Complex Correlations Between Desmin Content, Myofiber Types, and Innervation Patterns in the Human Extraocular Muscles

Jing-Xia Liu[1] and Fatima Pedrosa Domellöf[1,2]

[1]Department of Integrative Medical Biology, Section for Anatomy, Umeå University, Umeå, Sweden
[2]Department of Clinical Science, Ophthalmology, Umeå University, Umeå, Sweden

Correspondence: Fatima Pedrosa Domellöf, Department of Clinical Sciences, Ophthalmology, Umeå University, 901 85 Umeå, Sweden; fatima.pedrosa-domellof@umu.se.

PURPOSE. To investigate whether the distribution of intermediate filament protein desmin is related to the different patterns of innervation in the human extraocular muscles (EOMs).

METHODS. EOM samples were analyzed with immunohistochemistry using antibodies against desmin, vimentin, different myosin heavy chain (MyHC) isoforms, and fetal and adult acetylcholine receptor (AChR) subunits. Neuromuscular junctions (NMJs) were identified with α-bungarotoxin or with antibodies against neurofilament and synaptophysin.

RESULTS. Desmin was present in the vast majority of myofibers, but it was weakly present or absent in a limited area in the close vicinity of the single en plaque NMJs in less than half of these myofibers. Desmin was either present or lacking in MyHCsto/I myofibers displaying multiple en grappe endings but present in MyHCsto/I myofibers receiving spiral nerve endings. In MyHCeom myofibers displaying multiterminal en plaque endings, desmin was either present or absent irrespective of AChR subunits or EOM layer. Vimentin did not substitute for the lack of desmin.

CONCLUSIONS. The results indicate that the human EOMs have a more complex cytoskeletal organization than other muscles and suggest additional signalling mechanisms from the NMJs to the myofibers.

Keywords: extraocular muscles, desmin, intermediate filament, neuromuscular junctions, multiple en grappe endings, multiterminal en plaque endings, acetylcholine receptor subunit

The skeletal muscle cytoskeleton comprises a complex network of proteins organized to link and anchor structural cell components such as myofibrils, nuclei and sarcolemma, regulating cell shape and providing transmission of myofibril movement to the surrounding tissues.[1–3] Desmin, a type III intermediate filament protein,[4] is the major cytoskeletal protein present in adult skeletal muscle. It plays a crucial role in the organization of the extra-sarcomeric cytoskeleton of muscle fibers by linking adjacent myofibrils at the Z-discs and linking peripheral myofibrils to the sarcolemma and the nuclear membrane.[2,3] Desmin is the first muscle-specific protein detected during muscle development and it is such a fundamental component of muscle fibers that it is routinely used as a marker of muscle tissue.[5] In desmin knockout animals, although myoblasts differentiate and build muscle fibers, the highly used muscles show a typical dystrophic morphology, assigning desmin a crucial role in the maintenance of muscle fiber integrity.[6,7] Desmin is particularly enriched in the folds of the neuromuscular junctions (NMJs) of normal limb myofibers, suggesting that desmin is needed for maintaining the structural and functional integrity of the postsynaptic apparatus at NMJs.[8,9] Desmin was considered ubiquitous in skeletal myofibers until we recently reported that desmin is normally lacking in a subgroup of myofibers in the human extraocular muscles (EOMs)[10] and soft palate muscles.[11]

EOMs have a number of striking features that set them apart from other skeletal muscles, including complex myosin heavy chain (MyHC) composition and distinctive innervation patterns that are not found in adult limb muscles.[12,13] In human EOMs, three major fiber types can be identified on the basis of MyHC composition, that is, myofibers containing MyHCI and MyHCslow tonic (MyHCsto/I), myofibers containing MyHCIIa, or myofibers lacking these isoforms but containing MyHC extraocular (MyHCeom).[14,15] Large single en plaque motor endplates are present in all three types of EOM myofibers, whereas small multiple en grappe endplates are only present in myofibers containing MyHCsto/I.[15–17] Traditionally, these two groups of myofibers have been referred to as singly innervated muscle fibers (SIFs) and multiply innervated muscle fibers (MIFs). In addition, palisade endings or myotendinous cylinders near the tendon are also present exclusively in MIFs in the global layer of EOMs.[13,18–21] The nerve fibers of palisade endings on several neighboring myofibers arise from a single myelinated axon branching out from a nerve bundle of the muscle belly, passing from muscle to tendon, turning back and cupping the tips of the myofibers.[22] Another type of

Investigative Ophthalmology & Visual Science

TABLE. Primary and Secondary Antibodies Used

	Primary Antibodies			Secondary Antibodies
Name	**Epitope**	**Host**	**Dilution**	**Name**
D33[*]	Desmin	Mouse	1:100	Donkey anti-mouse Alexa fluor 488[†]
				Donkey anti-mouse Alexa fluor 647[†]
M0762[*]	Neurofilament	Mouse	1:5000	Donkey anti-mouse RhRed-X[†]
SY38[‡]	Synaptophysin	Mouse	1:50	
mAb168[§]	ε AChR	Rat	1:5	Donkey anti-rat FITC[†]
GTX74890[‖]	γ AChR	Mouse	1:5	Donkey anti-mouse Alexa fluor 488[†]
				Donkey anti-mouse RhRed-X[†]
V9[*]	Vimentin	Mouse	1:100	Donkey anti-mouse Alexa fluor 488[†]
MYH14/7b[¶,47]	MyHCsto	Rabbit	1:300	Donkey anti-rabbit Alexa fluor 647[†]
				Donkey anti-rabbit RhRed-X[†]
BA-D5[**]	MyHCl	Mouse	1:10	Goat anti-mouse Alexa Flour 594[††]
A4.74[**]	MyHCIIa	Mouse	1:40	Donkey anti-mouse RhRed-X[†]
				Donkey Anti-Mouse DyLight 405[†]
N2.261[**,15,48]	MyHCl+IIa+eom	Mouse	1:50	Donkey anti-mouse RhRed-X[†]

RhRed-X: Rhodamine Red-X.
[*] DAKO, Glostrup, Denmark.
[†] Jackson ImmunoResearch Europe Ltd., Newmarket, UK.
[‡] Boenhringer Mannheim Biochemica, Indianapolis, Indiana, USA.
[§] Dr. Socrates J. Tzartos, University of Patras, Rio, Greece.
[‖] Gentex, Landskorna, Sweden.
[¶] Gift from Prof. Stefano Schiaffino, CNR Inst. of Neuroscience, Padova, Italy.
[**] Developmental Studies Hybridoma Bank, Iowa City, IA, USA.
[††] Molecular Probes, Inc., Eugene, OR, USA.

peculiar nerve endings in the EOMs are the so-called simple spiral nerve endings that surround single myofibers with three or four coils before terminating.[23,24] We very recently reported a novel type of large multiterminal en plaque motor endplates which are abundant and exclusively present in myofibers containing MyHCeom in the human EOMs.[15] Some of these myofibers may in fact be polyinnervated, as adjacent endplates show different acetylcholine receptor (AChR) subunits.[15] Because of this novel type of multiterminal innervation, we try not to use the terms MIFs and SIFs to avoid misinterpretation of the data related to the myofibers containing MyHCsto/I and the myofibers containing MyHCeom.

We have reported that a subgroup of muscle fibers containing MyHCsto in the human EOMs strikingly lack desmin or contain only trace amounts of this protein.[10] The preserved basement membrane and the well-organized cytoskeletal architecture confirm that these desmin lacking myofibers are intact,[10] indicating that the human EOMs differ from other skeletal muscles with respect to the intermediate filament composition of their cytoskeleton. In the present study, we sought to elucidate whether the desmin content of the myofibers in the human EOMs correlated with the distinct and complex innervation patterns of the myofibers.

