Fascicular Specialization in Human and Monkey Rectus Muscles: Evidence for Anatomic Independence of Global and Orbital Layers

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Purpose. Connective tissue pulleys inflect the extraocular muscles (EOMs) and receive insertions from some fibers. The authors investigated insertions and anatomic relationships of fiber fascicles within rectus EOMs to clarify the relationship to their pulleys.

Methods. Two human and two monkey orbits were removed intact, serially sectioned in the coronal plane, histologically stained, and digitally photographed. The authors traced representative fascicles in the human medial rectus (MR) and inferior rectus and monkey lateral rectus and superior rectus muscles. In the human MR, the authors computed average collagen fractions in the orbital layer (OL) and the global layer (GL).

Results. In human and monkey, OL fascicles remained distinct from each other and from the GL throughout. Most OL fascicles were inserted into the pulley through short tendons. Most GL fascicles bypassed the pulley without insertion. Collagen content in the human MR OL increased from 29% ± 5% (SD) in midorbit to 65% ± 9% in the anterior orbit but slightly decreased from 26% ± 6% to 23% ± 1% in the GL. Tracing of every fiber in a human MR OL fascicle demonstrated terminations on pulley tendons without myomyous junctions.

Conclusions. Fibers in the primate rectus OL lack myomyous or GL junctions, but nearly all insert on the pulley through a broad distribution of short tendons and dense intercalated collagen. Fibers in the GL generally do not insert on pulley tissues and are associated with less collagen. These features support the distinct role of the OL in anteroposterior positioning of connective tissues proposed in the active pulley hypothesis and substantial mechanical independence of the OL and GL. (*Invest Ophthalmol Vis Sci.* 2007;48:3089–3097) DOI:10.1167/iovs.06-0692

A recent fundamental insight is that rectus extraocular muscles (EOMs) do not follow straight paths from their origins to their scleral insertions.[1] Rectus EOM paths are constrained by connective tissue pulleys, whose existence as such was first recognized by Joel M. Miller.[2] Miller's insight about rectus path stability supplanted the 19th century notion that the connective tissues were responsible only for bowing of rectus EOM paths away from the orbital center.[3,4] The rectus pulleys consist of rings of dense collagen encircling the EOM, with focal deposition of elastin. Smooth muscle is present in the pulley suspension,[1,5] particularly in the inferomedial region.[6] Anterior to the pulleys, rectus EOM paths follow the scleral insertions in eccentric gaze.[7] The pulleys thus act as functional origins of the rectus EOMs.[7–9]

It has recently been determined that the fundamental kinematic properties of eye movements are mechanically determined.[10] Listing's law (LL) of ocular torsion, which states that eye position can be reached from a primary position by a rotation about a single axis that lies in Listing's plane,[11–14] is a mechanical phenomenon rather than a neural control strategy. Not only do single-unit neural recordings from the cyclovertical motor neurons of monkeys fail to demonstrate the torsional signals that would have been required to command an explicit neural implementation of LL,[15] but direct electrical stimulation of the abducens nerve in alert monkeys elicits eye movements conforming to LL.[16] Even before these primate physiological observations, it had been proposed, based on magnetic resonance imaging (MRI) and histologic evidence, that LL is implemented by large active shifts in the anteroposterior positions of the rectus EOM pulleys.[8,17,18] This active pulley hypothesis (APH) relied on insertion of some of the rectus EOM fibers on the pulleys to implement shifts in pulley positions.

The oculorotary EOMs are bilaminar.[19] The global layer (GL), containing a maximum of approximately 10,000 to 15,000 fibers in the mid-length of the EOM in humans, is located adjacent to the globe in rectus EOMs and in the central core of the oblique EOMs.[20,21] In the rectus EOMs and the superior oblique (SO) muscle, the GL anteriorly becomes continuous with the terminal tendon that inserts on the sclera.[8] The orbital layer (OL) of each rectus EOM contains 40% to 60% of all the EOM fibers. The OL terminates well posterior to the sclera, and at least some of its fibers are known to insert on connective tissue pulleys.[8,20–22] The OL is located on the orbital surface of the rectus EOM and constitutes the concentric outer layer of the oblique EOMs. The OL contains fibers of small diameter with many mitochondria and abundant vessels. The GL contains fibers of larger diameter with variable mitochondrial content and fewer vessels.[20,23]

Although substantial evidence supports OL insertion on the pulleys,[1,5,8,20,21,24] some recent authors have challenged this with the alternative view that connective tissues merely insinuate into the OL, that OL fibers may terminate on the scleral tendons, or that GL fibers may also attach to pulleys.[25] Others have generally challenged the existence of any role of pulleys in constraining EOM paths.[26,27] Because of their anatomically distributed nature, some controversy persists regarding the structure of the pulleys, with the alternative supposition that the penetrations of the rectus EOMs through Tenon fascia are mechanically insignificant or that the connective tissues serve only to limit ocular rotations.[28] It has also been proposed that the OLs and GLs of rectus EOMs are in such intimate anatomic relationship that independent action is impossible.[29] Myomy-

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ous junctions, within or between EOM layers, might be terminations for some fibers proposed not to extend the full length of the EOM.[30,31] We performed this study to seek further evidence of selective insertion of OL fibers on the pulley compared with the scleral tendon and to clarify the histologic basis for possible mechanical independence of OL and GL fibers. We considered the possibility that a division of insertional fates between the OL and the GL might not be absolute but instead relative. Quantitative anatomic analyses were performed to permit a numerical estimate of the insertional fates of EOM fibers, particularly in borderline regions between the OL and the GL. We chose human and monkey specimens for generalization. Although tracing of individual EOM fibers is highly labor intensive and has rarely been performed,[31] we used this method in a sampling of EOM fascicles in both the OL and the GL of the human medial rectus (MR) muscle.

METHODS

Our laboratory has in the past decade accumulated a library of serially sectioned, whole human and animal orbits for prospective anatomic study. Each orbit, embedded whole and undisturbed in paraffin, requires 2000 to 4500 large serial sections of 10-μm thickness for completion. Representative sections have been stained and examined for selected studies, with a large number of unstained sections reserved for future study, including the present report. The specimens examined in detail here are representative of some 10 human and 25 monkey orbits processed and qualitatively examined in similar manner, and they were selected because of their particularly excellent preservation and histologic quality. The present study consisted of a highly detailed examination of features, not previously reported, that could be studied in densely sampled serial sections of the best anatomic material we have accumulated. This approach is consistent with a previous serial sectioning study in rabbit EOM.[31]

Human right orbits were obtained from the cadavers of a 17-month-old and a 4-year-old boy, in conformity with legal requirements and in compliance with the tenets of the Declaration of Helsinki. Whole heads frozen in liquid nitrogen shortly after death were obtained from a tissue bank (IIAM, Scranton, PA). After midsagittal hemisection, the right half of each head was slowly thawed in situ in refrigerated 10% neutral, buffered formalin. The right orbit was then removed intact.

