An Altered Phenotype in a Conditional Knockout of Pitx2 in Extraocular Muscle


PURPOSE. To determine the temporal and spatial expression of Pitx2, a bicoid-like homeobox transcription factor, during postnatal development of mouse extraocular muscle and to evaluate its role in the growth and phenotypic maintenance of postnatal extraocular muscle.

METHODS. Mouse extraocular muscles of different ages were examined for the expression of Pitx2 by RT-PCR, q-PCR, and immunostaining. A conditional mutant mouse strain, in which Pitx2 function is inactivated at postnatal day (P0), was generated with a Cre-loxP strategy. Histology, immunostaining, real-time PCR, in vitro muscle contractility, and in vivo ocular motility were used to study the effect of Pitx2 depletion on extraocular muscle.

RESULTS. All three Pitx2 isoforms were expressed by extraocular muscle and at higher levels than in other striated muscles. Immunostaining demonstrated the presence of Pitx2 mainly in extraocular muscle myonuclei. However, no obvious expression patterns were observed in terms of anatomic region (orbital versus global layer), innervation zone, or muscle fiber types. The mutant extraocular muscle had no obvious pathology but had altered muscle fiber sizes. Expression levels of myosin isoforms Myh1, Myh6, Myh7, and Myh13 were reduced, whereas Myh2, Myh3, Myh4, and Myh8 were not affected by postnatal loss of Pitx2. In vitro, Pitx2 loss made the extraocular muscles stronger, faster, and more fatigable. Eye movement recordings found saccades to have a lower peak velocity.

CONCLUSIONS. Pitx2 is important in maintaining the mature extraocular muscle phenotype and regulating the expression of critical contractile proteins. Modulation of Pitx2 expression can influence extraocular muscle function with long-term therapeutic implications. (Invest Ophtalmol Vis Sci. 2009;50: 4531–4541) DOI:10.1167/iovs.08-2950

Extraocular muscle is fundamentally distinct from other skeletal muscle and demonstrates specific anatomic divisions, unique muscle fiber types, diverse myosin isoform expression patterns, and a distinct genomic profile, as well as a differential involvement by neuromuscular disorders. Its unique properties are driven by the requirements of the visual system for rapid, coordinated eye movements as well as fatigue resistance.1,2 The maintenance of the mature extraocular muscle phenotype relies on cell-autonomous and non–cell-autonomous regulatory mechanisms. Dissection of these regulatory mechanisms may make it possible to exploit them to modify extraocular muscle contractile properties and influence eye movements. Achievement of such a goal may lead to treatments for strabismus and other disorders of ocular motility. Appreciation of phenotypic regulation may also lead to an understanding of disease characteristics, such as the sparing of extraocular muscle by most muscular dystrophies3 and their preferential involvement in Graves’ ophthalmopathy4 and neuromuscular transmission disorders, such as myasthenia gravis.5

Signaling cascades controlled by transcription factors direct extraocular muscle expression of a diverse array of traits that adapt to the wide dynamic physiologic range required by eye movement control systems. The paired-like homeodomain transcription factor 2 (Pitx2) plays a critical developmental role in formation of extraocular muscle.6–10 The Pitx2 protein was detected as early as E13.5 in mouse extraocular muscle and Pitx2 expression by extraocular muscle precursors is essential for early development, as deletion by homologous recombination leads to complete agenesis of mouse extraocular muscle.6 Haploinsufficiency of Pitx2 has also been identified as the cause of the human disorder Axenfeld-Rieger syndrome, in which patients exhibit abnormal development of the anterior segment and are at high risk of glaucoma. The degree of ocular motility or extraocular muscle abnormalities in this syndrome have not been well-characterized,11 and their exact relationship to the Pitx2 deficit is uncertain, since visual loss itself could alter extraocular muscle development.12–14

Genomic profiling demonstrated that Pitx2 is expressed at high levels in adult rodent extraocular muscle.15 Thus, we hypothesized that Pitx2 plays a role in maintaining the mature extraocular muscle phenotype. To lay the groundwork for testing the hypothesis, we examine temporal expression pattern of Pitx2 mRNA and Pitx2 protein distribution in mature mouse extraocular muscle. We then tested our hypothesis directly by characterizing extraocular muscle in a mouse strain with a conditional knockout of Pitx2, which allows for the normal expression of Pitx2 to occur until approximately P0, but then is absent in the adult.

METHODS

Animal Husbandry

Two mouse strains were crossed to generate the Pitx2 conditional knockout mice: The muscle creatine kinase (Mck)-Cre mouse strain was obtained from Ronald Kahn (Harvard University).16 The Mck-Cre mice, which are backcrossed to the C57BL/6 for more than three
generations, express CRE recombinase under the direction of the Mck promoter.16 The Pitx2\textsuperscript{loxP/loxP} mouse strain, in which the DNA binding homeodomain exon 4 of the Pitx2 gene is flanked with the LoxP sequence, was obtained from Philip Gage (University of Michigan).17 MCK-CRE is expressed predominantly in skeletal and cardiac muscle at the time of normal expression of the Mck gene, which in mice is around the time of birth.18,19 In rodents, Mck expression in extraocular muscle is lower than in cardiac and limb skeletal muscle20,21 but is still sufficient to trigger loxP excision of Pitx2 within the extraocular muscle. Genotyping was performed by PCR using genomic DNA isolated from tail tips. Identical PCR were also used to confirm the excision of genomic DNA isolated from extraocular muscle of mice that were both Pitx2\textsuperscript{loxP/loxP} and Cre positive (referred to as the conditional mutant, Pitx2\textsuperscript{Cre+}) and Pitx2\textsuperscript{A/} mice; their littermates Pitx2\textsuperscript{loxP/+} mice were used as control animals. Animals were housed and handled in accordance with National Institutes of Health (NIH) guidelines for animal care. All procedures involving mice were approved by Institutional Animal Use and Care Committees at Case Western Reserve University, University of Kentucky, and Saint Louis University. All experiments were conducted in accordance with the principles and procedures established by the NIH and the Association for Assessment of Laboratory Animal Care and in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Tissue Preparation and General Histology

