

Significant roles of microtubules in mature striated muscle deduced from the correlation between tubulin and its molecular chaperone α B-crystallin in rat muscles

Hyunseok Jee · Takashi Sakurai · Shigeo Kawada · Naokata Ishii · Yoriko Atomi

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Abstract To elucidate the significance of cytoskeletal microtubule networks in striated muscles, we analyzed correlation between the content of tubulin (building block of microtubules) and α B-crystallin (a molecular chaperone for tubulin) in a variety of striated muscles expressing different myosin heavy-chain (MHC) isoforms. The content of both tubulin and α B-crystallin was larger in MHC-I dominant soleus muscle and in MHC- α dominant cardiac (atrium and ventricle) muscles; intermediate in MHC-IIc dominant masseter, tongue, and diaphragm muscles; and smaller in MHC-IIb dominant plantaris, gastrocnemius, psoas, extensor digitorum longus, and tibialis anterior muscles. Since the muscles of slow-type MHC (MHC-I/ α) show the most economical features in their function and metabolism, which suit for continuous activity required to sustain posture and blood pumping, the present results afforded additional support to our hypothesis that microtubule networks transduce mechanical environmental demands to morphological and biochemical responses that eventually evolve adaptive transformation in the function and metabolism of the mature muscles. The comparison of

tubulin/ α B-crystallin ratios across the muscles of varied MHC isoforms further suggested that mechanical stress fluctuating at the rhythmic frequency of walking and breathing efficiently activates the hypothesized dynamic function of microtubules.

Keywords Heat shock protein · Adaptation · Cytoskeleton · Myosin heavy chain · Muscle

Introduction

Cytoskeletons are generally considered to transduce mechanical environmental demands to morphological and metabolic adaptation of cells and organs [15, 17, 20]. Among the major cytoskeletons (actin filaments, intermediate filaments, and microtubules), microtubules form characteristically dynamic network transmitting tensile force, transporting proteins and maintaining mitochondria [3], Golgi apparatus [28], and cell shape [9, 15, 16, 29]. In the case of striated muscles, microtubules are shown to be essential for differentiating myotubes [16]. However, their significance in mature muscle is still unknown. Since passive and active mechanical stresses are well documented to induce morphological as well as metabolic adaptation in striated muscles [5, 12, 29], we infer that microtubules would play significant roles especially in slow-twitch oxidative skeletal muscles (slow muscles) [29].

Soleus muscle is a typical slow skeletal muscle, which continuously keeps posture against gravity through neural and contractile activities. Compared with fast-twitch oxidative and fast-twitch oxidative glycolytic skeletal muscles (fast muscles), slow muscles are functionally characterized by slower contractile kinetics, larger oxidative metabolic

H. Jee · T. Sakurai · N. Ishii
Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153-8902, Japan

S. Kawada
Department of Human and Engineered Environmental Studies, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Chiba, Japan

Y. Atomi (✉)
Integrated Research System for Sustainability Science (IR3S), The University of Tokyo, Faculty of Engineering Bldg.3, Bunkyo-ku, Tokyo 113-8656, Japan
e-mail: atomi@bio.c.u-tokyo.ac.jp

capacity, and lower fatigability [11]. These functional features suit for their continuous activities to meet the mechanical demands from environment.

Slow muscles are morphologically as well as biochemically distinct from fast muscles. Morphologically, slow muscles are abundant in extracellular collagenous matrices [33], capillary vessels, myoglobin, and mitochondria [11]. Their sarcomeres show wider Z-disks and diffuse M-lines on electron micrographs [1, 31]. Biochemically, sarcomeres of slow muscles are composed of specific myoproteins. Myosin heavy chain (MHC) is dominantly MHC-I in slow muscles, while MHC-IIa, IId, or IIB in fast muscles [11, 13]. M-lines are dominantly composed of EH-myomesin in slow muscles, while M-proteins in fast muscles [30, 31]. Protein turnover rate would be higher in slow muscles because higher protein synthesis [5] and higher chaperone content are reported in slow muscle [7]. α B-crystallin is reported as one of the chaperone proteins [14, 27] that are rich in soleus muscle [5, 18, 29].

All of these specific features of slow muscles could be viewed as adaptive responses of the muscle to mechanical demands from the environment. We hypothesized that the general mechanical stress transducer, the microtubule network, plays a pivotal role even in mature striated muscle to induce the adaptive responses. The following findings support our hypothesis: (1) Soleus muscle was rich in microtubules and their constituent tubulin [9, 28, 29]. (2) In soleus muscle of hind-limb suspended rats, passive stretch prevented atrophy as well as specific decrease of α B-crystallin [5, 6, 29]. α B-crystallin was localized at Z-bands and M-lines, which would transmit mechanical stress between extracellular matrices and contractile myofilaments [6]. (3) α B-crystallin interacted with microtubules and α -tubulin in vitro [4, 14, 27] and in the lysate of mature muscle cells [29]. (4) α B-crystallin has a chaperone-like protecting effect on tubulin dimers against heat denaturation [4, 27], and on microtubules against depolymerizing factors including Ca^{2+} [14]. (5) Mechanical stress such as lengthening contraction induced translocation of α B-crystallin to Z-disks [18].