Fixed cadaver heads of a 2-year-old male rhesus (*Macaca mulatta*) and an 11-year-old male nemestrina (*Macaca nemestrina*) monkey were obtained through tissue sharing from Lawrence T. Tychsen at Washington University School of Medicine. All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Washington University Animal Care and Use Committee. After overdose of anesthesia, 400 mL of a solution containing 2.6% paraformaldehyde, 0.1 M lysine-HCl, 0.8% $NaIO_4$, and 0.8% iodoacetic acid, pH 7.4 (modified PLP-fixative) was infused by a pump through the left ventricle. Magnetic search coils previously implanted were removed by minimal dissection not involving the region of the pulleys.

As previously described,[1,5,8] the human and monkey orbits, with periorbita unopened, were decalcified for 24 hours, dehydrated, embedded in paraffin, and serially sectioned in the coronal plane at 10-μm intervals. Whole orbital sections were mounted on 50 × 75-mm gelatin-coated glass slides. Frequent sections were processed with Masson trichrome stain alternating with van Gieson elastin stain. Care was taken to achieve uniform staining of sections. To maximize chromatic uniformity, sections were processed in batches of 25 to 50 slides. Each section was numbered by the distance from the anterior corneal surface. This sectioning required the cutting of 3600 sections in human and 2500 sections in monkey orbits.

Digital light micrographs were taken using a large field microscope (Eclipse E800; Nikon, Tokyo, Japan) fitted with high-resolution digital camera (D1X; Nikon, Tokyo, Japan). To measure the collagen fraction, we selected six regions in the peripheral part of the 17-month-old human MR muscle that had staining features and location typical of the OL and three regions in the middle of the EOM with location and staining features typical of GL. In human and monkey, fibers in the OL are typically smaller and more darkly stained than are those in the GL.[18,20,32] Photographs were taken with up to 40× magnification. Nevertheless, absolute precision in distinguishing the border between the OL and the GL is impossible at the level of individual fibers, so borders were determined based on our best estimate of all relevant features, anticipating that borderline fascicles might have variable or even dual fates. With the use of a commercial imaging-editing program (Adobe Photoshop 7.0; Adobe Systems, San Jose), designated regions were selected and cropped to exclude artifacts, large vessels, and nerves. When measuring collagen fractions in the peripheral margin of the EOM, we excluded the collagen on the peripheral surface of the fascicle because this might have included part of the pulley. We included only the collagen between EOM fascicles.

After digital micrography, the color range function of an image-editing program (Adobe Photoshop; Adobe Systems) was used to select the red muscle or blue collagen, respectively. When there was local staining variation within a single section so that single chromatic segmentation omitted some tissue regions, we merged multiple digital selections as necessary for the same tissue. After converting segmented images of collagen and muscle into grayscale, images were threshold discriminated to determine the number of pixels separately representing collagen and muscle. Special care was taken during the procedure to match with the original micrograph to select EOM or collagen. Separate collagen and EOM images were imported to ImageJ software (ver. 1.34s; available by ftp at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) for counting of EOM and collagen pixels. Collagen fractions were calculated for each region as the number of pixels representing collagen divided by the number of pixels containing collagen and muscle fibers. We computed average collagen fractions on six regions in the OL and three in the GL in multiple sections along the MR path.

The number of EOM fibers and collagen fractions were investigated along a single OL fascicle in the younger human MR. We selected a fascicle in the OL at 23.59 mm posterior to the corneal surface in the posterior orbit. After taking photographs with 20× magnification of each section, tracing and fiber counting were performed anteriorly as long as fibers remained distinguishable. Collagen fractions were was calculated as described.

In the 17-month-old human MR, the 4-year-old human inferior rectus (IR), the rhesus LR, and the nemestrina superior rectus (SR) muscles, we used serial tracing to determine whether the GL and OL fascicles terminated in the pulley tendon or the insertional tendon. In the 17-month-old human MR, a representative sample of multiple EOM fiber fascicles was selected from the deep MR belly 17.97 mm posterior to the anterior corneal surface (Fig. 1). In the rhesus monkey LR, fascicles were selected 15.57 mm posterior to the anterior corneal surface. The border between the OL and the GL was delineated first according to the morphologic criteria of EOM fiber size and color, with OL fibers smaller and darker staining. In the human MR, we selected eight fascicles in the OL and six in GL (Fig. 1). In the rhesus monkey LR, we selected one fascicle each in the OL and GL, respectively. In the 4-year-old human IR, one OL fascicle was selected from the orbital surface in the middle of the EOM width 20.61 mm posterior to the corneal surface. In the nemestrina monkey SR, one OL fascicle was selected from the orbital surface in the middle of the EOM width 21.81 mm posterior to the corneal surface. After taking micrographs with 20× magnification, each selected fascicle was traced anteriorly to its termination, with higher-magnification photography as necessary. We used digital outlining to mark the borders of fascicles to facilitate reliable tracing and the following conservative rules to resolve ambiguities. When a significant part of a traced fascicle separated from the parent fascicle and joined to a small and well-defined neighboring fascicle, we incorporated the neighboring fascicle into the tracing and followed it and the parent fascicle anteriorly. If only a few fibers from a fascicle separated from the parent, we disregarded these departing