Extraocular muscle and other muscles were dissected from C57BL/6 mice after euthanatization by CO\textsubscript{2} asphyxiation at postnatal day (P)0, 3 weeks, and 6 weeks and from Pitx2\textsuperscript{loxP/loxP} mice and control littermates Pitx2\textsuperscript{loxP/+} at 3 weeks, 6 weeks, and 3 months of age. Pitx2\textsuperscript{loxP/loxP} mice were not studied at P0 because such mice would not have complete Cre-mediated recombination. After dissection, the muscles were immediately frozen in liquid N\textsubscript{2}-cooled 2-methylbutane and stored at −80°C until use. Ten-micrometer sections were prepared and analyzed with hematoxylin and cosin or Grumori’s trichrome.22,23

Antibodies

The P2Y4 antibody (provided by TH) or P2R10 (provided by Philip Gage) was used to identify Pitx2 protein expression. The antibody detects an epitope of Pitx2 encoded by a region in exon 4 that precedes the homeodomain and common to all three isoforms. P2R10 has been used in previous studies of several tissues, including muscle.24–28 The sources and working dilutions for antibodies against myosin heavy chain isoforms were as follows: mouse anti-extraocular muscle-specific myosin29 (1:100, provided by Neal Rubinstein, University of Michigan); anti-capital muscle myosin, C\textsuperscript{a} (mice); their littermates Pitx2\textsuperscript{loxP/+} mice were used as control animals). Animals were housed and handled in accordance with National Institutes of Health (NIH) guidelines for animal care. All procedures involving mice were approved by Institutional Animal Use and Care Committees at Case Western Reserve University, University of Kentucky, and Saint Louis University. All experiments were conducted in accordance with the principles and procedures established by the NIH and the Association for Assessment of Laboratory Animal Care and in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

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RNA Extraction and RT-PCR

Total RNA was extracted from the tissues (TRizol reagent; Invitrogen) and reverse transcription was performed (Superscript First-Strand Synthesis System; Invitrogen) according to the manufacturer’s instructions. Amplification of cDNA was performed (TaqMan cDNA polymerase; Invitrogen). Two sets of PCR reactions were designed to detect evidence of recombination with a common reverse primer (5’ GCC AGG CTC GAG TTA CAT G7 3’) from the C-terminal region of Pitx2. Set 1 with the forward primer (5’ GCC AGG CGT TGA ATG TCT CTG 3’) from the N-terminal region of the Pitx2c isoform amplified a truncated product of 590 bp from extraocular muscle tissue of Pitx2\textsuperscript{loxP/loxP} mouse compared to the 798-bp wild-type product. Set 2 with the forward primer (5’ AAG ATG AGA AGG GCC AGT TGA ATG TCT CTG 3’) from within the homeodomain region produced no product from extraocular muscle tissue of Pitx2\textsuperscript{loxP/loxP} mice, while amplification from Pitx2\textsuperscript{loxP/+} control mice produced a 658-bp product. Aliquots of PCR products were typically resolved by using 1% agarose gel electrophoresis with ethidium bromide (Sigma-Aldrich) for visualization and confirmation of product size.

Quantitative Real-Time PCR (qPCR)

Transcript-specific primers (Supplementary Table S1, http://www.iovs.org/cgi/content/full/50/10/4531/DC1) were designed (Primer Express 2.0 software; Applied Biosystems, Inc. [ABI], Foster City, CA) and specificity confirmed by BLAST (www.ncbi.nlm.nih.gov/blast) provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD). qPCR was performed with SYBR green PCR core reagent (ABI) in a 2×-µL reaction volume, with a sequence detection system (Prism model 7000; ABI). GAPDH was used as an internal positive loading control. Relative transcript abundance was normalized to the amount of GAPDH and calculated as the ratio to a normalized to the amount of GAPDH and calculated as the ratio to a keyhousekeeping gene using the 2\textsuperscript{−ΔΔCT} method.30

Contractility of Extraocular Muscle

The contractile function of control and conditional mutant extraocular muscles was studied as described previously.14 Whole extraocular muscles from Pitx2\textsuperscript{loxP/loxP} and age-matched Pitx2\textsuperscript{loxP/+} mice were