To test our hypothesis over a wider range of muscle types, we aimed in the present study to analyze the correlation between α -tubulin and α B-crystallin in various skeletal as well as non-skeletal striated muscle tissues expressing different MHC isoforms.

Methods

Animals

Adult female Wistar rats (age, 8 weeks; body weight, 195 ± 15 g; $n = 6$) were used. All animals were provided

with standard rat chow and water ad libitum. They were housed at 22–24°C with a 12:12-h light–dark cycle. Rat care and all experimental procedures employed were in accordance with the policy statement of the University of Tokyo on research with experimental animals. The study was approved by the Ethical Committee for Animal Experiments at the University of Tokyo.

At the end of the familiarization period (~1 week), the rats were euthanized by cervical dislocation under anesthesia with diethyl ether (50 ml/kg body weight). Cardiac muscles (ventricle and atrium), tongue, masseter muscle, diaphragm, soleus muscle, plantaris muscle, gastrocnemius muscle, extensor digitorum longus muscle, tibialis anterior muscle, and psoas muscle were then immediately dissected and stripped of all impurities (i.e., blood) with flushing buffer. These isolated muscle samples were frozen immediately in liquid nitrogen and stored at -80°C until analysis.

Western blotting

The frozen muscle samples stored at -80°C were weighed. They were then crushed into powder in liquid nitrogen inside mortars. The powder was diluted with homogenization buffer to five to six times in weight. The homogenization buffer contained 20 mM KCl, 2 mM sodium phosphate (pH 6.8), 2 mM EGTA, 5 mM EDTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, a protease inhibitor cocktail (1 mM phenylmethylsulfonyl fluoride, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor), and a phosphatase inhibitor cocktail (10 nM okadaic acid and 10 mM sodium β -glycerophosphate). The homogenate was solubilized with solubilizing sodium dodecyl sulfate (SDS) sample buffer [0.1 M Tris–HCl (pH 6.8), 8% glycerol and 2% SDS] and boiled for 2 min. The homogenate was centrifuged at $15,000\times g$ at 4°C for 10 min. The supernatant was collected, and its protein concentration was determined using a protein determination kit (Bio-Rad, Richmond, CA) [4]. Bovine serum albumin (Sigma, St Louis, MO) was used as the standard. Then, the total concentration of all the homogenate samples was set to 1 mg/ml with SDS sample buffer with 16% 2-mercaptoethanol and 0.032% bromophenol blue. The homogenate samples were again boiled for 2 min and centrifuged at $15,000\times g$ at 4°C for 10 min. The supernatant was collected for western blotting. The following primary antibodies were used for immunoblotting: anti- α -tubulin, 1:1,000 dilution (Sigma-Aldrich Corp., St Louis, MO) and anti- α B-crystallin, 1:5,000 dilution [14]. After incubation with a secondary antibody, the membrane was incubated with an enhanced chemiluminescence kit (Amersham Biosciences Corp., Buckinghamshire, UK; Millipore, Billerica, MA). Purified α B-crystallin expressed

by *Escherichia coli* [14, 27, 32] and tubulin isolated from porcine brain were obtained as described by Fujita et al. [14]. The purified proteins (α B-crystallin, 10, 20, 30, and 40 ng; α -tubulin, 5, 10, 15, and 20 ng) were used as protein standards for the calibration of band density (determined by NIH imaging software). Samples of various volumes were loaded with the protein standards on each gel so that one of the sample band intensity fell into the linear part of the calibration curve for the standard proteins.

Analysis of MHC isoforms

The MHC isoforms of muscles were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). An amount of 1.6 μ g of protein was separated by SDS-PAGE (8.5% polyacrylamide gels) for 24 h at a constant voltage of 150 V at 4°C. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 dye. These procedures yielded five distinct bands corresponding to MHC isoforms I, α , IIa, IIc, and IIb [2]. Band densities were determined by using NIH imaging software, and the percentage of each MHC isoform was calculated.

Statistical analysis

All data were presented as mean \pm SD. Differences among groups were examined with one-way analysis of variance followed by the Bonferroni–Dunn post-hoc test. Pearson’s correlation coefficient test was also used to examine the correlation between the quantity of α B-crystallin and that of α -tubulin in striated muscles. The statistical significance level was set at $P < 0.05$ and $P < 0.01$ for all analyses.