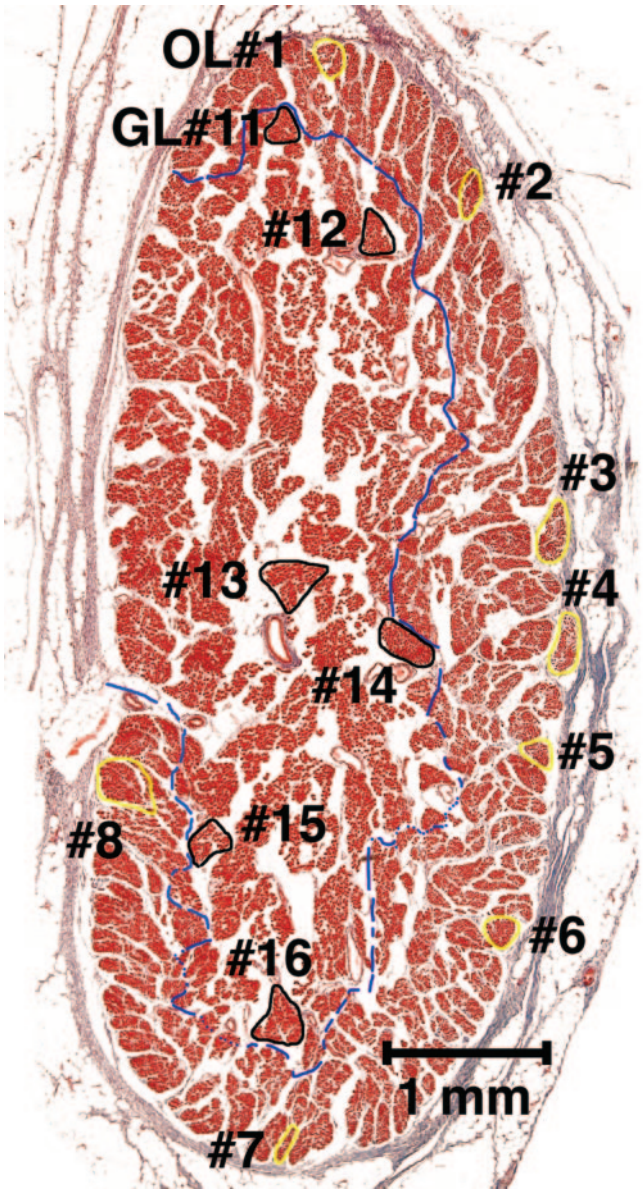

FIGURE 1. Fascicles sampled in OL and GL of a 17-month-old human MR muscle (17.97 mm posterior to the corneal surface). All numbered fascicles were initially selected in this section, and each was traced anteriorly to its insertion. Serial tracing showed that seven of eight fascicles initially classified as OL (1–8) inserted on the pulley by way of short tendons distributed over a wide anteroposterior extent. The exception, fascicle 8, was a global surface fascicle that inserted on the insertional tendon and that might have been misclassified because 8 is actually on the global surface. Of six fascicles initially classified as GL (11–16), five bypassed the pulley without insertion, and GL 11, located at the OL-GL border, inserted on the pulley (*blue line*; OL-GL border, based on fiber size and color).

fibers. If an ambiguous border was encountered during tracing, we compared the shape and number of fibers in the fascicle in the adjacent section with those of the surrounding fascicles. Five to 21 sections per millimeter were examined during the tracings. These laborious methods were adequate to determine the insertional fate of every fascicle traced, though we cannot account for the fates of small numbers of fibers departing from large fascicles.

To determine the terminal insertions of individual fibers, we selected one GL fascicle from the 17-month-old human MR (Fig. 1; #12). After taking serial photomicrographs at 10- to 60-μm intervals with 40× magnification from midorbit, 16.99 mm posterior to the anterior corneal surface, to termination, serial images were traced to identify the terminal insertion of every EOM fiber, and to seek evidence of myomyous junctions if any fibers terminated on other fibers. When we found the end of an individual EOM fiber completely encircled by collagen, we considered it to have terminated in connective tissue without the possibility of a more anterior myomyous junction. Occasionally the margins of the traced fiber fascicles became partly indistinct; this was resolved by incorporating, and continuing to trace, any ambiguous fiber groups at the margins of the reference fascicle that could have contained the continuation of the reference fascicle. This approach to occasional ambiguous borders was adequate to determine the insertional fate of the reference fascicle. Although in some cases it was difficult to trace the same fascicle from the origin to insertion, the approach was adequate to determine whether each fascicle terminated in the pulley tissues or bypassed these tissues to continue anterior to the pulley toward the scleral insertion.

RESULTS

In the 17-month-old human MR, the OL contained a higher proportion of collagen than did the GL, particularly anteriorly. Collagen fractions of OL and GL are plotted in Figure 2. The collagen fraction of the OL was 0.29 ± 0.05 (mean ± SD) at 20.19 mm posterior to the corneal surface and progressively increased anteriorly to 0.65 ± 0.09 at section 11.19 mm posterior to the corneal surface, with a relative plateau between sections 17.19 mm to 12.17 mm posterior to the corneal surface. In the GL, the collagen fraction remained almost constant along the MR length, ranging from 0.25 ± 0.06 in the posterior section 20.19 mm posterior to the corneal surface to 0.23 ± 0.01 in the anterior section 11.19 mm posterior to the corneal surface (Fig. 2).

The collagen fraction in one selected EOM fascicle in the OL of the 17-month-old human MR increased as traced anteriorly from 0.04 in the deep orbit at 23.59 mm posterior to the anterior corneal surface to 1.0 in the anterior orbit at 14.39 mm posterior to the anterior corneal surface (Fig. 3). The number

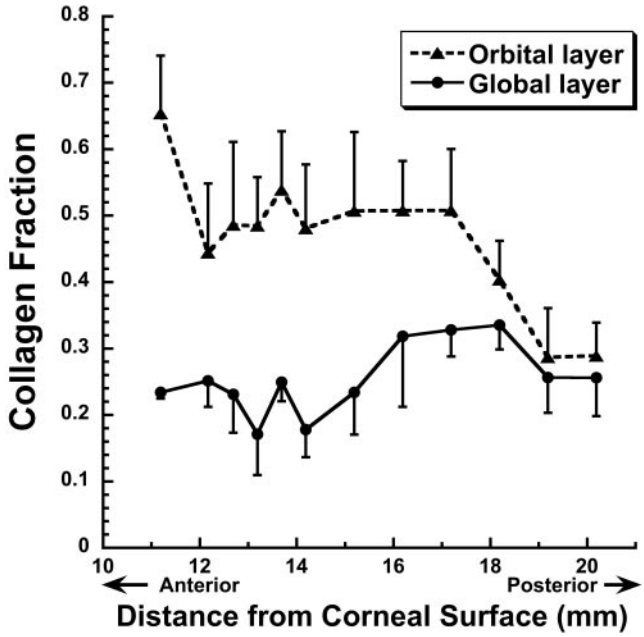

FIGURE 2. Collagen fraction in the GL and OL of a 17-month-old human MR muscle. Collagen content in the OL increased from 29% ± 5% in midorbit to 65% ± 9% in anterior orbit but slightly decreased from 26% ± 6% to 23% ± 1% in the GL.

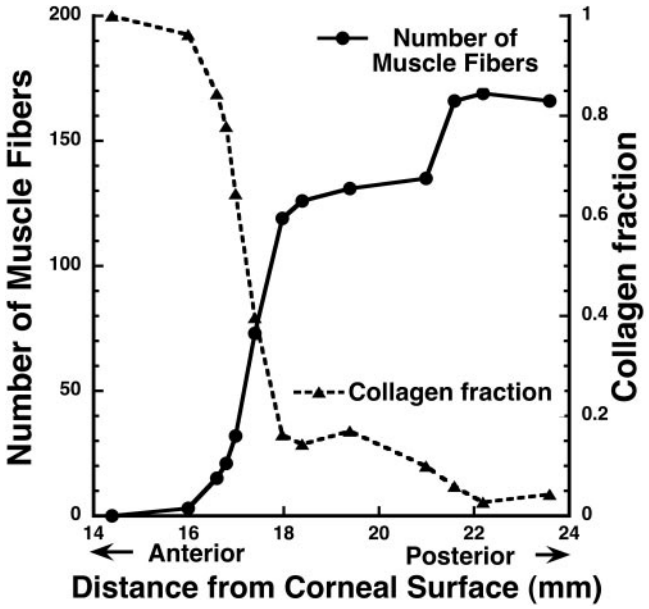

FIGURE 3. Collagen fraction plotted in comparison with muscle fiber number in a representative fascicle from a 17-month-old human MR OL. When traced anteriorly, the collagen fraction increased as the number of muscle fibers decreased to zero, and the fiber ultimately terminated in a tendon continuous with the pulley.

of EOM fibers in the same selected fascicle decreased from 166 to 0. As the collagen fraction increased anteriorly, fiber number commensurately decreased.