10% normal goat serum. To assess normal expression of Pitx2 and a correlation with known differentially expressed proteins, the sections were immunostained with the P2Y4 antibody, to detect Pitx2 expression, or doubly labeled with mouse anti-dystrophin, Alexa Fluor 488 labeled α-bungarotoxin, or MHC isoform antibodies. Cryosections from Pitx2\textsuperscript{loxP/loxP} and Pitx2\textsuperscript{loxP/+} mice were immunostained with antibodies against MHC isoforms. Sections were examined with a fluorescence microscope (Diaphot Nikon Instruments Inc., Melville, NY) and images captured with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI) and software (SpotAdvanced; Diagnostic Instruments) before processing with Photoshop (San Jose, CA).
isolated and firmly attached to a force transducer (AE801; SensoNor, Horten, Norway) and the movable arm of a servomotor (Aurora Scientific, Aurora, ON, Canada) and positioned between platinum electrodes inside a muscle chamber. The chamber was superfused with a physiological salt solution (in mM): 137 NaCl, 5 KCl, 2.0 CaCl2, 1.0 MgSO4, 1.0 Na2HPO4, 24 NaHCO3, 11 glucose, and 0.026 α-tubocurarine, bubbled with a 95% O2 and 5% CO2 gas mixture to maintain pH at 7.4 at 25°C. The muscles were stretched to the length that gave maximum tetanic force (optimal length, L0). Force measurements (P0, in Newtons) were normalized to muscle cross-sectional area (in square centimeters). The unloaded shortening velocity (V0 in L0 seconds−1) was determined with slack tests. Fatigue was induced by stimulating muscles at a frequency giving approximately one half of maximum tetanic force (50–70 Hz) for 500 ms, followed by a 1.5-second interval between contractions until force declined to 50% of initial force or 10 minutes. All results are presented as the mean ± SD of eight muscles per group. Statistical significance was determined at the 95% confidence level by using Student’s t-tests for paired samples as indicated; the treatment effect in the fatigue runs was determined by analysis of variance.

**Ocular Motility Recording**

Eye movement recordings were performed as described previously with the modification that since recordings were performed in the light only, animals were not treated with the ophthalmic physostigmine as ordinarily administered in our studies to limit pupil dilation in darkness, and this limits any concerns that the physostigmine may have subtle effects at the neuromuscular junction. Data were collected to generate plots of saccade peak eye velocity versus saccade amplitude, the saccade main sequence. Two different stimuli to generate plots of saccade peak eye velocity versus saccade amplitude in darkness, and this limits any concerns that the physostigmine as ordinarily administered in our studies to limit pupil dilation in darkness, and this limits any concerns that the physostigmine may have subtle effects at the neuromuscular junction. Data were collected to generate plots of saccade peak eye velocity versus saccade amplitude, the saccade main sequence. Two different stimuli to generate the saccades were used. In the standard main-sequence stimulus, a turntable was rotated manually to produce saccades largely initiated from near-stationary eye positions. In the fatiguing stimulus, saccades were generated by rotating the animal sinusoidally at 0.2 Hz, unlike other skeletal muscle, has a great deal of regional and fiber-type and neuromuscular synaptic diversity, and there-

**RESULTS**

**Expression of Pitx2 in Extraocular Muscle and Other Muscle**

The Pitx2 gene utilizes an upstream promoter to express the Pitx2a and Pitx2b isoforms that differ from each other as a result of alternative splicing, and Pitx2c is expressed by a downstream promoter located in intron 3. Alternative translation initiation (Pitx2Cbeta) and alternative mRNA splicing (Pitx2b2) variants were identified but not evaluated in the present study. Transcripts of all three murine isoforms were expressed in mature extraocular muscle (Fig. 1A) and show a distinct temporal expression pattern. Postnatal Pitx2c expression declines in extraocular muscle, in marked contrast to Pitx2a and Pitx2b, which remain relatively constant, with the Pitx2a isoform being the most abundantly expressed isoform (Fig. 1B). We compared Pitx2 isoform expression across several adult skeletal muscles and heart (Fig. 1C). We arbitrarily chose heart to compare the differences. Extraocular muscle expressed all three isoforms at much higher levels than did any of the other muscles.

**Temporal and Spatial Distribution of Pitx2 within Extraocular Muscle**

We evaluated the temporal and spatial distribution Pitx2 protein in postnatal mouse extraocular muscle. Extraocular muscle, unlike other skeletal muscle, has a great deal of regional and fiber-type and neuromuscular synaptic diversity, and there-

**TABLE 1. Fold Differences of Pitx2 Isoforms among Muscles**

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Pitx2a</th>
<th>Pitx2b</th>
<th>Pitx2c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.0 ± 0.18</td>
<td>1.0 ± 0.13</td>
<td>1.0 ± 0.16</td>
</tr>
<tr>
<td>Masseter</td>
<td>1.4 ± 0.1*</td>
<td>-6.0 ± 0.5***</td>
<td>-3.9 ± 2.1*</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>4.0 ± 0.1***</td>
<td>-3.9 ± 0.9***</td>
<td>-1.2 ± 0.2***</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>3.7 ± 0.3***</td>
<td>-2.7 ± 0.4***</td>
<td>-2.2 ± 0.8**</td>
</tr>
<tr>
<td>Quadriceps</td>
<td>1.1 ± 0.1</td>
<td>1.8 ± 0.3*</td>
<td>-3.8 ± 0.9***</td>
</tr>
<tr>
<td>Tibialis anterior</td>
<td>1.2 ± 0.04</td>
<td>-8.4 ± 1.8***</td>
<td>-1.8 ± 0.1***</td>
</tr>
<tr>
<td>Extraocular muscle</td>
<td>84.7 ± 5.0***</td>
<td>17.9 ± 1.4***</td>
<td>9.2 ± 2.5***</td>
</tr>
</tbody>
</table>