MATERIAL AND METHODS

Human Muscle Samples

Nineteen EOM samples including medial rectus, superior rectus, lateral rectus, inferior rectus, and superior oblique muscles were collected from 11 subjects (9 males and 2 females) with a mean age of 59.5 years (range 34-82 years), at autopsy with the approval of the Regional Ethical Review Board in Umeå, Sweden, following the recommendations of the Declaration of Helsinki. None of the subjects was known to suffer from neuromuscular disease. In addition, samples

from biceps brachii and vastus lateralis muscles collected from 2 males (18 and 24 years) were also used to detect desmin immunoreactivity at NMJs.

The muscle samples were mounted and rapidly frozen in propane chilled with liquid nitrogen and stored at –80°C until processed. Serial cross or longitudinal sections, 5 µm thick, were prepared at –23°C in a Reichert Jung cryostat (Leica, Heidelberg, Germany).

Immunofluorescence

Sections were processed for double, triple, or sequential immunostaining[15] by using a battery of well-characterized antibodies (Table). Motor endplates were identified by rhodamine-conjugated α-bungarotoxin labeling (α-Btx; Molecular probes Inc., Eugene, OR, USA) or by labeling with a mixture[15] of monoclonal antibodies against neurofilament protein and against synaptophysin. In brief, the tissue sections were air-dried, rehydrated in 0.01M PBS, and then blocked with 5% normal serum for 15 minutes. Sections were then incubated with the first primary antibody (desmin or AChR subunit) at 4°C overnight. Thereafter, the sections were washed in PBS, additionally blocked with 5% donkey serum for 15 minutes and then incubated for 30 minutes at 37°C with the appropriate secondary antibody (Table). Thereafter, the second primary antibody (neurofilament + synaptophysin or AChR subunit) was applied (37°C for 60 minutes), followed by incubation with the appropriate secondary antibody for 30 minutes at 37°C. Subsequently, immunolabeling for the third or fourth primary antibody against desmin or MyHCs was performed, followed by incubation with the appropriate secondary antibody for 30 minutes at 37°C, as previously described.[15] Control sections were treated as previously mentioned, except that the primary antibodies were omitted. No staining was observed in control sections.
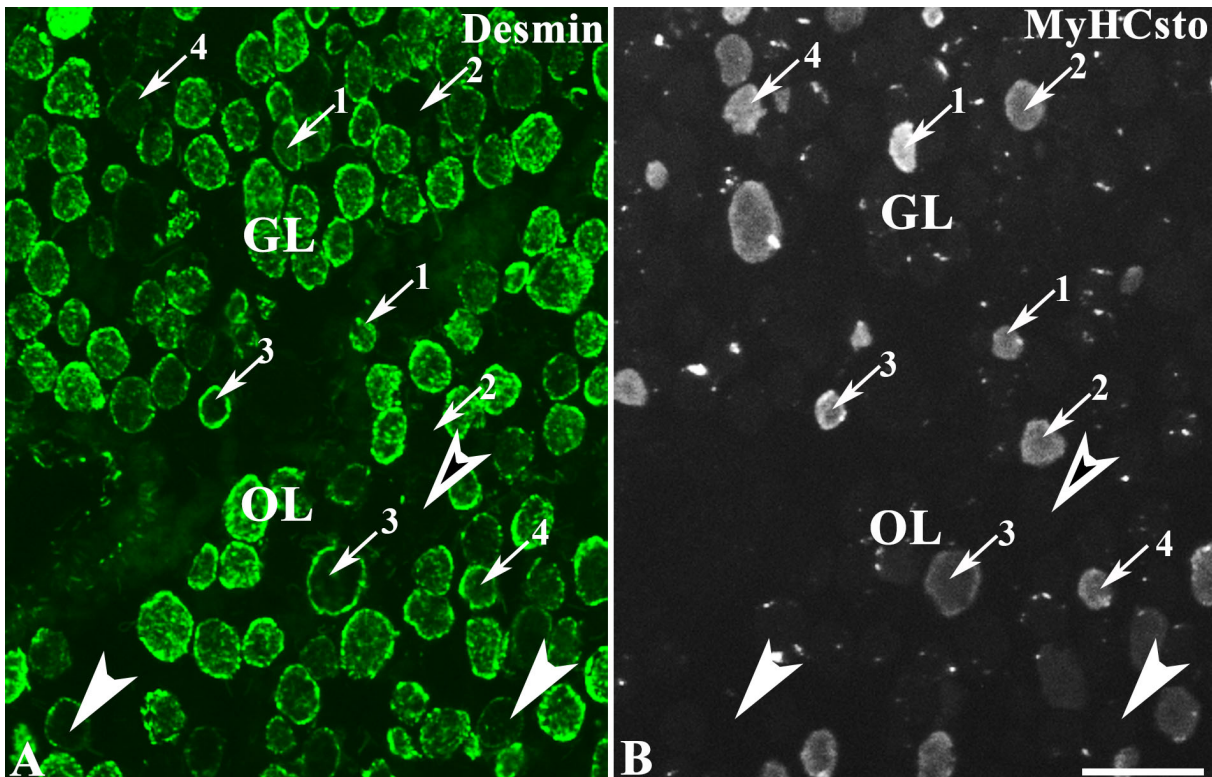
FIGURE 1. Cross section of EOM treated with the antibodies against desmin (**A**, *green*) and MyHCsto (**B**, *gray*). In general, desmin was present in the whole myofiber cross section of MyHCsto fibers (arrows 1), but desmin was found totally absent in the whole myofiber cross section (arrows 2), present subsarcolemmally only (arrows 3) or was detected weakly both subsarcolemmally and inside the myofiber (arrows 4) in both orbital (OL) and global (GL) layers. Note that weak (**A**; *arrowheads*) or absent labeling (**A**; *open arrowhead*) with desmin was also found in myofibers that did not contain MyHCsto (**B**; *arrowheads*, *open arrowhead*) (i.e., in myofibers containing fast myosin isoforms [MyHCIIa or MyHCeom]). *Bar scale* = 50 µm.

The sections were examined and photographed with a Nikon microscope (Eclipse, E800, Tokyo, Japan) connected to a Spot camera (RT KE slider, Diagnostic Instruments, Inc., MI, USA). The images were processed using the Adobe Photoshop software (Adobe System, Inc., Mountain View, CA, USA).

Myofiber Typing in Human EOMs

Three types of muscle fibers were classified in the human EOMs on the basis of their MyHC isoform content, as previously described.[10,15] In brief, the muscle fibers strongly labeled with the antibody against MyHCsto and in the vast majority, also strongly labeled with the antibody against MyHCI, were classified as muscle fibers containing MyHCsto/I. The myofibers strongly or moderately labeled with the antibody against MyHCIIa were classified as muscle fibers containing MyHCIIa. The myofibers unlabeled with the two antibodies above, but labeled with antibody N2.261 which recognizes MyHCI+MyHCIIa+MyHCeom, were classified as muscle fibers containing MyHCeom.

Motor Endplate Evaluation

The samples were evaluated with respect to the presence of desmin, vimentin, fiber types, motor endplates, and AChR subunits. Motor endplates were evaluated in sections double-labeled with the antibody against desmin and either α-Btx (desmin+α-Btx) or neurofilament and

synaptophysin (desmin+NF+Syn). In addition, triple- or quadruple-labeled sections combining antibodies against fetal γ AChR subunit, adult ε AChR subunit, and antibodies against desmin or one of the MyHC isoforms were also evaluated. When necessary, following microscopic evaluation and photographing, a sequential immunolabeling procedure with an additional MyHC antibody was performed to allow typing of all myofibers. For example, a muscle section that had been immunolabeled with antibodies against adult ε AChR subunit (FITC, green), against fetal γ AChR subunit (Rhodamine Red-X, IgG, red), and against desmin (Alexa flour 647) was, after evaluation and photographing, additionally incubated with the antibody against MyHCI (Alexa flour 594, IgG2b, red) and later on with the antibody against MyHCIIa (Alexa flour 594, IgG1, red). Thereby, we could examine the section and photograph it again and classify the myofibers into those containing MyHCI or MyHCIIa or, in case they were unlabeled by both antibodies, myofibers containing MyHCeom.