The human MR pulley contains a distributed sleeve of collagen encircling the EOM over an extensive anteroposterior distance of 10 to 20 mm; within this sleeve is a focally dense ring of collagen reinforced with a heavy encirclement of elastin fibers demonstrated here using van Gieson stain (Fig. 4). The entire encircling sheath is considered part of the pulley system. However, it cannot be known from histologic examination alone whether the focally dense ring of elastin-rich collagen corresponds to the sharp inflection in MR path observed by MRI in living subjects. Based on mechanical considerations, however, it seems likely that the elastin-rich ring is the actual locus of EOM path inflection. Six of eight selected EOM fascicles in the OL inserted on the orbital surface of the elastin-rich ring (Fig. 4; Movie 1, movies are online at http://www.iovs.org/cgi/content/full/48/7/3089/DC1). Fascicle 2 from the OL terminated on the collagenous muscle sheath anterior to the elastin-rich pulley ring in a region not containing significant elastin fibers. Fascicle 2 did not terminate on any tendon that had continuity with the sclera (Fig. 1). One EOM fascicle, OL 8, originated near the border between the OL and the GL in the posterior EOM belly and terminated on the scleral tendon (Movie 2).

One of six selected GL EOM fascicles in the 17-month-old human MR definitely bypassed the pulley. Four additional fascicles were assumed to bypass the pulley because they were still deep inside the EOM in the anterior part of the pulley system, though the fascicles did not remain individually distinguishable more anteriorly. One fascicle, GL 11, selected from near the border between the OL and the GL, inserted on connective tissue of the pulley or tendon sleeve. It was more difficult to trace individual EOM fascicles in the GL than in the OL because in the GL individual fascicles were not as well demarcated by connective tissues, and GL fascicles more frequently split and joined other fascicles than did fascicles in the OL.

Tracing revealed that some EOM fiber fascicles fused with other fascicles. Sometimes part of a fascicle in the OL or the GL separated from its parent and joined a nearby fascicle that appeared to be in the same layer. However, most fibers remained associated in defined fascicles that could be traced anteriorly.

OL fascicles fanned out in a C-shaped array on the orbital surface to insert over a broad anteroposterior extent on the pulley connective tissues but did not do so on the global side. The densest part of the pulley sleeve contained a dense condensation of collagen forming an encircling ring around the EOM. We presume this dense encirclement to be the pulley ring. The insertional tendons from the OL attached to this ring and to the connective tissue sleeve anterior and posterior to the ring. Fascicles originating from the orbital surface of the OL in the deep orbit inserted into the pulley tissues more posteriorly than did those originating from the more central part of the OL, and the fascicles originating from the OL near its junction with the GL inserted into the pulley sleeve anterior to the dense pulley ring.

To determine the insertional fate of individual fibers in the OL, including the possibility of myomyous junctions, we performed a detailed study of one OL fascicle from the 17-month-old human MR, OL 4 in Figure 1. We traced each of the 32 EOM fibers in this fascicle anteriorly to its termination. Moving anteriorly, most of the fibers tapered to a smaller diameter. Thirty of 32 fibers became surrounded with collagen and terminated within it to form a short tendon (Fig. 5). We could not determine the termination in two EOM fibers but did not identify any myomyous junctions between fibers.

We traced one fascicle on the orbital surface of the 4-year-old human IR from 20.61 mm posterior to the corneal surface, anteriorly to its termination (Fig. 6). This fascicle remained well delineated in the OL throughout and terminated in a short tendon that ultimately fused with the dense collagen of the IR pulley. In the anterior part of the insertional tendon of this fascicle, EOM fibers and tendinous collagen coexisted for a short transitional distance (Figs. 6D, 6E). This fascicle did not approach the GL or scleral tendon. Although not specifically traced, it was clear that other fascicles adjacent to the traced fascicle also terminated in the IR pulley by their own short tendons.

We traced EOM fascicles in the 2-year-old macaque monkey LR. One fascicle each from the GL and the OL was selected 15.57 mm posterior to the corneal surface and was traced anteriorly as far as possible. The OL fascicle could be traced to 10.46 mm posterior to the corneal surface, where it inserted on the pulley. One GL fascicle was traced to 11.96 posterior to the corneal surface, where it remained in the deep portion of the EOM and consequently could not insert on the pulley (Fig. 7). More anteriorly, the GL fascicle became poorly defined, but all fibers in its vicinity inserted on the scleral tendon.

We traced one fascicle on the orbital surface of the SR of an 11-year-old nemestrina monkey from 21.81 mm posterior to the corneal surface, anteriorly to its termination (Fig. 8). This fascicle remained well delineated in the OL throughout and terminated in a short tendon that ultimately fused with the dense collagen of the SR pulley. Tissue preservation was optimal because of fixative perfusion, highlighting distinct fiber morphology between the OL, GL, and levator palpebrae superioris (Fig. 8C). The presence of a wide fissure between the OL and the GL in the anterior part of the SR highlighted the functional separation of the fibers of this fascicle from the GL, to which there was never any attachment. Anteriorly, the OL fascicle transitioned to a short, collagenous tendon that fused with the collagen of the SR pulley.