**FIGURE 1.** Expression of all three Pitx2 isoforms in postnatal extraocular muscle. (A) RT-PCR with Pitx2a, Pitx2b, and Pitx2c isoform-specific primers on mRNA isolated from adult extraocular muscle. H2O represents cDNA blank control with all three pairs of primers mixed. (B) Relative expression levels of the three Pitx2 isoforms compared to GAPDH in P0, P21, and adult extraocular muscle with qPCR analysis. Arbitrary units were used. Pitx2c expression declined over time, whereas Pitx2a and Pitx2b remained about the same level. Pitx2a was the most abundantly expressed isoform in adult extraocular muscle. (C) Table represents fold differences of Pitx2 isoform expression among striated muscles by qPCR. Data are expressed as the mean ± SD of the difference compared with expression in heart; n = 3. *P < 0.05; **P < 0.01; ***P < 0.001.
Therefore, we evaluated whether a correlation of Pitx2 expression could be made with extraocular muscle anatomic divisions (global versus orbital, midportion versus distal ends), myosin isoform expression, or innervation. Our hypothesis was that Pitx2 would be associated with anatomic region, known differentially regulated muscle proteins, or innervation patterns. Pitx2 was expressed at P0 and appeared predominantly in the orbital layer (Fig. 2A), starting to be more expressed in the global layer at 3 weeks (Fig. 2B) and evenly distributed in global and orbital layer at adult (Fig. 2C). Cross-section of extraocular muscle (Fig. 2D) was immunolabeled with antibody to dystrophin (green) to reveal the myofiber boundary and with antibody to Pitx2 (red). Counterstain with DAPI (blue) identifies all nuclei. Pitx2 expression is localized within the boundary of dystrophin immunoreactivity, indicating that the majority of Pitx2 immunoreactive nuclei (showing purple) are myonuclei. Occasional Pitx2 immunoreactive nuclei were outside the sarcolemma, but closely adjacent, and are likely to be satellite cells (arrows). Longitudinal section of adult extraocular muscle (Fig. 2E) was immunolabeled the same way as in (Fig. 2D). As appreciated in (Fig. 2D), not all myonuclei are Pitx2 immunoreactive as evidenced by DAPI-only positive nuclei (arrows). Scale bar: (Fig. 2A–C) 100 μm; (Fig. 2D, E) 50 μm.

Figure 2. Differential expression of Pitx2 by extraocular muscle. Immunostaining with antibody against Pitx2 showed that Pitx2 was mostly expressed in the orbital layer at P0 (A), starting to be more expressed in the global layer at 3 weeks (B) and evenly distributed in global and orbital layer at adult (C). Cross-section of extraocular muscle (D) was immunolabeled with antibody to dystrophin (green) to reveal the myofiber boundary and with antibody to Pitx2 (red). Counterstain with DAPI (blue) identifies all nuclei. Pitx2 expression is localized within the boundary of dystrophin immunoreactivity, indicating that the majority of Pitx2 immunoreactive nuclei (showing purple) are myonuclei. Occasional Pitx2 immunoreactive nuclei were outside the sarcolemma, but closely adjacent, and are likely to be satellite cells (arrows). Longitudinal section of adult extraocular muscle (E) was immunolabeled the same way as in (D). As appreciated in (D), not all myonuclei are Pitx2 immunoreactive as evidenced by DAPI-only positive nuclei (arrows). Scale bar: (A–C) 100 μm; (D, E) 50 μm.

Figure 3. No obvious pattern was observed between Pitx2 expression and neuromuscular junction or myosin-expressing muscle fibers. Cross-sections of normal adult extraocular muscle were doubly stained with rabbit anti-Pitx2 (red) and mouse anti-extraocular muscle MHC (A, green) or Alexa Fluor 488-conjugated bungarotoxin (B, green). Nuclei were revealed by counterstain with DAPI (blue). Pitx2 was colocalized in nuclei and thus showed purple. White arrows: a muscle fiber positive for both EOM-MHC and Pitx2 in (A) and to a neuromuscular junction that is also positive for Pitx2 in (B). Yellow dashed arrows: a muscle fiber that is positive only for EOM-MHC in (A) and a neuromuscular junction that is not stained with Pitx2 in (B). Scale bar, 50 μm.
muscle, Pitx2 immunoreactivity was identified in the global and orbital regions with no clear predominance (Fig. 2C). Furthermore, there was no predominance of Pitx2 immunoreactivity in the midsection or distal ends of the muscle.

Pitx2 immunoreactivity was localized exclusively to the nucleus (Fig. 2D), which is consistent with its role as a transcriptional factor. Most Pitx2-positive nuclei appeared to be myonuclei, as evidenced by their localization within the ring of dystrophin immunoreactivity, which marks the periphery of the muscle fibers. We found a few immunoreactive nuclei immediately adjacent but outside the myofibers (Fig. 2D, arrows), and those were likely to be nuclei of satellite cells. Pitx2 immunoreactive nuclei were identified along the length of fibers (Fig. 2E); however, all myonuclei of a given fiber were not uniformly Pitx2 immunoreactive (Fig. 2E, arrows).

Because the Myb13 gene (extraocular muscle–specific MHC or EOM-MHC) does contain a potential Pitx2 binding domain in its cis-regulatory domain, we expected that Pitx2 expression would be associated with EOM-MHC expression. However, no such association was found. Pitx2-positive (Fig. 3A, arrow) and -negative (Fig. 3A, yellow dashed arrow) nuclei were found in EOM-MHC-expressing fibers. The developmental (d)MHC (Myh3) is exclusively expressed in the orbital layer of rat extraocular muscle and we found the same to be true in mouse. Again, both Pitx2-positive and -negative dMHC fibers were found in normal extraocular muscle serial sections (data not shown). Fibers expressing the type I MHC and type II MHC also contained a mixture of Pitx2 immunoreactive and negative nuclei (data not shown). Therefore, in mouse extraocular muscle, there is no correlation between Pitx2 expression and the MHC isoforms examined.

