons

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Purpose. To determine whether structural protein composition and expression of key regulatory genes are altered in strabismic human extraocular muscles.

Methods. Samples from strabismic horizontal extraocular muscles were obtained during strabismus surgery and compared with normal muscles from organ donors. We used proteomics, standard and customized PCR arrays, and microarrays to identify changes in major structural proteins and changes in gene expression. We focused on muscle and connective tissue and its control by enzymes, growth factors, and cytokines.

Results. Strabismic muscles showed downregulation of myosins, tropomyosins, troponins, and titin. Expression of collagens and regulators of collagen synthesis and degradation, the collagenase matrix metalloproteinase (MMP)2 and its inhibitors, tissue inhibitor of metalloproteinase (TIMP)1 and TIMP2, was upregulated, along with tumor necrosis factor (TNF), TNF receptors, and connective tissue growth factor (CTGF), as well as proteoglycans. Growth factors controlling extracellular matrix (ECM) were also upregulated. Among 410 signaling genes examined by PCR arrays, molecules with downregulation in the strabismic phenotype included *GDNF*, *NRG1*, and *PAX7*; *CTGF*, *CXCR4*, *NPY1R*, *TNF*, *NTRK1*, and *NTRK2* were upregulated. Signaling molecules known to control extraocular muscle plasticity were predominantly expressed in the tendon rather than the muscle component. The two horizontal muscles, medial and lateral rectus, displayed similar changes in protein and gene expression, and no obvious effect of age.

Conclusions. Quantification of proteins and gene expression showed significant differences in the composition of extraocular muscles of strabismic patients with respect to important motor proteins, elements of the ECM, and connective tissue. Therefore, our study supports the emerging view that the molecular composition of strabismic muscles is substantially altered.

Keywords: strabismus, extraocular muscle, growth factor, cytokine, gene expression, PCR array, proteomics, extracellular matrix, collagen, tendon

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Strabismus is a major problem in ophthalmology.[1] Both genetic and environmental factors contribute to strabismus,[2–4] but in contrast to the rare forms of congenital strabismus,[5] the genetics and molecular mechanisms of common strabismus are not well understood.[6] The etiology of strabismus involves multiple components of the oculomotor system, from the extraocular muscles (EOMs) to visual cortex.[6,7]

Numerous attempts have been made to identify structural differences between normal and strabismic EOMs, at the level of histology[8–13] and at the ultrastructural level.[8,9,14–20] Unless specific molecules were probed, most abnormalities were subtle and to some extent also present in normal control tissues, or they could not be identified at all. However, failure to demonstrate alterations at the level of histology and even ultrastructure does not rule out the possibility of differences in molecular composition. Recent work has shown that strabismic muscles display alterations in their gene expression for myosins, myogenic regulatory factors, as well as the extracellular matrix (ECM).[21,22]

To determine whether dysfunctionality in strabismic EOMs may have correlates in protein and gene expression that are difficult to document and quantify at the level of histology and ultrastructure, we used both proteomics and gene expression analysis to compare strabismic with normal human EOMs. Because of the key role of the ECM for viscoelasticity in muscles and tendons,[13,23,24] we focused on ECM components and their regulating enzymes, muscle- and tendon-derived signaling molecules, and cytokines. We show that major structural components of EOMs and their tendons are quantitatively altered at the protein level. Furthermore, ECM components known to control muscle and tendon composition, such as metalloproteases, growth factors, and cytokines, were dysregulated at the gene expression level. Surprisingly, most signaling molecules we examined were expressed primarily in the tendon rather than the muscle component, indicating that

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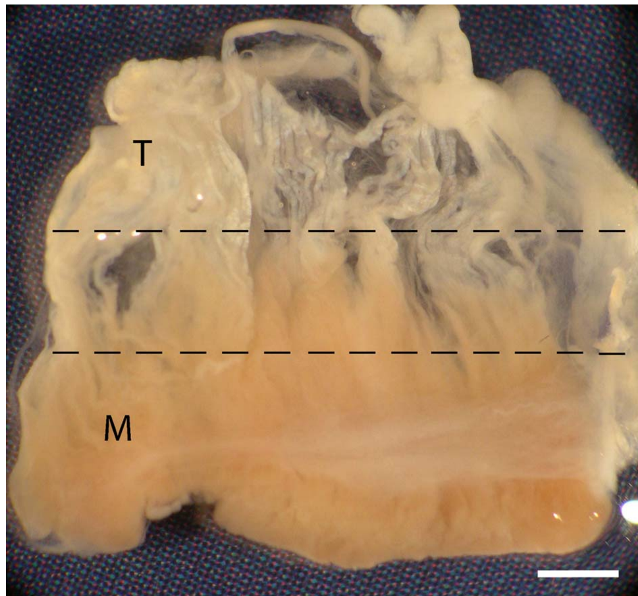

Figure 1. Representative example of a tissue sample from a human extraocular muscle (lateral rectus) at the myotendinous transition area. The primarily tendinous (T) and the primarily muscle-containing components (M) are indicated. *Scale bar*: 2 mm.

important aspects of signaling for EOM adaptation and plasticity occur within the tendon region. This work provides new support for the emerging view that strabismic muscles are altered at subcellular and molecular levels, determines which plasticity-mediating signaling pathways are imbalanced, and thereby may help to identify new targets for therapeutic strategies.