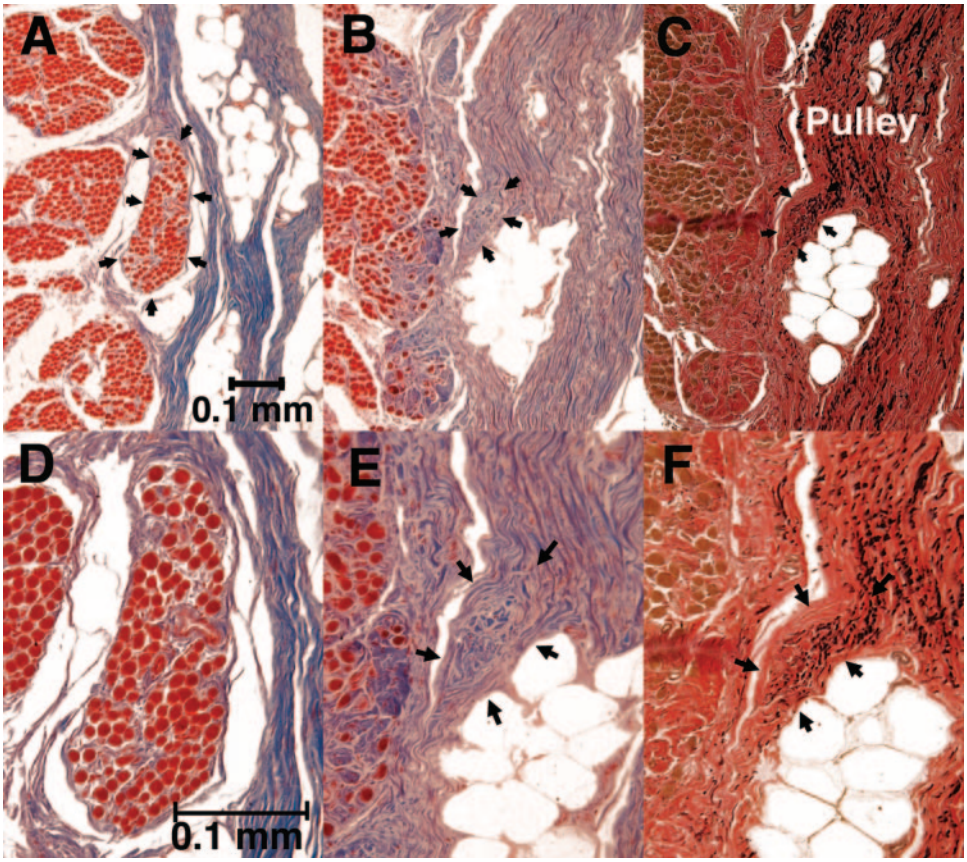

FIGURE 4. Fascicle 4 in a 17-month-old human MR OL inserts on the pulley by way of a short tendon. Scale bars apply to corresponding rows (Masson trichrome stain, Movie 1). (**A**) 17.97 mm posterior to the corneal surface, the fascicle consists of a well-demarcated bundle (*arrows*) containing 120 individual fibers. (**B**) 13.79 mm posterior to the corneal surface, all muscle fibers terminated in a pulley tendon (*arrows*). (**C**) 13.70 mm posterior to the corneal surface, van Gieson elastin stain shows heavy deposits of black elastic fibers (*arrows*) at the site of the fascicle's insertion into the pulley tendon. (**D**) Higher magnification of fascicle in (**A**). (**E**) Higher magnification of fascicle termination in pulley tendon in (**B**). (**F**) Higher magnification of elastin fibers (*black*) at fascicle insertion into the pulley tendon.

DISCUSSION

The recent recognition of the pulley system has provided valuable insight into EOM structure and function.[1,2,20,24,33] In this study, we investigated by fiber tracing the insertional fates of OL and GL fibers at the microscopic level to clarify controversies concerning the mechanical actions of rectus EOMs on the sclera and pulley system. The present study exploited the fascicular organization of rectus EOM fibers, which allowed consideration of manageable numbers of fibers. This histologic investigation of a human MR muscle demonstrated that OL fascicles insert almost exclusively on the pulley system. As the OL coursed anteriorly, the number of EOM fibers decreased with corresponding increases in collagen content. This quantitative finding is consistent with the notion that individual fascicles and fibers inserted at varying anteroposterior distances on short pulley tendons (Fig. 3). In the same region of the pulley sleeve in the GL, collagen fraction remained relatively constant, consistent with the notion that GL fibers passed through the pulley without insertion or transition to the tendon at that level. Because the necessity of defining the EOM perimeter required exclusion of the far peripheral portion of the OL, the OL collagen fraction was, if anything, underestimated.

Davidowitz et al.[31] traced 94 fibers in four fascicles of the SR of a juvenile rabbit and found five instances of myomyous

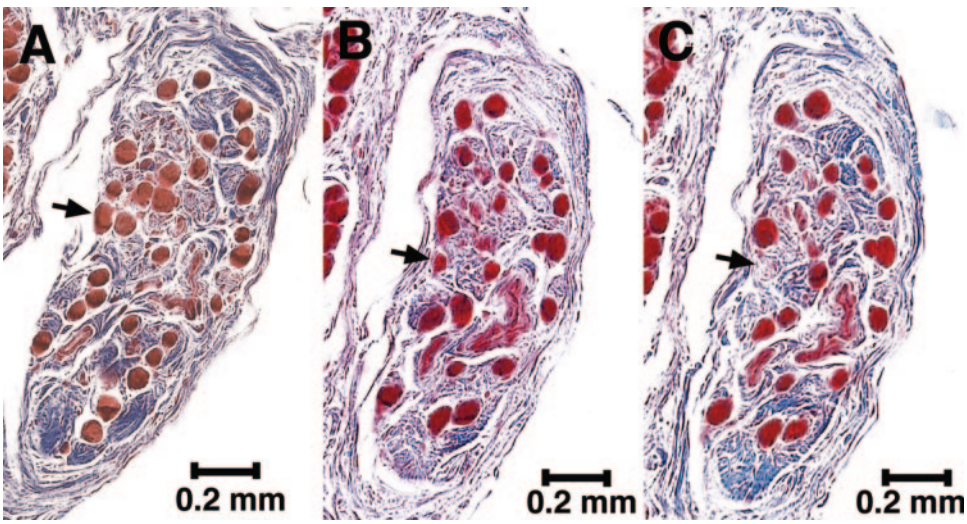

FIGURE 5. Masson trichrome stain showing tracing of individual fibers in a 17-month-old human MR OL to their terminations onto a pulley tendon. The transition to tendon is gradual, with individual fibers replaced by collagen at varying distances. *Arrow*: muscle fiber 14. (**A**) 16.99 mm posterior to corneal surface, fiber 14 has large diameter and is not heavily invested with collagen. (**B**) 16.77 mm posterior to corneal surface, fiber 14 becomes smaller and heavily invested by collagen. (**C**) 16.76 mm posterior to corneal surface, fiber 14 is no longer visible but is replaced by collagenous pulley tendon.