We used labeled α-bungarotoxin to mark the location of neuromuscular junctions and evaluated whether Pitx2 expression was related to innervation. Again, both Pitx2-positive (Fig. 3B, arrow) and -negative (Fig. 3B, yellow dashed arrow) endplates were found, indicating no relation existed between the neuromuscular junctions and Pitx2 immunoreactivity.

In summary, Pitx2 was present in the extraocular muscle myonuclei, but not in a fiber-type or region-specific pattern. Furthermore, there was no specificity with respect to MHC expression, point of innervations, or whether a fiber was singly or multiply innervated.

**Generation of Pitx2 Conditional Knockout Mouse**

We exploited Cre-loxP-mediated gene recombination to inactivate Pitx2 in postnatal extraocular muscle. The goal was to allow normal prenatal extraocular muscle development and then turn off Pitx2 expression at birth. Figure 4A depicts a typical result of PCR amplification of DNA from a Pitx2flox/flox mouse showing a single floxed band of 1092 bp. The same PCR amplification reaction of DNA extracted from extraocular muscle of Pitx2flox/flox/mice, which also express MCK-CRE demonstrated that the majority of DNA was recombined, and amplification produced a 495-bp mutant band (Fig. 4B, lane 1). As expected, RT-PCR using a forward primer binding within the homeodomain region and a reverse primer to the C-terminal common region of Pitx2c isofrom did not amplify any product (Fig. 4C, lane 2) from extraocular muscle tissue of Pitx2flox/flox/mice, which also express MCK-CRE demonstrated that the majority of DNA was recombined, and amplification produced a 495-bp mutant band (Fig. 4B, lane 1). As expected, RT-PCR using a forward primer binding within the homeodomain region and a reverse primer to the C-terminal common region of Pitx2c isofrom did not amplify any product (Fig. 4C, lane 2) from extraocular muscle tissue of Pitx2flox/flox/mice. RT-PCR with the forward primer from the N-terminal region of Pitx2c isofrom and the reverse primer from the C-terminal common region produced a truncated product of 590 bp (Fig. 4D, lane 2) from the extraocular muscle of Pitx2flox/flox/mice.

We examined the expression of Pitx2 protein in extraocular muscle of Pitx2flox/flox/mice at p0, p21, and 3 months of age with the antibody P2R10. At p0 there was no difference in the number and pattern of Pitx2-positive nuclei between Pitx2flox/flox/mice and Pitx2flox/flox/mice. By p21, a dramatic reduction in the number of positive nuclei was observed, and at 3 months only rare nuclei were immunoreactive in the Pitx2flox/flox/mice (data not shown). Thus, we successfully generated a Pitx2 conditional mutant mouse strain with no functional Pitx2 RNA or protein.

**General Phenotypic Evaluation**

The Pitx2flox/flox/mice demonstrated no gross phenotypic differences from control littersmates. Biweekly observation of their activity levels showed no differences among littersmates. Body weights were not different between Pitx2flox/flox/mice and...
Pitx2flox/flox mice from 3 weeks (males, 10.37 g ± 0.49 g vs. 10.1 g ± 0.8 g, \(P = 0.78\); females 10.9 g ± 2.0 g vs. 9.8 g ± 2.0 g, \(P = 0.32\)) to 6 months (males, 34.4 g ± 1.0 g vs. 34.5 g ± 1.1 g, \(P = 0.97\); females 26.4 g ± 3.9 g vs. 25.6 g ± 1.7 g, \(P = 0.61\)) of age. Extensive necropsies by an Animal Research Center veterinarian of three Pitx2flox/flox mice and three Pitx2flox/flox littermates at 3 months of age found no gross abnormalities. We did identify cardiac abnormalities through specialized assessment, which will be described in a separate publication.

Histology of Pitx2flox/flox Extraocular Muscle

We performed standard histologic evaluation of extraocular muscle at 3, 8, and 12 weeks of age. Extraocular muscle had no signs of disease; distinct global and orbital regions were identified (Fig. 5A, 5B). The number of fibers was no different in extraocular muscle from Pitx2flox/flox and Pitx2flox/flox mice in either the global layer (control, 337 ± 19; mutant, 354 ± 27; \(P = 0.43\)) or the orbital layer (control, 525 ± 53; mutant, 513 ± 62; \(P = 0.81\)). Central myonuclei, a sensitive marker of muscle disease, were not observed.

An obvious alteration of extraocular muscle fiber size was present in 8-week- and 3-month-old Pitx2flox/flox mice (Figs. 5B, 5D). Some muscle fibers appeared to be larger while others appeared smaller than those in control mice (Fig. 5A, 5C). An assessment of fiber size distribution found a shift toward smaller fibers in both the global and orbital layers of Pitx2flox/flox extraocular muscle (Fig. 5E). Within the global layer, however, a small percentage of fibers shifted toward larger sizes. Extraocular muscles of 3-week-old mice.

**FIGURE 5.** Altered extraocular muscle fiber sizes in Pitx2flox/flox. H & E (A, B) and Gomori’s trichrome staining (C, D) of extraocular muscle sections from 3-month-old Pitx2flox/flox (B, D) and littermate control Pitx2flox/flox (A, C) showed no centralized nuclei. However, in the Pitx2flox/flox mice, the muscle fiber size was altered. Fiber size measurement (E) confirmed that myofiber sizes for both orbital and global layers are significantly smaller than in the control extraocular muscle (\(P < 0.001\) for the nonparametric Kolmogorov-Smirnov two-sample test) despite the fact that a small number of fibers in the global layer in the mutant are extremely large. Scale bar, 100 μm.
were identical between \(\text{Pitx2}^{\alpha\text{floxed}}/\text{floxed}\) mice and the \(\text{Pitx2}^{\alpha\text{floxed}}/\text{floxed}\) control.