RESULTS

We confirmed our previous findings[10] that the majority of myofibers in the human EOMs showed even and strong immunoreactivity with the antibody against desmin regardless of fiber type. We also confirmed that immunolabeling for desmin was absent or very weak in a subgroup of myofibers containing MyHCsto/I (Fig. 1). In addition, weak or absent
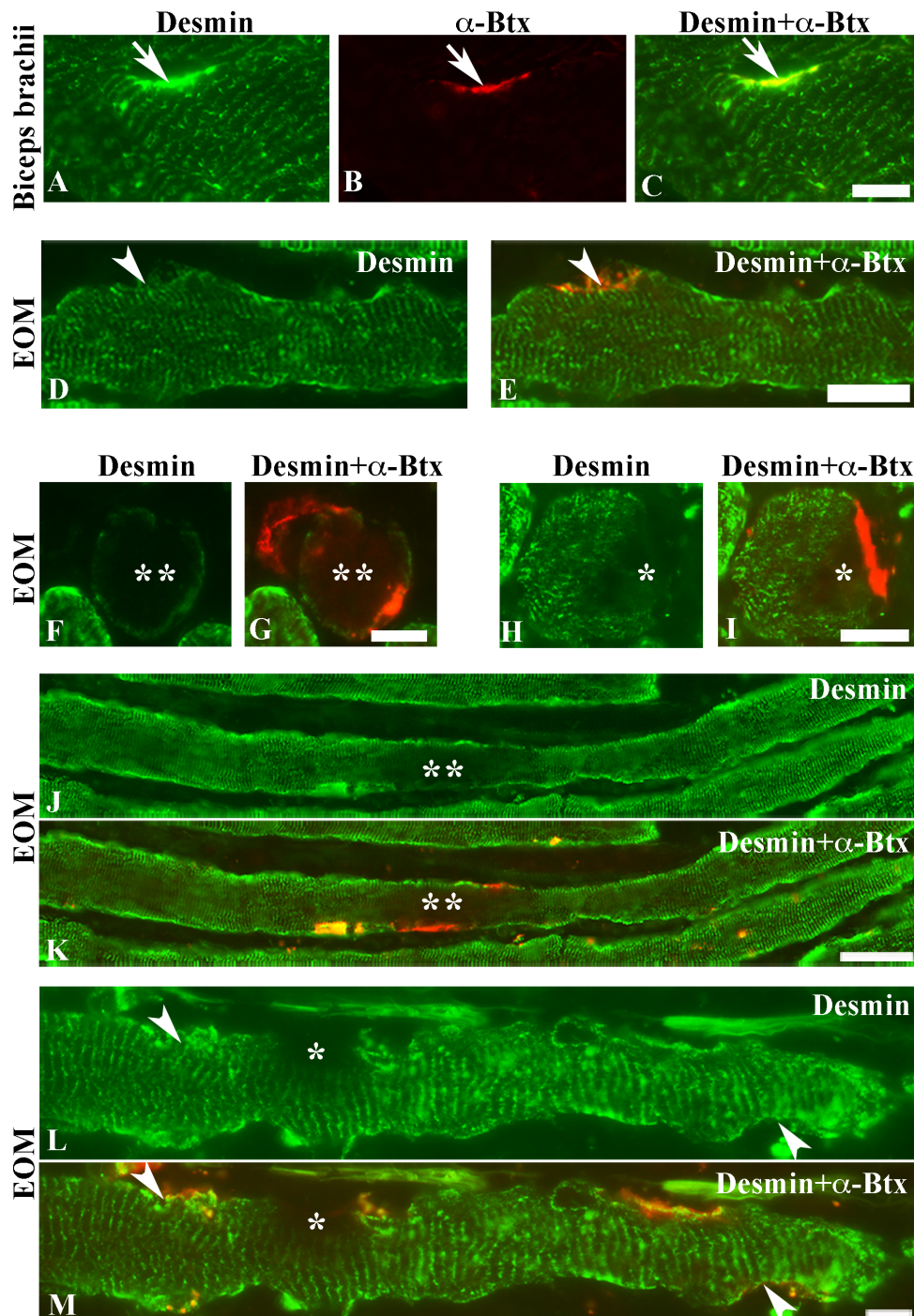
Figure 2. Immunoreactivity with the antibody against desmin (*green*) at NMJs identified with α-bungarotoxin (α-Btx, *red*) in cross sections of biceps brachii (**A-C**) and EOMs (**F-I**) and in longitudinal sections of EOMs (**D, E, J-M**). The desmin content was generally highly increased at NMJs (*arrows* in **A-C**) in samples from the biceps brachii muscle but it was not increased at NMJs in the EOMs (*arrowheads* in **D, E, L, M**). Note that desmin was absent in practically the whole myofiber cross section (** in **F, G, J, K**) or in a more limited region (* in **H, I, L, M**) in the close vicinity of NMJs in both cross and longitudinal sections of EOMs. *Bar scale* = 20 µm.

desmin labeling was also found in a subgroup of myofibers that did not contain MyHCsto/I (Fig. 1).

In cross sections, four different desmin staining patterns were observed in all types of myofibers of the human EOMs: (1) desmin was present in the whole myofiber cross-section; (2) desmin was totally absent in the whole myofiber cross-section; (3) desmin was present subsarcolemmally only;