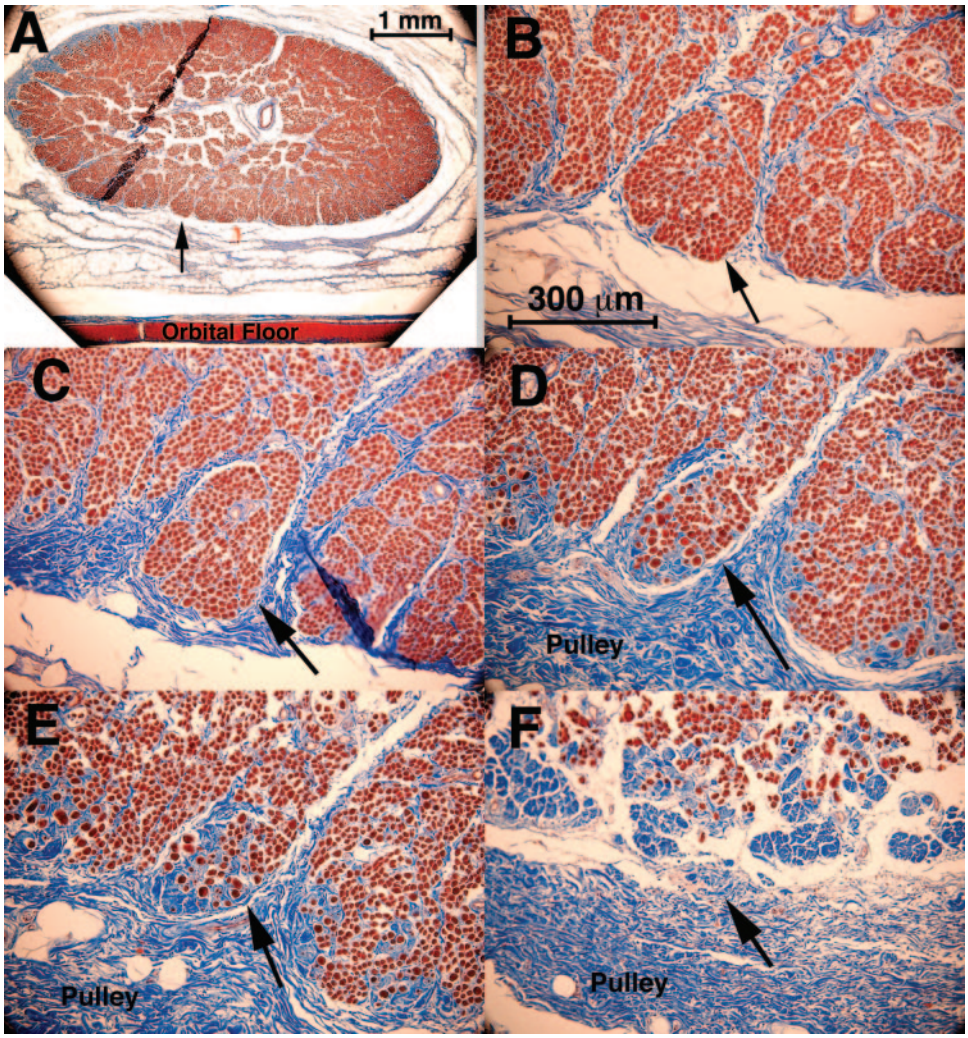

FIGURE 6. Serial tracing of fascicle in the inferior rectus muscle of a 4-year-old human, in coronal 10-μm–thick sections stained with Masson trichrome. (**A**) 20.61 mm posterior to corneal surface, low-power view shows the selected wedge-shaped fascicle (*arrow*) in the middle of the orbital surface of the muscle. Bone of the orbital floor stains *red*. (**B**) 20.61 mm posterior to corneal surface, higher-power view shows fascicle delineated by *arrow*. Scale bar, 300 μm (**B**–**F**). (**C**) 19.01 mm posterior to corneal surface, the fascicle (*arrow*) was well delineated by a collagen sheath. (**D**) 18.11 mm posterior to corneal surface, some fibers within the fascicle (*arrow*) were replaced by collagenous tendon, while many remaining fibers were individually surrounded by collagen. (**E**) 17.62 mm posterior to the corneal surface, more fibers were replaced by collagenous tendon (*arrow*). (**F**) 15.01 mm posterior to the corneal surface, the tendinous termination of the fascicle merged with the collagen of the IR pulley (*arrow*).

junctions, thus constituting approximately 5% of fibers. Most of these myomyous junctions were close to the EOM origin,[31] outside the more anterior region studied here. Although this study involved high-power examination of hundreds of serial sections, no myomyous junctions between fibers were ever observed from the midbelly anteriorly, nor were junctions of only one to several fibers to the pulley observed, as Ruskell[22] has suggested. Fascicles in the medial rectus OL typically contained tens to hundreds of fibers, and, in all four rectus EOMs, OL fascicles transitioned gradually to collagenous tendon (Figs. 4–8). We propose that the view that groups of only one to several fibers are the basic unit of OL insertion into pulley tendons[22] is an artifact of insufficient sampling of fibers along the length of the EOM. If we consider only single sections, it could appear that only one to three fibers insert into the typical pulley tendon. Rather, it is apparent from our frequent serial sections that OL fibers insert on the pulley system gradually over a wide anteroposterior extent and that, over this extent, individual fascicles have a gradually diminishing number of EOM fibers with diminishing terminal diameters. Precise counting of the number of fibers in individual fascicles was difficult because of the gradual decrease in their sizes as they coursed anteriorly. Consistent with our previous observations[8,18,20,21,32,34] and those of Ruskell,[22] the MR OL has a substantial number of fibers that insert directly on the pulley system, as required by the active pulley hypothesis (APH). According to McClung et al.,[25] the posterior border of the LR sheath insinuates into the anterior third of the EOM belly, and the anterior border of the sheath blends into the sides of the portal in Tenon capsule. The observation of McClung et al.[25] is consistent with the current quantitative findings of a transition in the OL between EOM fibers to collagenous tendon as the OL fibers insert on the pulley system (Figs. 4–8). Although McClung et al.[25] did not stain their specimen for elastin, the present study demonstrates that the OL insertion into the pulley contains a dense deposit of elastin fibrils (Fig. 4), consistent with a proposed mechanical requirement of high stiffness at the pulley.

Davidowitz et al.[31] reported an anterior termination of the lateral part of the OL relative to its center in the rabbit SR. Felder et al.[35] have similarly described in the rat that the anterior termination of the OL is C shaped, with the peripheral fibers inserting more anteriorly than the central fibers. This morphology in rat is consistent with the present finding of a wide anteroposterior extent of insertion of the OL fibers on the pulley system, though our sampling was not designed to identify a possible C shape to the OL termination.