**Figure 6.** Alteration of gene expression in Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice. Cross-sections of 3-month-old extraocular muscle from Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice (B, D) and Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) control mice (A, C) were immunolabeled with antibodies specific to MHC 1 (A, B) and to EOM-MHC (C, D). The immunoreactivity was dramatically decreased for MHC 1, and moderately decreased for EOM-MHC. Scale bars, 50 \(\mu\)m. (E) Selected gene expression alterations in Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice determined by qPCR. Data represent mean ± SD of the differences between Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice versus age-matched Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice, \(n = 3\). Changes ≤ 2-fold were not considered to be of biological significance. *\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\).

**Altered Gene Expression in Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) Mice**

Myogenic regulatory factors are thought to act downstream of Pitx2 during extraocular muscle development.\(^6,34\) We measured the expression of selected myogenic regulatory factors (\(\text{Myf5}, \text{Myog}, \text{and Myod}\)) to evaluate the impact of Pitx2 loss at 3 weeks, 8 weeks, and 3 months (Fig. 6E). Transcript levels of \(\text{Myf5}, \text{Myog}, \text{and Myod}\) were decreased dramatically in the \(\text{Pitx2}^{\alpha\text{floxed}}/\text{floxed}\) mice compared with Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) littermates. We then assessed transcript levels of major MHC isoforms. \(\text{Myh} 1\) (type IIX), \(\text{Myh} 6\) (\(\alpha\)-cardiac), \(\text{Myh} 7\) (MHC 1 or slow MHC), and \(\text{Myh} 13\) (EOM-MHC) transcripts were reduced, most dramatically at 3 months. In contrast, \(\text{Myh} 2\) (2A), \(\text{Myh} 3\) (developmental), \(\text{Myh} 4\) (2B), and \(\text{Myh} 8\) (neonatal) expression were not significantly affected.

Comparison of relative expression levels of myogenic regulatory factors and \(\text{Myh}\) isoforms in extraocular muscle from Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) and Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice at 3-week, 8-week and 3-month time points revealed that genes with expression levels affected by Pitx2 fell into two categories (Fig. 7). The first category, which we defined as the direct effect, included myogenic transcription factors \(\text{Myf5}, \text{Myog}, \text{and Myod}\), whose expression was suppressed rapidly after Pitx2 loss. In the second, which we defined as the indirect effect, \(\text{Myh}\) isoforms were expressed at similar levels in both mouse strains at 3 weeks of age and then they decreased over time in the Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice.

**Myosin Heavy Chain Content**

We assessed MHC distribution by immunohistochemistry. MHC I immunoreactivity was greatly reduced in extraocular muscle from Pitx2\(^{\alpha\text{floxed}}/\text{floxed}\) mice at 3 weeks, 8 weeks (data not shown), and 3 months (Fig. 6B compared with 6A), consistent with decreased transcript abundance at this age (Fig. 6E). The distribution of type 2 MHC was not altered (data not shown) as assessed by pan-type 2 MHC antibody, although \(\text{Myh} 1\) (for MHC 2X fiber) had sevenfold reduction in its transcript level at 3 months. Furthermore, immunostaining with MHC 2A- and 2B-specific antibodies found no significant differences in the content of these isoforms at 3 weeks to 3 months of age.
between Pitx2^{ΔfloxFloX} mice and Pitx2^{ΔfloxFlox} littermates (data not shown). Consistent with the almost nine-fold reduction in Myh13 transcript level at 3 months, EOM-MHC immunoreactivity was greatly reduced in Pitx2^{ΔfloxFloX} extraocular muscle, although some positive fibers were identified (Figs. 6C, 6D). By qPCR, the transcript levels of both Myb7 and Myb13 genes were less than 1% of total myosin mRNA in wild-type mice (0.55% for Myb7 and 0.078% for Myb13 at 3 months of age). In the mutant mice, the percentage decreased to 0.014% for Myb7 and 0.004% for Myb13. Myb1, Myb2, and Myb4 are the predominant isoforms expressed making up more than 90% of all myosin gene transcripts.

**Evaluation for Cre Toxicity**

We examined whether the MCK-Cre transgene itself has an effect on the phenotypes we observed. We compared the histology, gene expression patterns, and MHC1 (Myb7) immunostaining of extraocular muscle from MCKCre/Pitx2^{+/+}, Pitx2^{ΔfloxFloX}, Pitx2^{ΔfloxFlox}, and Pitx2^{ΔfloxFlox} mice. We did not observe fiber size alterations in MCK-Cre/Pitx2^{+/+} mice; nor did we find differences in gene expression patterns by qPCR (data not shown). Expression of MHC1 (assessed by immunostaining) was identical in the extraocular muscle of MCK-Cre/Pitx2^{+/+}, Pitx2^{ΔfloxFlox}, or Pitx2^{ΔfloxFlox} mice. Therefore, the mere presence of the MCK-Cre transgene did not appear to affect extraocular muscle. The Pitx2^{ΔfloxFlox} mice, on the other hand, showed a phenotype intermediate between the Pitx2^{ΔfloxFloX} mice and their control littermates Pitx2^{ΔfloxFlox}, suggesting dose-dependent effects of Pitx2, as had been observed in developmental studies.6