and (4) desmin was detected weakly both subsarcolemmally and further inside the myofiber (Fig. 1). In longitudinally cut sections, a novel labeling pattern was observed: desmin was absent locally (i.e. only in a limited region of a subgroup of myofibers) irrespective of myofiber type (Fig. 2). In addition, it was also clear that desmin was absent along the whole examined length of myofibers containing either
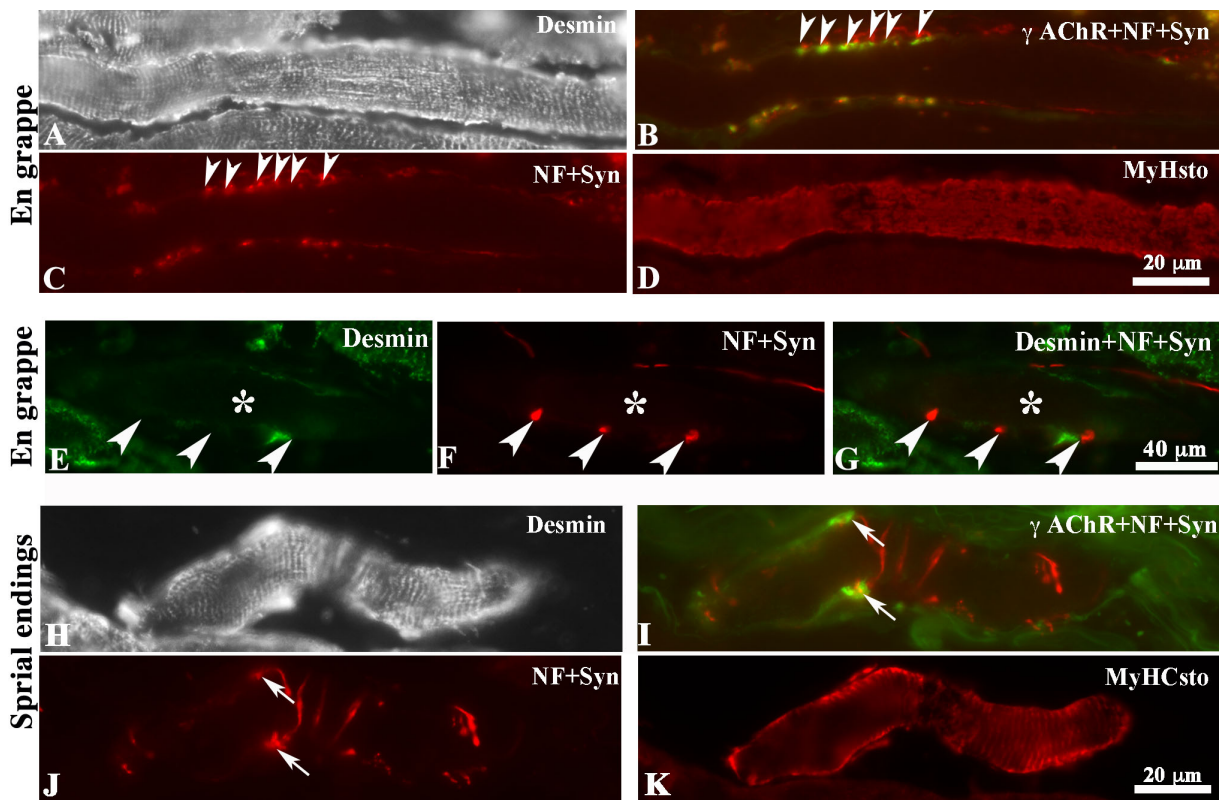
FIGURE 3. Immunoreactivity with the antibody against desmin in myofibers containing MyHCsto. Desmin (**A**, *gray*) was present in MyHCsto/I myofibers (**D**, *red*) displaying multiple en grappe endplates (*arrowheads* in **B, C**, *red*) containing fetal γ AChR subunits (**B**, *green*). Desmin was absent in myofibers (* in **E-G**) containing MyHCsto displaying multiple en grappe endplates (*arrowheads* in **F, G**, *red*). Desmin (**H**, *gray*) was present in MyHCsto/I myofibers (**K**, *red*) with spiral endings (**I, J**, *red*). Note the presence of fetal γ AChR subunits at endplates of spiral endings (*arrows* in **I**, *green*).

MyHCsto/I or MyHCeom (Figs. 3–5). To further explore the possible relationship between desmin content and innervation pattern, we systematically and carefully examined the pattern of desmin distribution in different types of motor endplates in all three types of myofibers in the human EOMs.

Desmin in Myofibers with Single en Plaque Motor Endplates

We confirmed previous findings[8,9] describing the ubiquitous presence of desmin with increased abundance at NMJs in limb muscle fibers (Figs. 2A–C). In contrast to the stronger desmin immunolabeling typically seen at NMJs in limb muscles, desmin immunoreactivity in the human EOMs was generally not increased at NMJs (Figs. 2D–E). Notably, approximately $43 \pm 13\%$ of myofibers displaying single en plaque endplates in longitudinal sections and containing desmin showed absent or weak labeling with the desmin antibody in a limited area, in the close vicinity of their NMJs (Figs. 2J–M). Clearly, these muscle fibers lacked desmin or contained only trace amounts of desmin in the close vicinity of typical en plaque motor endings, whereas desmin was present in the remaining portion of the same muscle fibers in both orbital and global layers, irrespective of myofiber type. We further investigated whether vimentin, a key cytoskeletal protein transiently present during muscle development, substituted for desmin. Immunolabeling with an antibody against vimentin was not found in the vicinity of NMJs

nor within the myofibers (not shown), although vimentin labeling was readily present in connective tissue and blood vessels, as we reported previously.[10] The remaining 57% of myofibers displaying single en plaque endplates in longitudinal sections and containing desmin showed neither increased nor absence of desmin at the NMJs.

Desmin in MyHCsto/I Myofibers with Multiple en Grappe Motor Endplates

In both orbital and global layers, the myofibers containing MyHCsto/I and displaying typical multiple en grappe motor endings were either labeled or unlabeled with the antibody against desmin along the whole fiber length examined (Figs. 3A–G). Such unlabeled MyHCsto/I myofibers were found more frequently in the orbital layer than in the global layer.

Desmin in MyHCsto/I Myofibers With Spiral Nerve Endings

Spiral nerve endings, tightly bound with three or more coils labeled with antibodies against neurofilament and synaptophysin as well as fetal γ AChR subunit, were exclusively found around myofibers containing MyHCsto/I (Figs. 3H–K). These MyHCsto/I myofibers receiving spiral endings were always labeled with the antibody against desmin along the whole fiber length examined
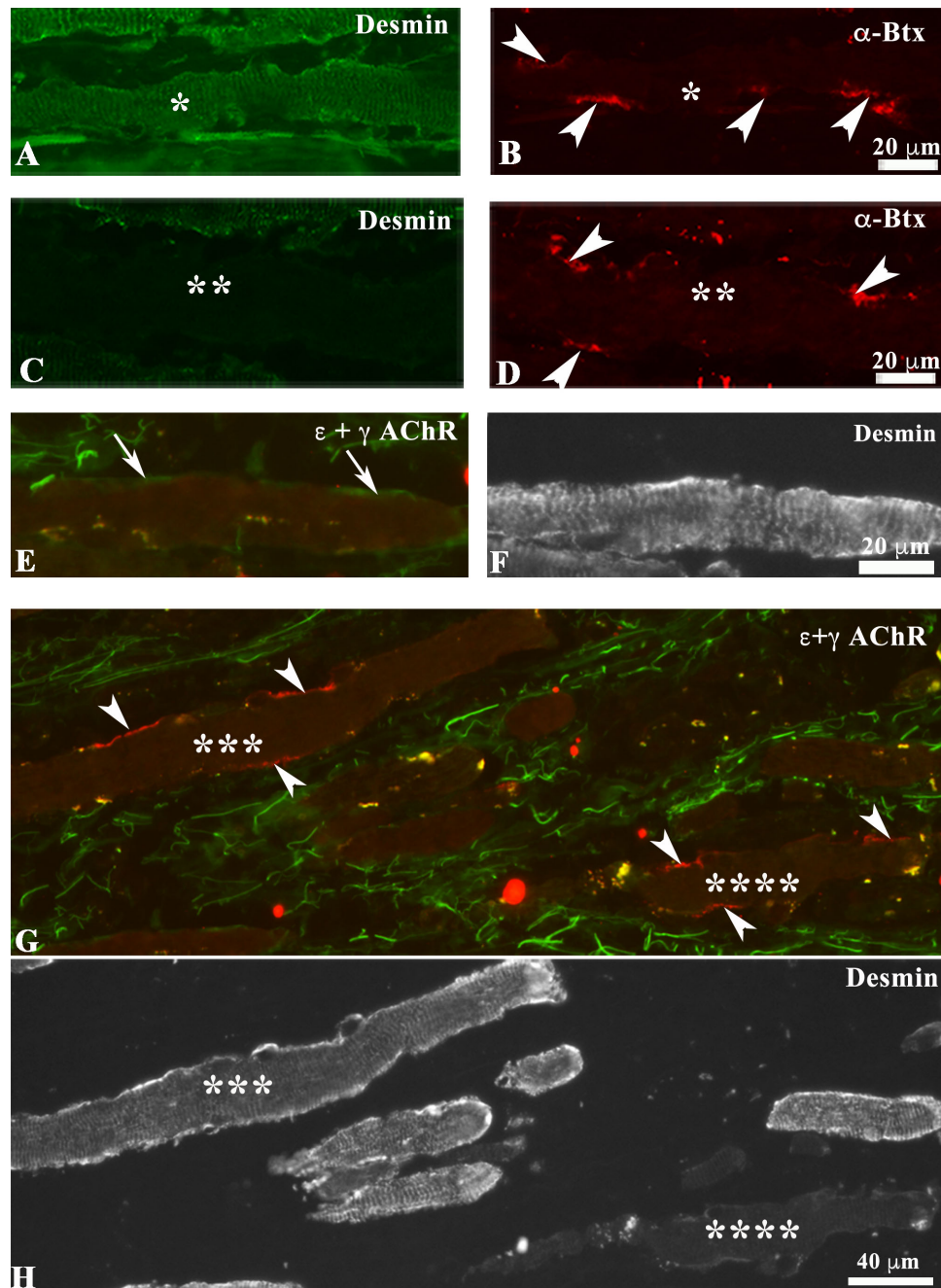
FIGURE 4. Immunoreactivity with antibodies against desmin in myofibers containing MyHCeom with multiterminal en plaque motor endplates. Desmin (*green* in **A, C**) was either present (* in **A**) or absent (** in **C**) in MyHCeom fibers displaying multiple en plaque endplates (*arrowheads* in **B, D**). Double immunolabeling with antibodies against adult ε (*green* in **E**) and fetal γ (*red* in **E**) AChR subunits showing two "en plaque" endplates containing adult ε AChR subunit only (*arrows* in **E**) in MyHCeom fiber containing desmin (*gray* in **F**). Sections treated with the antibodies against adult ε (*green in* **G**; please note that only unspecific labeling of connective tissue fibrils was seen) and fetal γ (*red*, *arrowheads* in **G**) AChR subunits and desmin (*gray* in **H**) showing two MyHCeom myofibers displaying multiterminal en plaque endplates in the global layer. Note two nearby MyHCeom myofibers (labeled *** and ****) each displaying three "en plaque" endplates (*arrowheads*) containing fetal γ AChR subunit only. Notice that one of these myofibers (***) was labeled with the antibody against desmin whereas the other myofiber (****) was unlabeled.