The present study confirms that most human MR OL fascicles insert at or close to the elastically stiffened pulley ring, as anticipated under the APH. Even the exceptional OL fascicles, such as fascicle 2, inserted at the border between the pulley sheath and the pulley ring (Fig. 1). This slight anatomic difference might have been caused by postmortem artifact or might have reflected an actual distribution of OL force to the sheath rather than to the pulley ring. It seems reasonable to consider the pulley sheath broadly as part of a pulley system and to

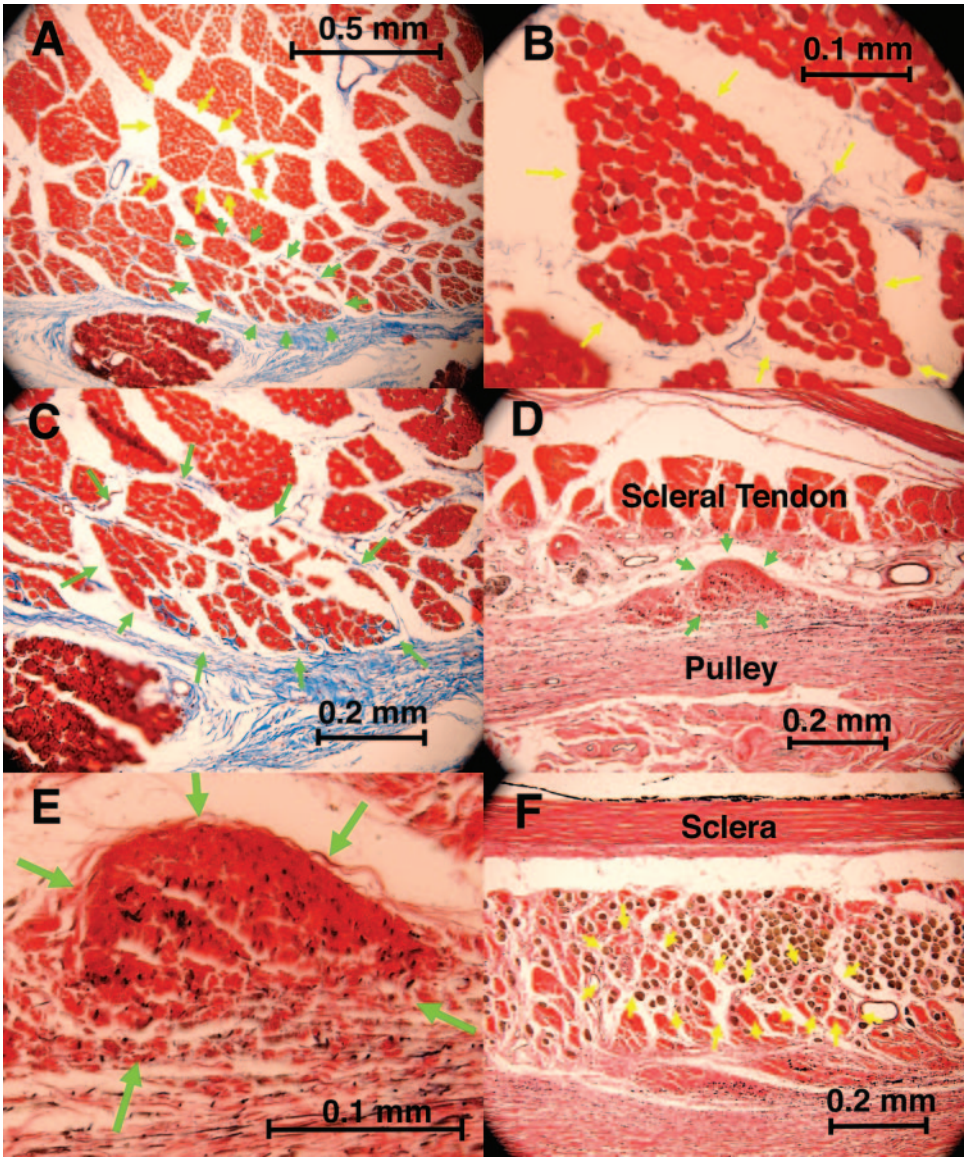

FIGURE 7. Serial tracing of fascicles in the lateral rectus muscle of a 2-year-old macaque monkey. (**A**) 15.57 mm posterior to corneal surface, we selected one OL fascicle (*green arrowheads*) and one GL fascicle (*yellow arrowheads*) for tracing. Masson trichrome stain. (**B**) Higher-power view of GL fascicle shown in (**A**). (**C**) Higher-power view of OL fascicle shown in (**A**). (**D**) 10.46 mm posterior to corneal surface, the OL fascicle inserted on the pulley through a short tendon (*green arrowheads*). van Gieson elastin stain shows dense, black-staining elastin anchoring the OL fibers in the pulley tendon. (**E**) High magnification of (**D**) shows abundant elastin fibers in the pulley. van Gieson elastin stain. (**F**) 11.96 mm posterior to corneal surface, the GL fascicle remained deep in the muscle and bypassed the pulley. Note the *black* elastin fibers in the connective tissue sleeve at this level (van Gieson elastin stain).

recognize that it would also be expected to shift anteroposteriorly during corresponding shifts of the pulley ring required by the APH.

In this study it was difficult to trace individual GL fascicles anteriorly to their terminations because the general merging of GL fascicles anteriorly abolished individual fascicle identity. Nevertheless, it was possible to trace most GL fascicles far enough anteriorly to be confident that they did not insert onto the pulley system and that the fibers with which they intermingled did become contiguous with the scleral insertional tendon. It seems a conservative presumption that the GL fibers in the fascicles traced did ultimately become contiguous with the scleral insertional tendons, as known from qualitative studies and from longitudinal sectioning.[1]

We observed little evidence from fascicle and fiber tracing of mechanical coupling between the GL and the OL. In the anterior part of the nemestrina monkey SR, for example, a substantial fissure separated the OL from the GL that would seemingly exclude significant direct coupling of force in that case. Though one fascicle in the human MR OL fused to the insertional tendon and one fascicle in the GL fused to the pulley tendon, GL and OL fibers did not generally intermingle, and their insertional fates were segregated to a high degree. Two sampled fascicles were deliberately selected from the OL-GL border to provide some insight into possible overlapping roles. Of course, this deliberate effort to seek ambiguity invites the possibility of erroneous delineation between these OL and GL fascicles. In the rectus EOMs, the C-shaped OL on the orbital surface is distinguished in most regions of the EOM from the central GL on the basis of smaller fiber size and darker red staining in the former and the larger, bright red fibers in the latter (Fig. 8).[20,23] The microscopic appearance of rectus EOMs is similar in humans and monkeys.[20,23] However, there are regions along human and monkey rectus EOMs in which the morphologic and staining characteristics of the OL and the GL are not so distinct, particularly near the neuromuscular junction, where a large fraction of EOM cross-section is occupied by nerve bundles. Numerous characteristics of EOM fibers vary along their lengths, including myosin isoform expression and ability to conduct action potentials,[19,30,36] illustrating the three-dimensional complexity emphasized by McLoon et al.[30] Such fundamental variations in EOM characteristics may limit the precision with which a border between the OL and the GL can even be defined at some midorbital locations. Nevertheless, the present study confirms the strong finding that the peripherally located EOM fascicles on the orbital surface insert