**Contractile Properties of Extraocular Muscle from Pitx2^{ΔfloxFloX} Mice**

We tested Pitx2^{ΔfloxFloX} extraocular muscle function in vitro to determine the influence of the histologic and gene expression changes on contractility. The Pitx2^{ΔfloxFlox} demonstrated contractile properties similar to those reported in wild-type mice.14 As shown in Table 1, the mutant extraocular muscle had a 20% higher peak tetanic force (P_{0}) and 9% greater maximum velocity of shortening (V_{0}). In contrast, the endurance time (to a 50% reduction in force from the start of

**Table 1. Contractile Characteristics of Pitx2^{ΔfloxFlox} Extraocular Muscle**

<table>
<thead>
<tr>
<th></th>
<th>Pitx2^{ΔfloxFloX}</th>
<th>Pitx2^{ΔfloxFlox}</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{0} (N/cm²)</td>
<td>11.1 ± 0.8*</td>
<td>10.2 ± 0.6</td>
</tr>
<tr>
<td>P_{0} (N/cm²)</td>
<td>6.9 ± 0.7**</td>
<td>5.7 ± 0.5</td>
</tr>
<tr>
<td>Fatigue index (% of force at time = 0)</td>
<td>48.0 ± 4***</td>
<td>59.1 ± 6</td>
</tr>
<tr>
<td>Endurance (fatigue duration, sec)</td>
<td>555 ± 50.1*</td>
<td>600 ± 0</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD; n = 8 animals for each group.

* P < 0.05; ** P < 0.01; *** P < 0.005, compared with control littermates.

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**FIGURE 7.** Comparison of gene abundance between Pitx2^{ΔfloxFloX} and Pitx2^{ΔfloxFlox} mice. Relative expression levels were normalized to GAPDH by qPCR analysis. Arbitrary units were used for the purpose of illustration. Levels of Myb isoform expression (shown are Myb1, Myb6, and Myb13) in the Pitx2^{ΔfloxFloX} are at similar or slightly lower level to their wild-type counterparts at early time points and decreased over time, suggesting that these genes may be indirect targets of Pitx2. Levels of myogenic transcription factors (Myf5, MyoG, and MyoD) were low from early time points and stayed low throughout; therefore, they may be direct targets of Pitx2 action.
the fatigue protocol) was 555 ± 50.1 seconds for the Pitx2<sup>flox/flox</sup> extraocular muscle with four of eight specimens unable to complete the fatigue protocol compared with 600 ± 0 seconds for Pitx2<sup>flox/flox</sup> littermates, all of which completed the protocol. The force of contraction at the end of the fatigue protocol was reduced to 48.0% of initial force.

**Eye Movement Recordings**

The oculomotor consequences of the Pitx2<sup>flox/flox</sup> mutation were evaluated in vivo. Saccades require the highest levels of extracellular muscle force and are thus particularly sensitive to alterations of extracellular muscle function. Therefore, saccade performance was used to assess ocular motility and quantified saccade peak velocity versus saccade amplitude. Pitx2<sup>flox/flox</sup> mice exhibited the main sequence of the usual mouse configuration (i.e., relationship of peak velocity to saccade amplitude). Pitx2<sup>flox/flox</sup> mice had dramatically lower levels of myogenic expression postnatally did not lead to degeneration of extraocular muscle as might have been expected. In fact, the extraocular muscle of the Pitx2<sup>flox/flox</sup> mice did not demonstrate any signs of muscle disease (i.e., fiber degeneration or centrally displaced myonuclei), but rather developed a “new” extracellular muscle with distinct morphology, gene expression, and functional characteristics. The findings demonstrate that Pitx2 in the mature extraocular muscle does not fulfill a critical maintenance role, but rather modulates the extraocular muscle phenotype.

**Understanding of the transcriptional control of the mature extraocular muscle phenotype is extremely limited and is based entirely on analogy with other striated muscle and developmental studies of extraocular muscle.** Despite Pitx2’s critical importance in development and its high expression in adult extraocular muscle, it is clear from our study that other transcription factors are essential for the maintenance of the adult extraocular muscle phenotype, given that a functional extraocular muscle is produced despite the absence of Pitx2. It is possible that Pitx3 compensates for the loss of Pitx2, as appears to be the case in skeletal muscle.39 Despite our extensive evaluation of the normal expression pattern of Pitx2, a correlation of expression could not be made to global and orbital regions, fiber type specificity, points of innervations, tendon insertions, all of which are sites of regional specialization in skeletal and extraocular muscle.1,40,41 Therefore, the expression pattern provided limited insight into Pitx2’s functional role.

The rapid reduction of myogenic regulatory factors in the Pitx2<sup>flox/flox</sup> mice would suggest that Pitx2 acts upstream of them, as has been hypothesized during development (Fig. 6).17,34 This notion is speculative; however, as there is very little information on target genes for Pitx2.25,42,45 The Pitx2<sup>flox/flox</sup> mice had dramatically lower levels of myogenic regulators detected at 3 weeks compared with the controls, which we defined as supporting a direct effect of Pitx2. Myogenic regulatory factors (MyoD, Myf5, myogenin) are not regulated in the Pitx2<sup>flox/flox</sup> indicating that they have no role in compensating for the absence of Pitx2.