(Fig. 3H). Sporadically, multiple en grappe endings were also seen in these myofibers, and in such cases desmin was also present along the whole fiber length examined (not shown).

It was also noted that in the close vicinity of myotendinous junctions in the global layer, myofibers containing MyHCsto/I and being surrounded by long nerve fibers were always labeled with the antibody against desmin along the whole fiber length present in the muscle section. Because the specimens available did not include the tendon, it was not possible to establish whether these MyHCsto/I myofibers received palisade endings. The presence of desmin in these
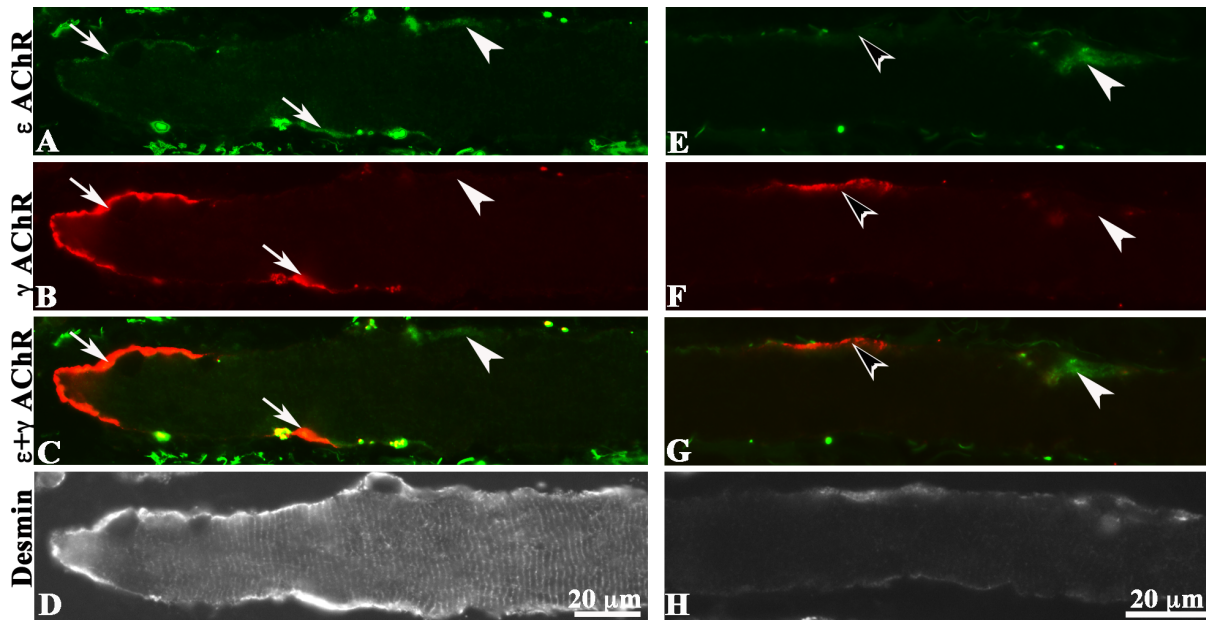
Figure 5. Immunoreactivity with the antibodies against adult ε (**A, E**, *green*), fetal γ (**B, F**, *red*) AChR subunits and desmin (**D, H**, *gray*) in two MyHCeom myofibers displaying multiterminal en plaque endplates, in the global layer. Merged images of (**A**) and (**B**) as well as (**E**) and (**F**) are shown in (**C**) and (**G**), respectively. Left panel shows a myofiber displaying three endplates with distinct AChR subunit composition: the endplate labeled with *arrows* contained adult ε and fetal γ AChR subunits, whereas the endplate labeled with the *arrowhead* contained only adult ε AChR subunit. Notice that desmin was present in this myofiber (**D**). Right panel shows a myofiber displaying two endplates. Note that the endplate labeled with arrowhead contained adult ε AChR subunit only, whereas the endplate labeled with the *open arrowhead* contained fetal γ AChR subunit only. Desmin was absent in this myofiber (**H**). The *dots* at the lower surface of the myofiber in E are unspecific labeling.

particular myofibers explained our previous findings very well,[10] as the desmin-negative MyHCsto/I myofibers were mostly present in the orbital layer, whereas these desmin-labeled MyHCsto/I myofibers were generally present in global layer.

Desmin in MyHCeom Myofibers with Multiterminal en Plaque Motor Endplates

The vast majority of myofibers containing MyHCeom were labeled with the antibody against desmin irrespective of having single or multiterminal en plaque motor endplates (Figs. 4A, B). However, approximately 35 ± 15% of the MyHCeom myofibers displaying multiterminal en plaque motor endplates were unlabeled with the antibody against desmin, irrespective of being in the orbital or the global layer (Figs. 4C, D). There was no straightforward relationship between desmin and innervation patterns in myofibers containing MyHCeom. Therefore, further examination using antibodies against different AChR subunits was performed on longitudinal sections to assess whether there was a correlation between desmin and AChR subunit composition of the multiterminal en plaque motor endplates. The subgroup of MyHCeom fibers displaying multiterminal en plaque endplates containing adult ε AChR subunits only was generally labeled with the mAb against desmin (Figs. 4E, F). The subgroup of MyHCeom fibers displaying multiterminal en plaque endplates containing exclusively fetal γ AChR subunit were either labeled or unlabeled with the antibody against desmin, regardless of location in the orbital or the global layer (Figs. 4G, H). Finally, in a subgroup of MyHCeom myofibers, the AChR subunit composition of the multitermi-

nal en plaque endplates varied from one endplate to another along the length of a single myofiber. These myofibers with varying AChR subunits among adjacent endplates either contained (Figs. 5A–D) or lacked (Figs. 5E–H) desmin, and no relation was apparent between type of AChR subunit and desmin content.