Methods

Sources of Tissues

Human EOM samples were obtained during strabismus correction surgery and from deceased organ donors. Experimental procedures for human tissue were conducted in compliance with the Declaration of Helsinki and conformed to the requirements of the U.S. Health Insurance Portability and Privacy Act. All patients consented, and the institutional review boards (IRBs) of the University of Nevada and the local hospitals approved the research involving human subjects. Samples consisted of distal segments of horizontal rectus muscles (including the myotendinous transition area, Fig. 1), and some were enriched for muscle or tendon. Most medial rectus samples were obtained from patients with exotropia, and most lateral rectus samples from patients with esotropia.

Muscle Samples

Samples were immersed in Allprotect (Qiagen 76405; Valencia, CA, USA) for proteomic analysis or in RNAlater (Ambion AM7022; Austin, TX, USA) for RNA extraction. Samples were collected either during surgery or, in case of normal EOMs, from organ donors within 1 to 4 hours of death. All samples were stored at –80°C until processed. Samples from organ donors with a history of strabismus, eye surgery, or muscle or neurologic disease were excluded from further analysis. From a total of 41 muscle samples collected for proteomics, 8 normal and 9 strabismic samples were selected for analysis, matched by EOM type and age. Proteomics samples were from patients with a mean age of 36.2 years (range, 6–71; male/female ratio: 5/4) and donors with a mean age of 25.6 years (range, 11–44; male/female ratio: 5/3). A total of 135 samples (111 strabismic and 24 normal) were collected for gene expression analysis; among these samples, 48 were selected in pairs for PCR arrays, matched by EOM type, RNA quality, and age at surgery. Polymerase chain reaction array samples were from patients with a mean age of 15.6 years (range, 2–45; male/female ratio: 11/17) and donors with a mean age of 19.4 years (range, 6–38; male/female ratio: 10/9). Some of the organ donor samples were large enough to be used for more than one pairwise comparison, while some patient samples were so small that they had to be combined with two or three patient samples of the same type and similar age. None of the patient samples were from individuals with a history of thyroid-associated orbitopathy, with paralytic conditions, or secondary corrective surgeries. The demographics of the muscle samples are provided in the Supplementary Table S1 (proteomics) and Supplementary Table S2 (PCR arrays).

Proteomics: Tagging, Mass Spectometry, and Data Analysis

Samples were obtained from nine different strabismic muscles (patients) and eight different normal donor muscles, matched by muscle type and age. For protein isolation, tissue samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer and centrifuged. Supernatants were subjected to ultracentrifugation; resulting supernatants were quantified in triplicate for protein using the EZQ protein assay (Invitrogen, Carlsbad, CA, USA), precipitated in acetone, and digested with trypsin. Peptides were mass tagged using Thermo-Fisher's TMT 10plex isobaric label kit (cat. no. 90061; Waltham, MA, USA). Three sets of independent experiments, with $n = 2$ or $n = 3$ samples per group (strabismic or normal), were performed. For experiment 2, the tagged peptides were subjected to fractionation by high pH reversed-phase chromatography followed by reversed-phase HPLC–tandem mass spectrometry. Tagged peptides from experiments 1 and 3 and peptide fractions from experiment 2 were then separated using an UltiMate 3000 RSLCnano system (Thermo Scientific, San Jose, CA, USA) on a self-packed UChrom C18 column (100 μm × 35 cm). Elution was performed using a 90-minute gradient of solvent B from 2% to 27% (solvent A, 0.1% formic acid; solvent B, acetonitrile, 0.1% formic acid) at 50°C using a digital Pico View nanospray source (New Objectives, Woburn, MA, USA) that was modified with a custom-built column heater and an ABIRD background suppressor (ESI Source Solutions, Woburn, MA, USA). Briefly, the self-packed column tapered tip was pulled with a laser micropipette puller P-2000 (Sutter Instrument Co., Novato, CA, USA) to an approximate inner diameter of 10 μm. The column was then packed with 1 to 2 cm of 5-μm Magic C18 followed by 35 cm of 1.8-μm UChrom C18 (120A) at 9000 psi using a nano LC column packing kit (nanoLCMS, Gold River, CA, USA). Mass spectral analysis was performed using an Orbitrap Fusion mass spectrometer (Thermo Scientific). TMT analysis was performed using an MS3 multinotch approach.[25] The MS1 precursor selection range was from 400 to 1400 m/z at a resolution of 120K and an automatic gain control (AGC) target of 2.0×10^5 with a maximum injection time of 100 ms. Quadrupole isolation was set at 0.7 Thompson for MS2 analysis using collision-induced dissociation fragmentation in the linear ion trap with a collision energy of 35%. The AGC was set to 4.0×10^3 with a maximum injection time of 150 ms. The instrument was set in a top-speed data-dependent mode with a most intense precursor priority. Dynamic exclusion was set to an exclusion duration of 60 seconds

with a 10-ppm tolerance. MS2 fragment ions were then captured in the MS3 precursor population. These MS3 precursors were isolated within a 2.5-Da window and subjected to high-energy collision-induced dissociation (HCD) with a collision energy of 55%. The ions were then detected in the Orbitrap at a resolution of 60,000 with an AGC of 5.0×10^4 and a maximum injection time of 150 ms. We compared identified protein levels between biological replicates, as well as across the two groups, to identify statistically significant differences between strabismic and control protein expression levels, with false discovery rates (to deal with multiple comparisons) at 1%. The MS data were extracted using Proteome Discoverer 2.1 and analyzed using Sequest (both Thermo Scientific) to validate protein identifications and provide quantitation using the ratio of TMT reporter ions within the isobarically labeled peptides. Samples from surgeries contained significantly more blood than donor samples; therefore, blood proteins were excluded after normalization. A simple Student's *t*-test was applied to the Master proteins quantified in all channels that had at least a 2-fold differential abundance across cohorts; each experiment was considered separately. To account for the multiple tests, we calculated false discovery rates.[26] Experimental sets 1 through 3 yielded a total of 167 proteins with an at least 2-fold differential abundance and an adjusted P value < 0.05. Fisher's combined probability test was used as a test for the significance of the three independent sets of experiments.[27]