Pitx2 was found in the majority of myonuclei, but without a specific pattern of expression related to region, fiber-type, MHC or innervation. In skeletal muscle, the region-specific expression of certain transcripts is well appreciated—for example, subsynaptic and myotendinous nuclei have enhanced mRNA expression for proteins that are preferentially found in those regions.44,45 Pitx2 is involved in myotendinous development in the forelimb,46 and again differential expression of Pitx2 to the insertion points of the extraocular muscle could have been expected. However, the link between transcription...
at individual myonuclei and ultimate protein expression is complex.47 For example, despite the requirement of troponin across the length of the myofiber, its gene is not expressed at all myonuclei.48 If Pitx2 expression were stochastic or pulsatile, our immunohistochemical studies would reflect the snapshot of expression of the transcriptional factor, which is likely to have a short half life.

Extraocular muscle fibers are generally smaller and show a broader range of fiber size than do other skeletal muscles. In the Pitx2-deficient extraocular muscle, the global region had a distinct prominence of large fibers, but quantitative assessment found that these large fibers made up only a small percentage of the total fibers and that the fiber size distribution was skewed toward smaller fibers in both the global and orbital layers. In skeletal muscle, fiber size is controlled by intrinsic factors and factors extrinsic to the muscle (i.e., mechanical load, hormones, diffusible factors, and motoneuron activity),49 and the same appears true in extraocular muscle, although the specific mechanisms are likely to differ.1 The alteration in fiber size distribution in the Pitx22flox/floxflox indicates that Pitx2 plays a role in fiber size.

MHC expression forms the basis of fiber-type classification of skeletal muscle and is a major determinant of a muscle’s contractile properties. Extraocular muscles are unique in their expression of nearly all known MHC and the coexpression of certain MHC in a single fiber.50 Pitx22flox/floxflox mice had significant reductions in gene and protein expression of MHC-I and extraocular muscle-specific MHC, the Myb13 gene product, and no alteration in MHC-2. Figure 6 illustrates the striking dissociation in the pattern of expression of Myb1 and Myb13. Myb1 in wild-type continued to demonstrate increased expression at 3 months compared with a decrease in mutant mice, in contrast to the uniformly lower and falling level of Myb13. The Myb1 gene has Pitx2 binding sites in the promoter region, and the absence of Pitx2 would be expected to reduce Myb13 mRNA levels, as observed. Consistent with the reduction in transcript level, the protein level of extraocular muscle-specific MHC is also downregulated, although we consistently observe a few positive fibers in the orbital layer. The pattern for the extraocular muscle-specific MHC to be relatively retained is consistent with the dissociation of protein expression and regulation of MHC genes based on promoter analysis.57,50 We did not attempt to classify extraocular muscle fibers of the Pitx22flox/floxflox mice because of the diversity in fiber size and MHC content, which are major components of the generally accepted classification.1

In vitro functional studies of extraocular muscle demonstrated Pitx22flox/floxflox extraocular muscle had higher velocity of shortening and greater peak tetanic force but fatigued more rapidly. The increased shortening velocity is consistent with the observed decrease in the slow MHC isoform. Force of contraction is dependent on number of contractile units per muscle cross-sectional area. We found no evidence of an increase in the number of the Pitx22flox/floxflox extraocular muscle fibers, but the presence of the population of larger fibers could have led to higher overall force generation. We found no reduction in the connective tissue matrix leading to an improved “packing” of contractile units. Extraocular muscle is highly resistant to fatigue.1,2,51 The susceptibility to fatigue of the Pitx22floxflox extraocular muscle was significantly increased: half the Pitx22floxflox extraocular muscles did not complete our standard fatigue protocol, whereas all the control muscles did.

In contrast to the increases in maximum tension and greater fatigability observed in vitro, the eye movements recorded in vivo were slightly slower (suggesting lessened peak tensions) and no more fatigable than in control animals. Although apparently contradictory, the in vivo results could arise if, in the in vivo condition, the extraocular muscles are equilibrated at what is the fatigued state of the in vitro experiment. In other words, since the extraocular muscles are continuously activated in the in vivo preparation, they may never achieve the rested, prefatigue state found of the in vitro experiment, in which the extraocular muscles are activated only intermittently. Alternatively, the fatigability of extraocular muscles of Pitx22floxflox revealed by the in vitro assay may not occur in vivo. These results suggest the hazards of relying purely on in vitro studies to assess extraocular muscle function in studies of therapeutic manipulation.

The Pitx22floxflox mouse ablates Pitx2 expression limited to tissues that express Mck, and not other tissues, such as midbrain motor neurons, leading to the retention of normal innervations, and therefore the Pitx2 effect is only related to its loss from the extraocular muscle.57,52 This effect is an important advantage of the conditional knockout over complete knockouts of Pitx2 during development that are compromised by potential loss of innervation as a contributor to muscle agenesis. Organotypic nerve-muscle cocultures have demonstrated that extraocular muscle explants will only survive long-term culture with oculomotor motor neurons and not spinal motor neurons.53 An in vivo parallel, the Wnt1CreERT mouse, shows loss of the midbrain, including oculomotor and trochlear motor neurons, aberrant extraocular muscle innervation, and impaired myogenesis.54 The motoneuron pool-specific requirement for extraocular muscle survival makes it clear that neurotrophic mechanisms direct key features of the extraocular muscle phenotype.

The results demonstrate that Pitx2 functions to sustain a specific extraocular muscle phenotype. Its loss does not lead to degeneration of extraocular muscle—as might have been expected—but rather to altered expression of key myogenic regulatory factors and muscle specific genes and proteins. Extraocular muscle anatomy is largely maintained, but contractile function is altered consistent with alterations in myosin isoform expression. Our results demonstrate that alterations of transcriptional expression can engineer modification of extraocular muscle contraction with the ultimate expectation that appropriate targeted gene expression intervention could lead to a therapeutic intervention.

References