Desmin in MyHCeom Myofibers with Multiterminal en Plaque and Multiple en Grappe Motor Endplates

A small number of myofibers containing MyHCeom displayed both large multiterminal en plaque motor endplates and small multiple en grappe motor endplates along their length. These myofibers were all moderately labeled with the antibody against desmin (not shown). In addition, sporadic myofibers containing MyHCeom or MyHCsto/I displayed clustered small "grape-like" endplates on either side of the muscle fiber, as described previously.[15] Desmin was always detected in these myofibers irrespective of myofiber type and location (not shown).

Discussion

The present study confirmed the absence of desmin in a subset of myofibers in the human EOMs and further revealed the lack of desmin in the close vicinity of the NMJs in a large proportion of myofibers of the human EOMs (Fig. 6), in contrast to the enriched desmin content at NMJs typically seen in limb muscles. A summary of the results is presented in Figure 6. In brief, (1) immunoreactivity with the antibody against desmin was decreased or absent in a short fiber
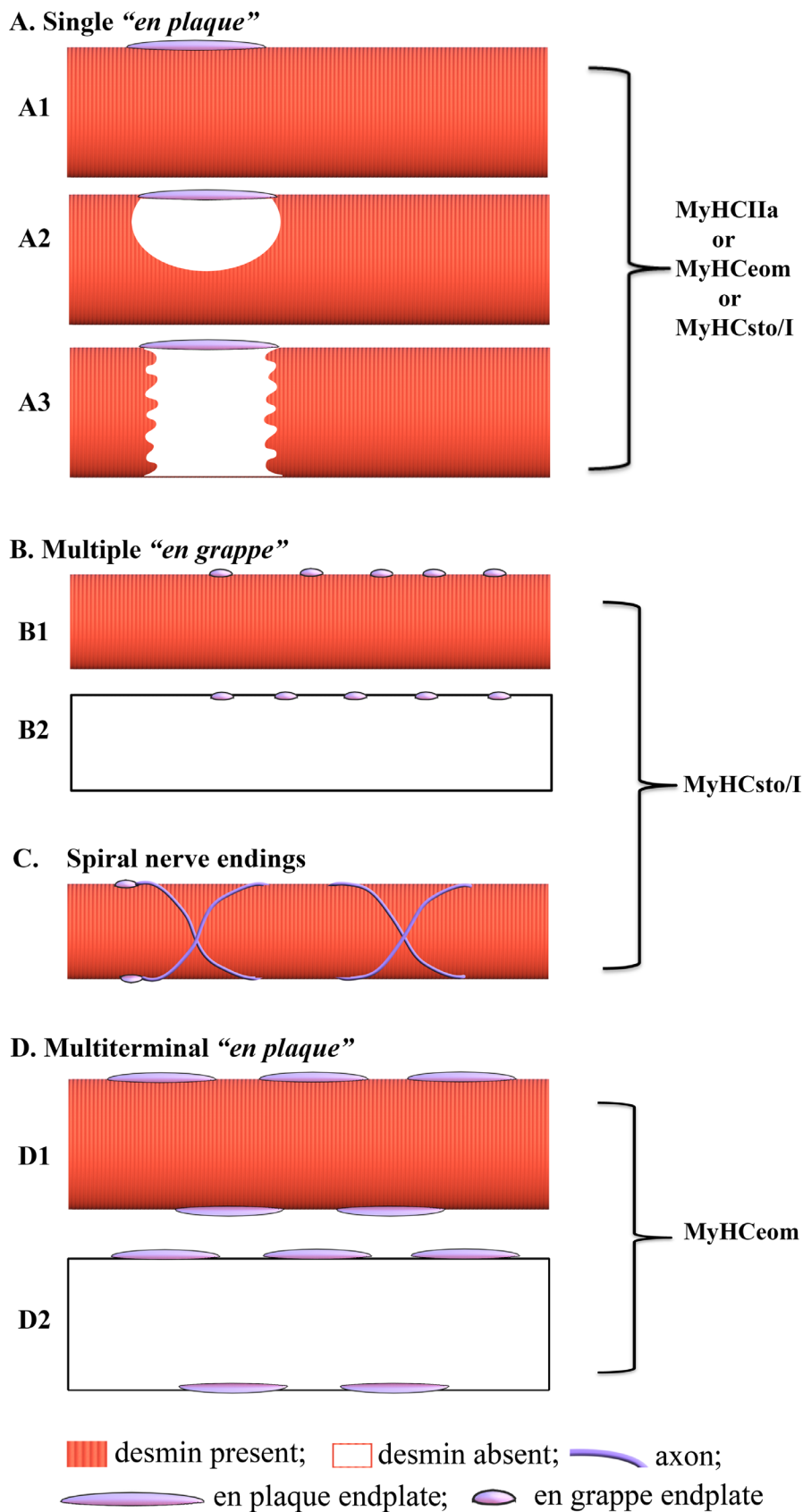
FIGURE 6. Schematic illustrations of patterns of desmin composition in the three major myofiber types with distinct motor endplates in the human EOMs. (**A**) Myofibers of all three types displaying single en plaque motor endplates showed either (**A1**) rather uniform staining with desmin; (**A2**) lack of desmin in a restricted; or (**A3**) larger area in the close vicinity of the endplates but uniform staining in the rest of the

myofiber. (**B**) Desmin was usually present in MyHCsto/I myofibers innervated with multiple en grappe endplates (**B1**) but was absent in a subgroup of these myofibers (**B2**). (**C**) Desmin was always present in myHCsto/I myofibers with spiral nerve endings. (**D**) Desmin was present (**D1**) or absent (**D2**) in myofibers containing MyHCeom with multiterminal en plaque endplates.

segment adjacent to en plaque endings of all fiber types in the EOMs; (2) desmin was lacking in a subgroup of MyHC-sto/I myofibers displaying multiple en grappe endings; (3) desmin was present in MyHCsto/I myofibers displaying spiral endings; and (4) no apparent correlations were seen between desmin content and innervation pattern in myofibers containing MyHCeom. Altogether, these results indicate that the EOMs have a more complex cytoskeleton organization than other muscles and suggest additional signalling mechanisms from the NMJs to the myofibers in the EOMs.

Desmin was Absent in the Vicinity of NMJs

The present results confirmed previous studies showing that desmin is ubiquitously present in limb muscle fibers and is highly concentrated at NMJs in human limb muscles.[8,9] The concentration of desmin at NMJs has been proposed to be vital for clustering of acetylcholine receptors and maintenance of NMJs.[8,9,25] Furthermore, Durmus et al.[26] have recently reported that NMJs become fragmented and have increased surface area in soleus muscle of homozygous R349P desmin knock-in mice, confirming that desmin is needed for maintaining the structural and functional integrity of the postsynaptic apparatus. However, the present study showed that desmin was absent or decreased in the close vicinity of NMJs in nearly one-half of myofibers with single en plaque endplates in the human EOMs, regardless of myofiber type, indicating that desmin does not play the same role in EOMs as in limb muscles.