RNA Collection

Muscle samples were thawed, weighed, wrapped in foil, pulverized in liquid nitrogen, transferred into chilled TRIzol (Life Technologies 15596-026; Grand Island, NY, USA), homogenized, and centrifuged. Chloroform was added to the supernatant; tubes were shaken vigorously and centrifuged. Supernatant was poured into microcentrifuge tubes; ethanol added, vortexed, and loaded onto columns for RNA isolation (RNAeasy Lipid Tissue Mini Kit; Qiagen 74804). The manufacturer's kit protocol for total RNA isolation was followed, including on-column DNase digestion. After analysis of quantity and quality on an Agilent 2100 BioAnalyzer (Santa Clara, CA, USA), RNA samples were stored at −80°C until used for reverse transcription and PCR array.

Reverse Transcription and PCR Arrays

Reverse transcription was performed using RT^2 First Strand Kit (SABiosciences 330401; Frederick, MD, USA). Similar amounts of RNA (750–880 ng) were added to each reverse transcriptase reaction in order to produce similar amounts of cDNA. The cDNA was used for PCR array immediately or after storage for 2 to 3 hours at −20°C. All experiments were conducted as pairs, usually with one normal and one strabismic sample (containing cDNA from between one and four muscles) per pair, except for pairs of muscle versus tendon. Expression of a total of 410 different genes was examined; 29 of these were from a custom PCR array (SABiosciences), and another 381 different genes were examined on five different types of Human SABiosciences arrays—Common Cytokines (PAHS-021ZC), Neurotrophins and Receptors (PAHS-031ZC), Tyrosine Kinases (PAHS-161ZC), Neurogenesis (PAHS-404ZC), and Myogenesis/Myopathy (PAHS-099ZC)—according to the manufacturer's protocol with SYBR Green/ROX quantitative PCR (qPCR) Master Mix (SABiosciences 330521). Results from a fraction of the samples evaluated on the Myogenesis array were included in a previous report.[21] All arrays were processed on Applied Biosystems (Carlsbad, CA, USA) 7900HT real-time PCR Systems. Data were collected using SDS 2.4 software (Foster City, CA, USA), applying the same baseline and threshold values for all samples.

Microarrays

Data from our previous microarray study[21] were reanalyzed for comparison with PCR array data and proteomics data.

Data Analysis for PCR Arrays

Data files were exported from SDS 2.4 and analyzed using web-based SABiosciences software (www.sabiosciences.com/pcrarraydataanalysis.php [in the public domain]), which calculates differences in relative gene expression using the ΔΔCt method. We normalized the expression data to reference genes that were most consistently expressed in the array plates, usually *ACTB*, *GAPDH*, and *RPLP0*. We considered any gene up- or downregulated by 2-fold or more (fold change ≥ 2.0 or ≤ 0.5) versus controls to be altered, as compiled for 35 genes in Results. The P values were calculated based on a Student's *t*-test of the replicate 2^(- Delta Ct) values for each gene in the donor and strabismic groups. To account for multiple comparisons and to control the false discovery rate, we calculated an adjusted P value of 0.023, using the method of Benjamini and Hochberg.[26] To measure the strength of a linear relationship when gene expression appeared unchanged, we calculated r values based on Pearson's R correlation test.[28]

Results

Proteomics

We identified and quantified a total of 2952 to 6098 proteins in human EOMs by liquid chromatography/mass spectrometry (LC/MS). Data are based on a total of nine strabismic and eight donor samples.

Myosins and Filament-Related Proteins. Myosins 2, 7, 7B, and 13 were reduced in strabismic muscles, but only myosins 2 and 7 were reduced with statistically significant P values (Table 1). The filament- and contraction-related proteins tropomyosin 1, 2, and 3 were significantly decreased. Similarly, troponin I, T, and C were reduced significantly, and the giant protein titin was also reduced with statistical significance at the protein level (Table 1).

Collagens. Collagens are the main constituents of tendon, and collagen types I, III, IV, and VI have been described in human EOMs.[13,29] In our strabismic samples, collagen XII (alpha 1) was increased nearly 4-fold, with strong statistical significance (Table 1), while collagen VI (alpha 3) was increased in one of three sets of experiments with statistical significance, and collagen VI (alpha 2) was increased with borderline significance in that set of experiments (Supplementary Table S3).

Proteoglycans. Proteoglycans space and lubricate tendons and contribute to fibril fusion and myogenesis.[23,30] The proteoglycan decorin was upregulated nearly 3-fold in strabismic muscle, but with borderline significance (Table 1). The adhesive glycoprotein thrombospondin 4 showed similar 3-fold upregulation and the ECM glycoprotein tenascin X was also increased, both of them with borderline statistical significance.