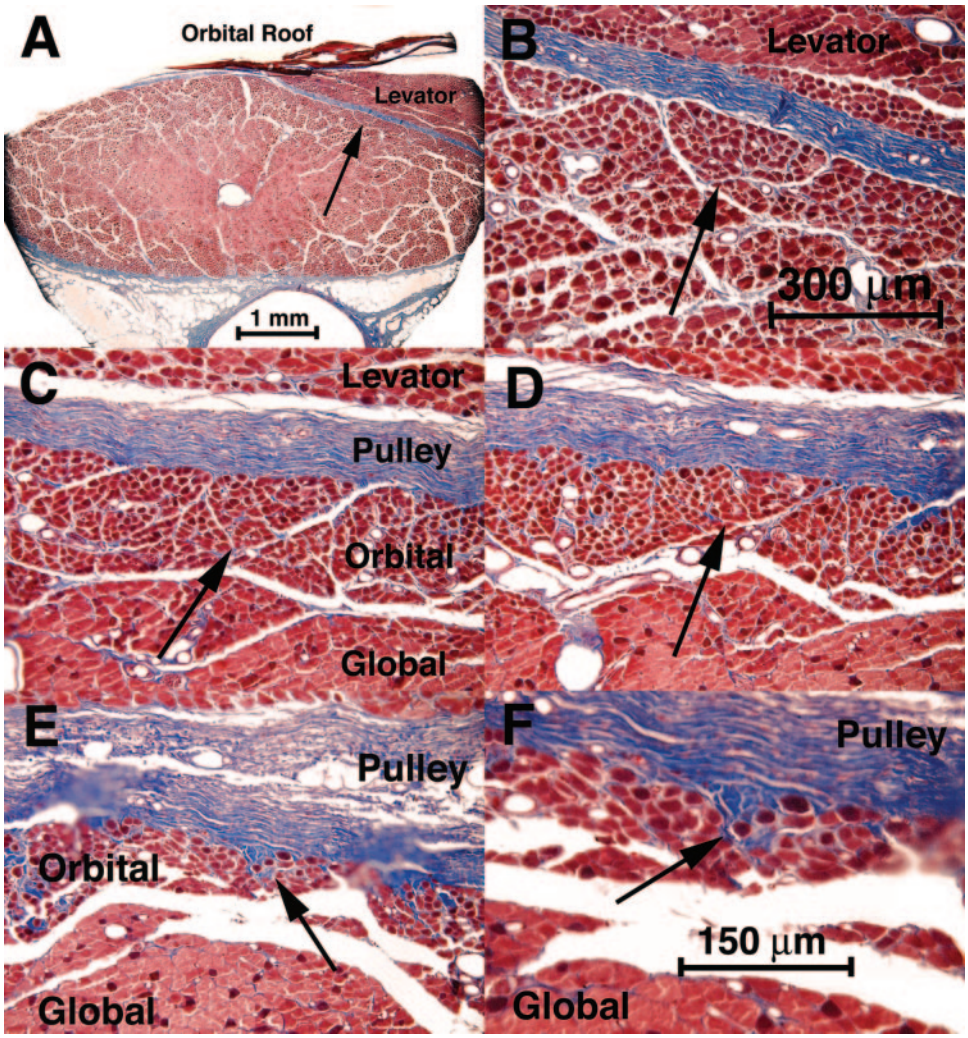

FIGURE 8. Serial tracing of fascicle in the superior rectus (SR) muscle of an 11-year-old nemestrina monkey, in coronal 10-μm–thick sections stained with Masson trichrome. (**A**) 21.81 mm posterior to the corneal surface, low-power view shows that the selected fascicle lies on the orbital surface of the muscle inferior to the levator palpebrae superioris muscle. Dilated superior ophthalmic vein lies inferior to the SR. (**B**) 21.81 mm posterior to the corneal surface, higher-power view shows selected fascicle (*arrow*) demarcated by thin fissure. Scale bar, 300 μm (**B**–**E**). (**C**) 17.42 mm posterior to the corneal surface, the selected fascicle (*arrow*) remains among the smaller and darker-staining fibers of the orbital layer (OL), which is demarcated from the larger and brighter-staining fibers of the global layer (GL) by a thin fissure. Note the similarity of the GL fibers to levator fibers, separated from the SR by the thick collagen sheath of the pulley. (**D**) 15.82 mm posterior to the corneal surface, the OL fascicle remains distinct from the GL by a wider fissure between them. (**E**) 14.22 mm posterior to the corneal surface, fibers of the traced fascicle transition to a short, collagenous tendon that begins to merge with the dense collagen of the pulley. The OL is separated from the GL by a wider fissure. (**F**) 14.22 mm posterior to the corneal surface at higher power, OL fibers of the fascicle are surrounded by dense collagen contiguous with the pulley.

with high specificity into the pulley system, whereas the more global fibers insert with high specificity on the scleral tendon. This suggests a high degree of functional independence of the two layers. Differences in morphologic, metabolic, and molecular characteristics of OL and GL fibers are consistent with their different mechanical loadings by the pulley system and globe, respectively. Expression levels differ by at least twofold for more than 180 genes between the OL and the GL in the monkey[37] and rat[36] compared with a difference of only 12 genes between the MR and the LR in the monkey.[37] Although some differences between OL and GL may be genetically programmed, it has been suggested that activity patterns during development may alter EOM fiber phenotype[38–40] so that some of these differences may reflect different mechanical loadings between the OL and the GL.

The absence of significant mechanical couplings between the OL and the GL in the human MR and monkey LR provides an anatomic basis for some degree of mechanical laminar independence in the rectus EOMs. However, Shall and Goldberg[41] have suggested, on the basis of EOM force measurements after partial myotomy, that interlaminar force coupling must be substantial. Physiological experiments of this kind are important but must be performed with strict attention to connective tissue anatomy that was unknown at the time of Shall and Goldberg's experiments. The strong possibility exists, for example, of noninsertional coupling of rectus pulley translation through pulley suspensions to exert a rotational action on the globe. Computational simulation suggests that even substantial direct coupling of force between the OL and the GL of the horizontal rectus EOMs would still be consistent with the major functional anatomic observations supporting the APH (Vijayaraghavan A et al. *IOVS* 2006;47: ARVO E-Abstract 5070). These simulations also suggest that static OL forces are greater than static GL forces, consistent with the specializations of the OL for fatigue resistance and oxidative metabolism.[8]

The present study involved a limited sampling and tracing of fascicles from human and monkey rectus EOMs from only two orbits of each. Although both specimens appeared typical in comparison with a much larger number of EOMs from human and monkey orbits, additional features might be determined from examination of additional specimens, such as features of the cyclovertical EOMs.

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