The present results easily explain why desmin is absent or decreased not only in myofibers containing MyHC-sto/I but also in sporadic myofibers containing MyHCIIa or MyHCeom in cross sections (Fig. 1) (i.e., desmin was absent or decreased in myofibers cut transversely at the level of the NMJs). These NMJs showed normal morphology and normal distribution of many crucial synaptic molecules, including AChR subunits; neurofilament and synaptophysin; laminin-α2, -α4, -α5, and -β2 isoforms; and various Wnt isoforms, neurotrophins, and their receptors, in healthy human EOMs.[27–30] Therefore, the lack of desmin at NMJs cannot be regarded as defective, but rather an intrinsic feature of the normal human EOMs. Transmission electron microscopy has revealed that the junctional folds at NMJs, typically present in trunk or limb muscles, are absent in rat EOMs or sparse in mouse EOMs.[31,32] In cat EOMs, the junctional folds are present at NMJs but their number is dramatically decreased in slow-twitch fibers and absent in slow-tonic fibers.[33] Therefore, the absence of an increased concentration of desmin at NMJs in EOMs might be related to the absence and sparseness of synaptic folds in these muscles. We have previously described important differences in the molecular composition of the NMJs in the EOMs compared with limb muscles, in particular with respect to gangliosides GQ1b, GT1a, and GD1b.[34] Taken together, all these findings suggest a different cytoskeletal organization and that there may be distinct signalling pathways from the cell surface to the nuclei at the NMJs of the EOMs.

Desmin was Absent Along a Subgroup of MyHCsto Myofibers with Solely en Grappe Endings but Present in MyHCsto Myofibers Receiving Spiral Endings

We have previously reported the absence of desmin along the fiber length of a subgroup of myofibers containing MyHCsto/I in human EOMs, yet the reason why desmin was lacking only in a subgroup of these myofibers was puzzling.[10] The present study partially revealed a correlation between desmin expression and innervation patterns in myofibers containing MyHCsto/I: desmin was absent in a subgroup of MyHCsto/I myofibers receiving multiple en grappe nerve endings solely but present in MyHCsto myofibers receiving spiral nerve endings with or without en grappe endings. The current findings indicate that desmin content in human EOMs was not only correlated to the type of myofiber (MyHCsto/I), but also correlated to the pattern of innervation (multiple en grappe endings, spiral nerve endings) in these myofibers. We can only speculate that the myofibers containing MyHCsto/I found in the close vicinity of myotendinous junctions and surrounded by long nerve fibers may have received palisade endings. Unfortunately, the present data are insufficient to draw conclusions on this subgroup of myofibers.

Desmin in Myofibers Containing MyHCeom

The present study revealed that lack of desmin also occurred in MyHCeom myofibers containing multiterminal en plaque motor endplates. However, in contrast to the MyHCsto/I myofibers mentioned previously, no direct correlation between desmin content and the type of motor endplates/AChR subunits was observed for MyHCeom myofibers. In orbital or global layer, desmin could be present in one MyHCeom fiber but absent in another in its close vicinity and although both myofibers displayed multiterminal en plaque motor endplates with identical AChR subunit composition. Because we observed no signs of abnormal myofibrillar organization, the absence or low levels of desmin found in myofibers containing MyHCeom was not an abnormality but rather a special property of these myofibers.

MyHCeom isoform, encoded by the MYH13 gene, is restricted to EOMs and laryngeal muscles.[35,36] MYH13 is thought to play a role in the extraordinary fast contraction speed of the EOMs, and proposed to be mainly involved in saccadic eye movements.[37] Indeed, although MyHCIIa myofibers in EOMs contract as fast as MyHCIIa fibers in limb muscles, MyHCeom myofibers in EOMs contract much faster than MyHCIIa fibers in limb muscles.[38] On the other hand, MyHCeom myofibers contract against a much lower load and thus a much lower tension output than most limb muscles. Furthermore, desmin is required for optimal active force development in skeletal muscle because lack of desmin affects the spacing and lateral force transmission between the contractile filament proteins, in zebrafish.[39] It has been shown that the absence of desmin in knockout mice affects mostly muscles that are weight-bearing or constantly used whereas other muscles are not significantly affected.[40,41]

Although the EOMs are highly used muscles, they are particularly low weight-bearing, thus low weight-bearing may be associated with desmin content (low/absence) in myofibers in EOMs, especially in those MyHCeom myofibers displaying multiterminal en plaque endplates. Whether the absence or presence of desmin is related to multiple or polyneuronal innervation remains to be further investigated.

Implications of Absence of Desmin in Myofibers of EOMs

Previous studies have shown that mice lacking desmin develop a skeletal myopathy, including muscular dystrophy and irregular organization of myofibrils.[6,7,40,42] We have recently studied the effect of lack of desmin on the structure and the distribution of crucial cytoskeletal proteins on the EOMs of desmin$^{-/-}$ mice.[43] In contrast to the severely (soleus) or slightly (gastrocnemius) affected limb muscles, the structure of the EOMs is well preserved.[43] In the EOMs, the myofibers are well aligned and desmin-binding proteins show a correct location in spite of the lack of desmin.[43] The present study further showed that vimentin did not compensate for the lack of desmin at NMJs.

The EOMs are relatively well-preserved in amyotrophic lateral sclerosis.[28–30,44,45] In particular, we have shown that the composition and the motor neuron occupancy at the NMJs are well preserved in the EOMs until late stages of the disease.[28,30,45] The present data further support previous studies indicating that the NMJs of EOMs have distinct properties but it is too early to propose that desmin has a particular role. Furthermore, it is noteworthy that lack of desmin has also been reported in a subpopulation of soft palate myofibers in healthy subjects,[11] but these muscles are affected in amyotrophic lateral sclerosis patients.[46]

Taken together, the present data confirmed that desmin is not ubiquitously present in human muscle fibers and indicate that the EOMs differ significantly from limb muscles with respect to intermediate filament composition. Further studies are needed to elucidate whether the distinct patterns of desmin composition in the different myofiber and NMJ types in the EOMs translate into different signalling pathways between the extracellular matrix and the nuclei and whether they have any implications for the particular resistance of these muscles to disease. However, because of the complexity of the EOMs and methodological limitations, this will be a very challenging question to answer.

References

1. Thornell LE, Price MG. The cytoskeleton in muscle cells in relation to function. *Biochem Soc Trans*. 1991;19(4):1116–1120.

2. Small JV, Furst DO, Thornell LE. The cytoskeletal lattice of muscle cells. *Eur J Biochem*. 1992;208(3):559–572.

3. Fuchs E, Weber K. Intermediate filaments: structure, dynamics, function, and disease. *Annu Rev Biochem*. 1994;63:345–382.

4. Szeverenyi I, Cassidy AJ, Chung CW, et al. The human intermediate filament database: comprehensive information on a gene. *Hum Mutat*. 2008;29(3):351–360.

5. Bar H, Strelkov S, Sjoberg G, Aebi U, Herrmann H. The biology of desmin filaments: how do mutations affect their structure. *J Struct Biol*. 2004;148(2):137–152.

6. Carlsson L, Thornell LE. Desmin-related myopathies in mice and man. *Acta Physiol Scand*. 2001;171(3):341–348.

7. Thornell LE, Carlsson L, Li Z, Mericskay M, Paulin D. Null mutation in the desmin gene gives rise to a cardiomyopathy. *J Mol Cell Cardiol*. 1997;29(8):2107–2124.

8. Askanas V, Bornemann A, Engel W. Immunocytochemical localization of desmin at human neuromuscular junctions. *Neurology*. 1990;40(6):949–953.

9. Carlsson L, Li Z, Paulin D, Thornell L. Nestin is expressed during development and in myotendinous and neuromuscular junctions in wild type and desmin knock-out mice. *Exp Cell Res*. 1999;251(1):213–223.

10. Janbaz AH, Lindstrom M, Liu JX, Pedrosa Domellof F. Intermediate filaments in the human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2014;55(8):5151–5159.

11. Shah F, Berggren D, Holmlund T, Levring Jäghagen E, Stål P. Unique expression of cytoskeletal proteins in human soft palate muscles. *J Anat*. 2016;228(3):487–494.