Other Proteins. Other protein levels were essentially unchanged, for example, actinin, annexin, calmodulin, caveolin, dermatopontin, gelsolin, laminin, nidogen, peroxiredoxin, and many others (Supplementary Tables S3, S4, S5). Table 1 compares the fold change for proteins along with the fold change for gene expression (from our previous microarray work and from our current PCR array data). Most protein expression changes were similar to gene expression changes.

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Table 1. Changes in Protein Expression in Strabismic Versus Normal Extraocular Muscles (Aggregates From Three Independent Experiments), Compared With Gene Expression Data

Gene Symbol	Protein	Fold Change Proteomics	Fisher's Combined *P* Value	Fold Change Microarray, Ref. 21	Fold Change PCR Array, Ref. 21
COL12A1	**Collagen type XII, alpha 1**	**3.72**	**0.0217**	**7.62**	
DCN	Decorin	2.57	0.0640	**2.42**	
MYH2	**Myosin 2**	**0.19**	**0.0124**	1.14	0.31
MYH7	**Myosin 7**	**0.27**	**0.0141**	0.82	
MYH7B	Myosin 7b	0.27	0.0747	1.22	
MYH13	Myosin 13	0.19	0.1464	**0.02**	
THBS4	Thrombospondin 4	2.53	0.0932*	7.52	
TPM1	**Tropomyosin 1 (alpha) isoform 1**	**0.04**	**0.0129***	1.10	
TPM2	**Tropomyosin 2, beta chain**	**0.12**	**0.0103**	0.76	
TPM3	**Tropomyosin 3, alpha 3**	**0.21**	**0.0066**	**2.00**	
TNNI2	**Troponin I, fast skeletal**	**0.20**	**0.0233**	0.60	**0.22**
TNNT3	**Troponin T, fast skeletal**	**0.14**	**0.0245**	**0.53**	**0.18**
TNNC2	**Troponin C, skeletal muscle**	**0.08**	**0.0081**	0.89	
TNXB	Tenascin-X	3.16	0.0752	**4.21**	
TTN	**Titin**	**0.36**	**0.0130**	0.79	**0.21**

Bold font, statistically significant change; regular font, not significant, but borderline or approaching borderline.

* Value calculated from experiments 1 and 3, protein not found in experiment 2.

PCR Arrays

We used PCR arrays to determine which signaling molecules (growth factors, receptors, transcription factors with potential effects on muscle plasticity) have altered gene expression levels in strabismic muscles (Table 2; Fig. 2).[21,22–24,31]

Myogenesis. Myogenesis genes with downregulation included satellite cell markers such as *PAX7*.[32] Downregulation of *PAX7* is consistent with alteration of myogenesis-related genes such as *MYOD1*, myosins, and filament-related muscle proteins (see proteomics, microarray data,[21] and qPCR data[22]).

Collagens and Their Regulators. Collagen synthesis and degradation is regulated by growth factors (transforming growth factor beta [TCGβ], connective tissue growth factor [CTGF], tumor necrosis factor alpha [TNFα]), through collagenases (matrix metalloproteinases, MMPs) and their inhibitors (TIMPs[23]). We found that expression of TGFβ (*TGFB1*) was not significantly increased in strabismic EOMs, although it was slightly increased by microarray, while CTGF was increased 6-fold and TNFα 3-fold (Table 2). Transforming growth factor β can signal directly or via CTGF to increase collagen synthesis, as well as via TIMPs (that inhibit collagenases). Most TIMPs were upregulated in strabismic EOMs: TIMP1 and TIMP2 were upregulated 6- to 7-fold, and based on microarray data approximately 3-fold. At the same time, the collagenase MMP2 was increased approximately 5-fold. Interleukins stimulate the formation of MMPs from ProMMPs.[23] Indeed, several interleukins and their receptors (*IL7*, *IL10RA*) were upregulated 2- to 4-fold. Most of the PCR array changes were independently confirmed by our microarrays (Table 2).

Other Cytokines and Growth Factors. Several cytokines were upregulated in strabismic EOMs, including *IL7* (up 2- to 3-fold, confirmed by microarray), the receptors *IL10RA* (up 4-fold, confirmed by microarray), *CXCR4* (up 3-fold), *NPY1R* (up 5-fold), and the prostaglandin receptor *PTGER2* (up 26-fold). Additional genes with significant changes were the neurotrophin receptors trkA and trkB (*NTRK1* and *NTRK2*, up 3- to 4-fold), and neuregulin1 (*NRG1*, down 4-fold), while the reduction of *CNTF* expression did not reach statistical significance.

Genes With Stable (Unchanged) Expression. Not all genes were changed in expression; many remained unchanged between normal and strabismic muscles. To confirm the lack of change, we calculated the r values as a measure of a true lack of differences (Table 2). Genes with weak or moderate linear correlations (r values below 0.50, or above −0.50) were BMP4, *NRCAM*, *TGFB1*, and *VEGFA* (Table 2). Genes with strong r values (above 0.50, or below −0.50) included *BDNF*, *CNTFR*, *FGF2*, *IGF1*, *IGFBP5*, *LIFR*, *MMP9*, and *NTF3*. A number of genes appear to be unaffected by the strabismic condition, further indicating that the changed ones are specific and differences are not due to global differences between groups.

Comparison of Different Age Groups. Although we compared gene expression in pairs that were roughly matched by age to eliminate potential effects of age,[31] we also analyzed our PCR array data by comparing a younger age group (2–9 years) with an older age group (14–45 years). The large majority of changes were similar in the two age groups, and we did not find any gene for which the directionality of change reversed with age (Supplementary Table S6). These data are consistent with the idea that once an EOM becomes strabismic, the altered gene expression is relatively stable.

Microarrays

In our previous microarray study,[21] we used a cutoff of 3-fold to report gene expression changes between normal and strabismic EOMs. Since this cutoff could have missed less dramatic but nonetheless functionally relevant gene expression changes, we reexamined all gene expression changes between 2.0- and 3.0-fold. Genes of interest in this context are compiled in Table 3. These data confirm our protein and PCR array data by showing decreases in myosins and myogenesis-related genes, as well as increases in collagen and ECM-related genes. Table 1 compares our data on protein changes with changes in gene expression determined by PCR array and microarray.

Localization of Gene Expression Between Muscle and Tendon

To determine whether genes of interest (signaling molecules) were expressed primarily in muscle or tendon, we further dissected EOM samples and compared gene expression levels between samples enriched for muscle and samples enriched for tendon. We found that the large majority of relevant ligands, receptors, and transcription factors were expressed in the tendon compartment (Table 2), while a smaller number were primarily expressed in muscle, along with genes known to be

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TABLE 2. Gene Expression Levels of Signaling Molecules in Strabismic Extraocular Muscles

	Fold Change							
	Increase*	None	Decrease†					
Gene Symbol	+	±0	−	***n***	***P* Value**	***r* Value**	**Confirmation**	**Reference**
BDNF§		**1.41**		**10**		**0.73**		
BMP4§		1.37		14		0.33	PCR array: ±0	21
CNTF‡			0.40	10	0.05018			
CNTFR§		**1.29**		**8**		**0.51**		
CTGF§	**6.39**			**5**	**0.00938**		MA: **7.02**	21
CXCR4||	**2.86**			**8**	**0.000256**		MA: **5.15**	21
DDR2	**3.11**			**7**	**0.000991**		MA: **3.49**	21
DYSF			0.36	5	0.032124			
FGF2		**0.83**		**10**		**−0.67**		
GDNF‡			**0.11**	**10**	**0.002096**		MA: **0.40**, qPCR: NS	21
IGF1		**0.94**		**5**		**0.80**	MA: NS, qPCR: **5.33**	21
							qPCR: most reduced	22
IGFBP5		**1.26**		**5**		**−0.57**	MA: **3.60**	21
IL7§	**2.37**			**7**	**0.018221**		MA: **3.78**	21
IL10RA§	**4.42**			**8**	**0.014755**		MA: **3.19**	21
LIFR		**1.02**		**3**		**−0.98**		
MMP2§	**4.63**			**5**	**0.000207**			
MMP9‡		**1.88**		**10**		**0.73**	PCR array: 0.68	21
MUSK‡			0.25	12	0.027601		PCR array: 0.31	21
NOTCH2§	4.96			7	0.027403		MA: **3.32**	21
NPFFR2§	**44.43**			**8**	**0.000138**		MA: **8.89**	21
NPY1R§	**4.51**			**8**	**0.000009**			
NRCAM‡	0.52			7		0.32	Gene loci	63
NRG1‡§			**0.25**	**10**	**0.000198**			
NTF3		**1.11**		**5**		**0.71**		
NTRK1§	**3.09**			**10**	**0.005728**			
NTRK2§	**3.71**			**10**	**0.004338**		MA: **3.34**	21
PAX7‡			**0.11**	**10**	**0.004492**		PCR array: 0.35	21
PTGER2§	**25.73**			**8**	**0.000195**			
SLIT2§	**3.51**			**7**	**0.000215**		MA: **3.15**	21
TGFB1§		1.31		17		0.45	MA: **2.15**	21
TIMP1§	**7.43**			**5**	**0.000459**		MA: **2.87**	21
TIMP2§	**5.81**			**5**	**0.002852**		MA: **3.61**	21
TNF§	**3.41**			**12**	**0.000083**		PCR array: 1.97	21
TNFRSF11B§	**11.15**			**7**	**0.000549**		MA: **11.74**	21
VEGFA||		0.67		9		−0.28		

Gene symbols are listed alphabetically, indicating the fold change (more than 2-fold) for the strabismic sample. The adjusted *P* value was $P < 0.023$ (threshold for significance taking into account multiple comparisons) according to Benjamini and Hochberg.[26] Significant differences in gene expression are shown in bold font. Likewise, genes and *r* values are bolded when $r \geq 0.50$ or $r \leq -0.50$, indicating a strong linear correlation (= no significant difference) between normal and strabismic muscles (Pearson's *R* correlation test). MA, microarray; *n*, number of independent experiments (pairwise comparison of muscle samples); NS, not significant. *NRG1* was expressed primarily in muscle in lateral rectus, but in tendon medial rectus.

* Gene expression increased more than 2-fold.

† Gene expression decreased more than 2-fold.

‡ Expressed primarily in muscle.

§ Expressed primarily in tendon.

|| Expressed equally in both tissues.

expressed in muscle, such as *MUSK*, *PAX7*, and *MYOD1*.[22,32] Interestingly, several members of the same family of genes were expressed primarily in either tendon or muscle, but very few were equally distributed in both, indicating specialized compartment-specific functions of distinct members (e.g., *MMP2* in tendon, *MMP9* in muscle). In addition, some ligands were expressed in one compartment while their receptors were expressed in the other compartment, suggesting that ligands produced in one compartment (e.g., muscle) signal to receptors in the other compartment (e.g., tendon). An example for such potential crosstalk across regions is muscle-produced ciliary neurotrophic factor (CNTF) signaling to CNTF receptors on tendons. Other signaling molecules were produced in tendon, and may signal in that same compartment: bone morphogenetic protein 4 (BMP4), CTGF, the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) to their neurotrophin receptors (trkA and trkB), TNF to TNF receptors, MMP2, and its inhibitors TIMP1 and TIMP2.