12. Fische M, Budak M, Bakay M, et al. Definition of the unique human extraocular muscle allotype by expression profiling. *Physiol Genom*. 2005;22(3):283–291.

13. Spencer RF, Porter JD. Biological organization of the extraocular muscles. *Prog Brain Res*. 2006;151:43–80.

14. Kjellgren D, Thornell LE, Andersen J, Pedrosa-Domellof F. Myosin heavy chain isoforms in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2003;44(4):1419–1425.

15. Liu JX, Pedrosa Domellöf F. A novel type of multiterminal motor endplate in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2018;59(1):539–548.

16. Sadeh M. Extraocular muscles. In: Engel AG, Franzini-Armstrong C , eds. *Myology*. New York, McGraw-Hill; 1994;119–127.

17. Bormioli SP, Torresan P, Sartore S, Moschini GB, Schiaffino S. Immunohistochemical identification of slow-tonic fibers in human extrinsic eye muscles. *Invest Ophthalmol Vis Sci*. 1979;18(3):303–306.

18. Ruskell G. The fine structure of innervated myotendinous cylinders in extraocular muscles of rhesus monkeys. *J Neurocytol*. 1978;7(6):693–708.

19. Alvarado-Mallart R, Pincon-Raymond M. The palisade endings of cat extraocular muscles: a light and electron microscope. *Tissue Cell*. 1979;11(3):567–584.

20. Lukas J, Blumer R, Denk M, Baumgartner I, Neuhuber W, Mayr R. Innervated myotendinous cylinders in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 2000;41(9):2422–2431.

21. Blumer R, Maurer-Gesek B, Gesslbauer B, et al. Palisade endings are a constant feature in the extraocular muscles of frontal-eyed, but not lateral-eyed, animals. *Invest Ophthalmol Vis Sci*. 2016;57(2):320–31.

22. Richmond F, Johnston W, Baker R, Steinbach M. Palisade endings in human extraocular muscles. *Invest Ophthalmol Vis Sci*. 1984;25(4):471–476.

23. Daniel P. Spiral nerve endings in the extrinsic eye muscles of man. *J Anat*. 1946;80:189–193.

24. Ruskell G. Spiral nerve endings in human extraocular muscles terminate in motor end plates. *J Anat*. 1984;139(Pt 1):33–43.

25. Agbulut O, Li Z, Périé S, et al. Lack of desmin results in abortive muscle regeneration and modifications in synaptic structure. *Cell Motil Cytoskeleton*. 2001;49(2):51–66.

26. Durmus H, Ayhan O, Cirak S, et al. Neuromuscular endplate pathology in recessive desminopathies: Lessons from man. *Neurology*. 2016;87(8):799–805.

27. Liu JX, Brannstrom T, Andersen PM, Pedrosa Domellof F. Different impact of ALS on laminin isoforms in human extraocular muscles versus limb muscles. *Invest Ophthalmol Vis Sci*. 2011;52(7):4842–4852.

28. Liu JX, Brannstrom T, Andersen PM, Pedrosa Domellof F. Distinct changes in synaptic protein composition at neuromuscular junctions of extraocular muscles versus limb muscles of ALS donors. *PLoS One*. 2013;8(2):e57473.

29. McLoon LK, Harandi VM, Brannstrom T, Andersen PM, Liu JX. Wnt and extraocular muscle sparing in amyotrophic lateral sclerosis. *Invest Ophthalmol Vis Sci*. 2014;55(9):5482–5496.

30. Harandi VM, Gaied AR, Brannstrom T, Pedrosa Domellof F, Liu JX. Unchanged neurotrophic factors and their receptors correlate with sparing in extraocular muscles in amyotrophic lateral sclerosis. *Invest Ophthalmol Vis Sci*. 2016;57(15):6831–6842.

31. Ogata T. Structure of motor endplates in the different fiber types of vertebrate skeletal muscles. *Arch Histol Cytol*. 1988;51(5):385–424.

32. Salpeter M, McHenry F, Feng H. Myoneural junctions in the extraocular muscles of the mouse. *Anat Rec*. 1974;179(2):201–24.

33. Pilar G, Hess A. Differences in internal structure and nerve terminals of the slow and twitch muscle fibers in the cat superior oblique. *Anat Rec*. 1966;154(2):243–251.

34. Liu JX, Willison HJ, Pedrosa-Domellof F. Immunolocalization of GQ1b and related gangliosides in human extraocular neuromuscular junctions and muscle spindles. *Invest Ophthalmol Vis Sci*. 2009;50(7):3226–3232.

35. Briggs M, Schachat F. Early specialization of the superfast myosin in extraocular and laryngeal muscles. *J Exp Biol*. 2000;203(Pt 16):2485–2494.

36. Lucas C, Rughani A, Hoh J. Expression of extraocular myosin heavy chain in rabbit laryngeal muscle. *J Muscle Res Cell Motil*. 1995;16(4):368–378.

37. Schachat F, Briggs M. Phylogenetic implications of the superfast myosin in extraocular muscles. *J Exp Biol*. 2002;205(Pt 15):2189–2201.

38. Sartore S, Mascarello F, Rowlerson A, et al. Fibre types in extraocular muscles: a new myosin isoform in the fast fibres. *J Muscle Res Cell Motil*. 1987;8(2):161–172.

39. Li M, Andersson-Lendahl M, Sejersen T, Arner A. Knockdown of desmin in zebrafish larvae affects interfilament spacing and mechanical properties of skeletal muscle. *J Gen Physiol*. 2013;141(3):335–345.

40. Li Z, Mericskay M, Agbulut O, et al. Desmin is essential for the tensile strength and integrity of myofibrils but not for myogenic commitment, differentiation, and fusion of skeletal muscle. *J Cell Biol*. 1997;139(1):129–144.

41. Li Z, Colucci-Guyon E, Pinçon-Raymond M, et al. Cardiovascular lesions and skeletal myopathy in mice lacking desmin. *Dev Biol*. 1996;175(2):362–366.

42. Paulin D, Li Z. Desmin: a major intermediate filament protein essential for the structural integrity and function of muscle. *Exp Cell Res*. 2004;301(1):1–7.

43. Rodriguez M, Liu J, Parkkonen K, Li Z, Pedrosa Domellof F. The cytoskeleton in the extraocular muscles of desmin knockout mice. *Invest Ophthalmol Vis Sci*. 2018;59(12):4847–4855.

44. Ahmadi M, Liu JX, Brannstrom T, Andersen PM, Stal P, Pedrosa-Domellof F. Human extraocular muscles in ALS. *Invest Ophthalmol Vis Sci*. 2010;51(7):3494–3501.

45. Tjust AE, Brannstrom T, Pedrosa-Domellof F. Unaffected motor endplate occupancy in eye muscles of ALS G93A mouse model. *Front Biosci (Schol Ed)*. 2012;4:1547–1555.

46. Pawlukowska W, Baumert B, Gołąb-Janowska M, et al. Comparative assessment and monitoring of deterioration of articulatory organs using subjective and objective tools among patients with amyotrophic lateral sclerosis. *BMC Neurol*. 2019;19(1):241.

47. Rossi A, Mammucari C, Argentini C, Reggiani C, Schiaffino S. Two novel/ancient myosins in mammalian skeletal muscles: MYH14/7b and MYH15 are expressed in extraocular muscles and muscle spindles. *J Physiol*. 2010;588(Pt 2):353–364.

48. Liu JX, Eriksson PO, Thornell LE, Pedrosa-Domellof F. Myosin heavy chain composition of muscle spindles in human biceps brachii. *J Histochem Cytochem*. 2002;50(2):171–184.