DISCUSSION

Our study is the most comprehensive quantitative study to date that surveys changes in expression of both proteins and genes in strabismic human EOMs. Previous studies on strabismic muscles have either examined small numbers of proteins[13,33,34] or various numbers of genes,[21,22] or a small

SIGNALING MOLECULES	INCREASED	UNCHANGED	DECREASED
Growth Factors	**CTGF, IL7,** NOTCH2, **SLIT2**, **TNF**	**BDNF,** BMP4, **FGF2, IGF1, NTF3,** TGFB1, VEGFA	CNTF, **GDNF, NRG1**
Receptors, Membrane Proteins	**CXCR4, DDR2, IL10RA, NPY1R, NTRK1, NTRK2, PTGER2, TNFRSF11B**	**CNTFR, IGFBP5, LIFR,** NRCAM	DYSF, MUSK
Transcription Factors			**PAX7**
Proteases, Inhibitors	**MMP2, TIMP1, TIMP2**	**MMP9**	

Figure 2. Summary of signaling molecules that are increased, unchanged, or decreased in strabismic versus normal human eye muscles. Genes that are significantly increased or decreased are shown in *bold*; those that are statistically almost significant are shown in *regular font*. Genes are bolded in the "UNCHANGED" column when $r \geq 0.50$ or $r \leq -0.50$, indicating a strong linear correlation (= no significant difference) between normal and strabismic muscles, while *regular font* indicates a modest linear correlation (Pearson's R correlation test).

number of proteins along with genes, but without normal controls.[31] Our study was designed to reveal major changes in protein and gene expression that may contribute to the implementation of adaptive responses.

Technical Issues and Limitations

We and others have examined the myotendinous transition area of strabismic EOMs, because those samples become available during corrective surgeries. It is currently controversial to what extent the muscle portion of horizontal human EOMs extends to the sclera in the myotendinous transition area. The distal tendons of the human medial and lateral rectus muscles have been reported to be 3.0 to 3.7 mm and 8.0 to 8.8 mm in length, respectively,[18,31] while another study reported that at least some myofibers of these horizontal muscles extend directly into the sclera with virtually no distal tendon component.[35] Because of the variability between individual muscles, our surgeons estimated the relative contributions of muscle and tendon in each EOM sample so strabismic samples could be closely matched with normal samples; we also dissected muscle and tendon to enrich for either of the two regions (as depicted in Fig. 1) and to determine the relative contributions of the two regions for gene expression. The conclusions of our study apply only to the myotendinous transition area of the horizontal EOMs, and may not be pertinent to the muscle belly or the region of the proximal insertion.

Previous work employed histology, immunohistochemistry, and Western blots to assess changes in selected proteins between strabismic and normal human EOMs[13,31,33]; the composition of normal EOMs has also been examined for select proteins.[32,36,37] In general, due to the variability of antibody quality and tissue processing, as well as concerns about linearity of the signal, immunocytochemistry is not considered an ideal approach for protein quantification.[31,38] In addition, antibodies may recognize multiple antigens, possibly expressed by distinct isoforms.[36,37,38] Therefore, we used LC/MS to quantify changes in protein composition in our study. This enables superior quantification of most structural proteins, but rare proteins such as transcription factors and growth factors and their receptors were expressed at levels too low for reliable quantification.

Myosins

Myosin heavy chain isoforms are instrumental in regulating contraction force and velocity of muscle fibers.[39] Previous work showed that myosin proteins in normal human EOMs are composed primarily of myosins MYH1, MYH2, and MYH13.[36,37] We found that myosin isoforms 2, 7, and 13 were reduced at either the protein or gene expression level in strabismic muscles, along with several filament- and contrac-

Table 3. Genes Altered in Strabismic Medial Rectus Muscle on Microarrays (Data From Ref. 21)

Symbol	Gene Name	Fold Change	Confirmation
Decreased			
ERBB3	**Erb-B2 receptor tyrosine kinase 3**	**0.38**	PCR array
GDNF	**Glial cell line–derived neurotrophic factor**	**0.40**	PCR array
MYH1	**Myosin, heavy chain 1**	**0.03**	
MYH13	Myosin, heavy chain 13	0.02	Proteomics
MYH14	**Myosin, heavy chain 14**	**0.45**	
MYO18B	**Myosin XVIIIB**	**0.38**	
Increased			
BMP6	**Bone morphogenetic protein 6**	**2.31**	
COL1A1	Collagen, type I, alpha 1	20.31	
COL1A2	Collagen, type I, alpha 2	12.10	
COL4A4	**Collagen, type IV, alpha 4**	**2.78**	
COL6A1	Collagen, type VI, alpha 1	3.12	
COL6A2	Collagen, type VI, alpha 2	5.31	Proteomics
COL8A1	Collagen, type VIII, alpha 1	7.51	
COL8A2	Collagen, type VIII, alpha 2	11.48	
COL11A1	Collagen, type XI, alpha 1	14.08	
COL11A2	Collagen, type XI, alpha 2	10.62	
COL12A1	Collagen, type XII, alpha 1	7.62	Proteomics
COL16A1	**Collagen, type XVI, alpha 1**	**2.06**	
CTGF	Connective tissue growth factor	7.00	PCR array
CXCR4	C-X-C chemokine receptor type 4	5.15	PCR array
CXCR7	C-X-C chemokine receptor type 4	4.03	
DCN	**Decorin**	**2.41**	Proteomics
DDR2	Discoidin domain receptor 2	3.49	PCR array
FGFR1	**Fibroblast growth factor receptor 1**	**3.06**	
FGF7	**Fibroblast growth factor 7**	**2.43**	
FGF9	**Fibroblast growth factor 9**	**2.19**	
IL15	**Interleukin 15**	**2.07**	
IL7R	**Interleukin 7 receptor**	**2.35**	
LUM	**Lumican**	8.21	
MAPK1	**Mitogen-activated protein kinase 1**	**1.96**	
MYH3	Myosin heavy chain 3	7.30	
NOTCH2	Neurogenic locus notch homolog 2	3.32	PCR array
TGFB1	**Transforming growth factor beta 1**	**2.15**	PCR array
TNC	Tenascin C	5.97	
TIMP1	**Tissue inhibitor of metalloproteinase 1**	**2.87**	PCR array
TPM4	**Tropomyosin 4**	**2.21**	

Bold font: 2- to 3-fold change; regular font: more than 3-fold change (Ref. 21).

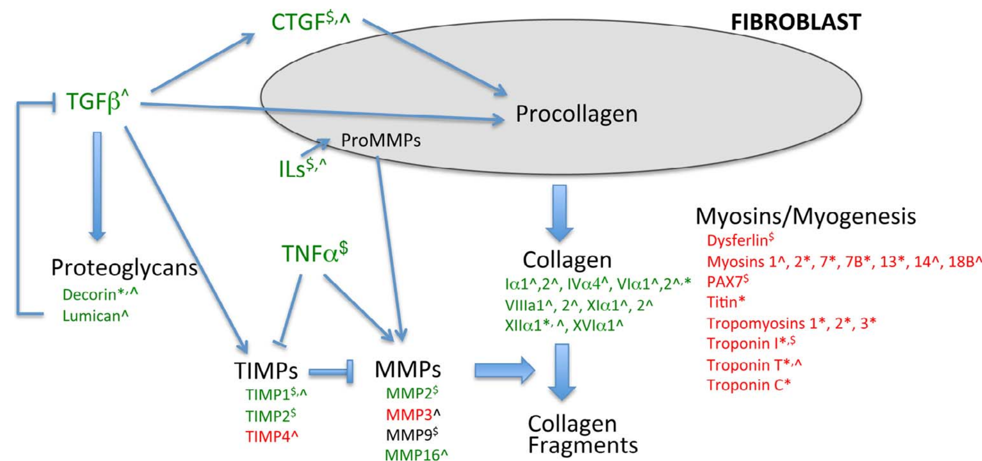

Figure 3. Synopsis of collagen, myosin, and proteoglycan signaling pathways in fibroblasts based on previous studies[23,24,30] (⊣, inhibition; →, stimulation), with our data on changes in protein composition and gene expression from extraocular muscles included. *Green*: upregulation; *red*: downregulation. CTGF, connective tissue growth factor; IL, interleukin; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha. Sources of data: *, from proteomics (current study); ^, from microarray[21]; $, from PCR array (current study).

tion-related proteins that included troponins, tropomyosins, and titin (Table 1). Titin confers passive elasticity to muscles[40–43]; accordingly, its downregulation may impact and compromise EOM stiffness. Analysis of mutant tropomyosins (causing nemaline myopathy) revealed reduced isometric force when measured in muscles of less than optimal length.[44] Collectively, these studies indicate that the motor protein composition of strabismic muscles is substantially altered, likely with functional consequences.

Myogenesis/Repair

Previous studies have shown that gene expression of proteins related to myogenesis, and specifically markers for activated satellite cells, such as *PAX7* and *MYOD1*,[32,45] were also altered in the strabismic condition.[33,34] While levels of these proteins were too low for quantification in our proteomic samples, our current study's and other studies' gene expression data confirmed that *PAX7* and *MYOD1* were significantly reduced at the transcript level.[22]

Collagens and Other ECM Molecules

Collagens are the main constituent of tendon and also present in muscle.[23] The composition of the ECM in tendons is crucial for force transmission and function.[24] Collagen types I, III, IV, and VI have been described in human EOMs at the protein level.[13,28] According to previous work using histology and immunolabeling, collagen (types I, IV, and VI) was increased in strabismic EOMs.[13,15] In our hands, collagen XII (and in some experiments, collagen VI, Supplementary Table S5) was increased in quantity in our proteomics analysis (Table 1), and these and additional collagens had increased gene expression levels (Table 3). The collagen receptor DDR2 was also increased at the gene expression level. Collagen synthesis and degradation are known to be controlled by growth factors (TGFβ, CTGF, TNFα), through collagenases (MMPs) and their inhibitors (TIMPs), as summarized in Figure 3. Consistent with reports of increased collagen,[13,15,19] we found that expression of CTGF, TNFα, and, to a lesser extent TGFβ was increased in strabismic EOMs. Transforming growth factor β can directly or via CTGF increase collagen synthesis, as well as via TIMPs (that inhibit collagenases).[23] We found that *TIMP1* and *TIMP2* were upregulated in strabismic EOMs. At the same time, some collagenases (*MMP2*) were also increased. Simultaneous or near-simultaneous activation of collagen degradation and synthesis is a common occurrence.[23] Matrix metalloproteinases are formed from ProMMPs that may be stimulated by interleukins. Indeed, some interleukins and their receptors (*IL7*, *IL7R*, *IL10RA*) were upregulated at the gene expression levels, and some of these increases were independently confirmed by microarray. The composition of muscle and tendon is crucial for muscle function and therefore tightly regulated, so muscle function can adapt to altered load or demand.[23,24,41] Proteoglycans such as decorin (increased in strabismic EOMs) are known to play an important role in myogenesis and muscle/tendon structure,[30] by interacting with TGFβ, CTGF, and collagens, by binding and inhibiting myostatin,[46] by regulating myofiber diameter, and by inhibiting angiogenesis via vascular endothelial growth factor (VEGF) receptors.

Signaling Molecules Involved in Muscle and ECM Adaptation

Several signaling systems have been implicated to play major roles in ECM-mediated muscle plasticity. These include signaling pathways activated by CTGF, glial cell line–derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF1), neurotrophins (BDNF), TGFβ, TNF, and VEGF. We here summarize how these key signaling molecules (ligands) behaved in strabismic horizontal eye muscles in terms of gene expression, and how our data relate to results from previous studies.

GDNF is relatively abundant in EOMs.[47] In animal models, it was shown to control the speed of EOM contraction[47] and induce strabismus when applied unilaterally in excess (McLoon LK, et al. *IOVS* 2016;57:ARVO E-Abstract 1395). *GDNF* was substantially downregulated in strabismic muscles, consistent with the report of a significantly slower contraction speed of strabismic muscle.[48]

Insulin-like growth factor 1 is an established growth factor that induces EOM hypertrophy in animal models.[49–51] However, IGF1 gene expression studies in strabismic human EOMs have yielded complex results, with microarrays showing no change,[21] qPCR showing upregulation[21] or a mixed result (some up, most down),[22] and now in PCR arrays overall no significant change (Table 2). Insulin-like growth factor 1 is a

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major factor that regulates EOM contractile strength, as demonstrated by effects of endogenous IGF1[49] and of exogenous IGF1,[50] as well as the fact that exogenous IGF1 can induce strabismus.[51] Variability in expression levels of IGF1 in strabismic muscles likely reflects variability in causes and/or phases of strabismus,[12] or a mismatch between gene expression and protein levels.[31,52]

Neurotrophins such as BDNF are expressed in EOMs and are known to promote survival of oculomotor neurons.[53,54] Unilateral oversupply of exogenous BDNF to a horizontal EOM did not induce strabismus in a primate animal model.[55] In our samples, BDNF gene expression was not significantly changed, while both specific receptors for NGF and BDNF, trkA (NTRK1) and trkB (NTRK2), were upregulated in strabismic muscles (Table 2), consistent with our previous microarray report.[21]

The TGFβ superfamily is a large muscle-relevant family of ligands including TGFβ and bone morphogenetic proteins (BMPs). We found that expression of TGFβ and BMP4 may be slightly increased, without statistical significance in the PCR arrays, but with a 2-fold increase for *TGFB1* with microarrays. This was surprising, since TGFβ is known to be a key regulator of collagen (signaling in part via CTGF, Fig. 3), collagens are known to be mostly upregulated in strabismic EOMs, and TGFβ and BMP4 both decrease muscle force when applied exogenously to EOMs.[56] Connective tissue growth factor was upregulated in strabismic muscles, consistent with our previous microarray study,[21] possibly due to expression in perimysial fibroblasts, where CTGF is heavily expressed in mouse EOMs.[57]

Tumor necrosis factor is considered a major regulator of skeletal muscle atrophy and weakness.[58] We found that both TNF and the TNF receptor (*TNFRSF11B* = osteoprotegerin, a collagen-interacting protein) were upregulated, consistent with a previous report based on microarray.[21] This receptor may be expressed primarily in perimysial fibroblasts, as shown for murine EOMs.[57]

Vascular endothelial growth factor A (VEGFA) regulates angiogenesis of skeletal muscle (and presumably of EOMs).[59] *VEGFA* appeared slightly downregulated (but in our data set statistically insignificant) in strabismic EOMs, consistent with a known decreased capillary density in strabismic EOMs.[10,48]

Localization of ECM Signaling: Is Tendon More Important Than Muscle?

There is a growing appreciation that connective tissue, including ECM and fibroblasts surrounding the EOMs, is critical for the function of EOMs and ocular alignment.[21,57,60,61] Our work provides novel support for this notion: The large majority of signaling molecules relevant for EOM plasticity and adaptation were expressed in the tendon component of the EOM rather than the muscle itself. This suggests that a major fraction of ECM-relevant signaling takes place in the myotendinous transition area and the tendon/connective tissues rather than within the muscle tissue proper—further emphasizing the importance of the connective tissues.[57,61] In order to use growth factors as a new or accessory strategy for treating underacting and overacting strabismic muscles, as has been suggested,[51,56,62] it is crucial to understand which signaling molecules control properties of muscle contractile force and contraction speed as well as stiffness and elasticity of the EOMs and their connective tissues. Our study was designed to make progress in defining normal and abnormal gene and protein expression in human strabismic EOMs and their connective tissues.

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