Results

Content of α B-crystallin and α -tubulin in various striated muscles

α B-crystallin and α -tubulin were detected in a variety of striated muscles by western blotting using antibodies (Fig. 1a, b). The mean content of eleven samples from different rats for α B-crystallin and α -tubulin are shown in Table 1. The α B-crystallin content ranged from 1.2 ng/ μ g in masseter and tibialis anterior muscle to 31.1 ng/ μ g in soleus

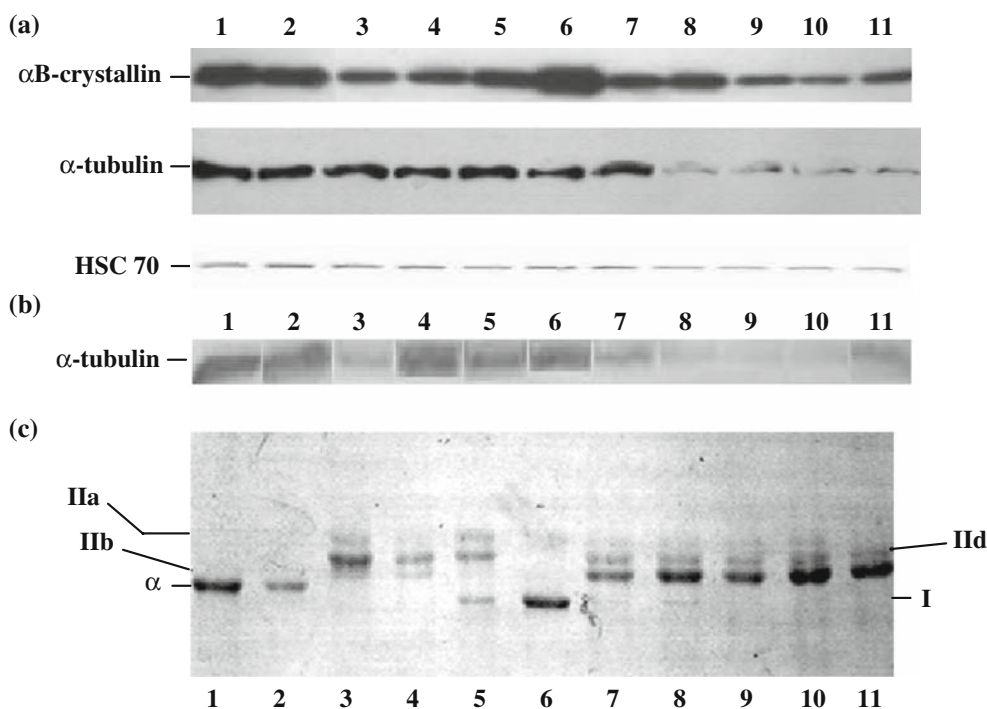


Fig. 1 Comparisons of protein contents of MHC-isoforms, α B-crystallin and α -tubulin in a variety of rat striated muscles. **a** Immunoblotting bands showing α B-crystallin, α -tubulin, and HSC 70 (control). Numbers labeled under the figure represent respective striated muscles; atrium (1), ventricle (2), masseter (3), tongue (4), diaphragm (5), soleus (6), plantaris (7), gastrocnemius (8), extensor digitorum longus (9), tibialis anterior (10), and psoas (11) muscles. Protein quantities per lane were 1.6, 50, and 20 μ g for α B-crystallin, α -tubulin, and HSC 70, respectively. **b** Immunoblotting bands

showing α -tubulin corresponding to the same striated muscles as in **a**. Protein quantities of total muscle extracts loaded per lane were lane 1 8 μ g, lane 2 8 μ g, lane 3 30 μ g, lane 4 13 μ g, lane 5 15 μ g, lane 6 8 μ g, lane 7 28 μ g, lane 8 25 μ g, lane 9 25 μ g, lane 10 40 μ g, lane 11 50 μ g. **c** Typical SDS-PAGE pattern showing MHC-isoform composition. IIa, IIc, IIb, α , and I indicate MHC-IIa, MHC-IIc, MHC-IIb, α -MHC, and MHC-I, respectively. Attached numbers are the same as in **a**. Protein loaded for each lane was 1.6 μ g

Table 1 Content of α B-crystallin and α -tubulin

Number	Striated muscles	α B-crystallin quantity (ng/ μ g protein)	α -Tubulin quantity (ng/ μ g protein)
1	Atrium	15.7 \pm 3.3	2.7 \pm 0.7
2	Ventricle	15.5 \pm 2.7	2.5 \pm 1.3
3	Masseter	1.2 \pm 0.2** ^{a,b}	0.8 \pm 0.3
4	Tongue	3.0 \pm 0.5** ^{a,b}	1.0 \pm 0.5
5	Diaphragm	12.3 \pm 2.5** ^{c,d,f}	0.9 \pm 0.5
6	Soleus muscle	31.1 \pm 6.7** ^{a-f}	2.4 \pm 1.3
7	Plantaris muscle	2.6 \pm 0.4** ^{a,b,e,f}	0.7 \pm 0.3* ^a
8	Gastrocnemius muscle	3.3 \pm 0.7** ^{a,b,e,f}	0.7 \pm 0.5
9	EDL muscle	1.6 \pm 0.4** ^{a,b,e,f}	0.6 \pm 0.3* ^a
10	TA muscle	1.2 \pm 0.5** ^{a,b,e,f}	0.3 \pm 0.2*** ^{a,b,f}
11	Psoas muscle	1.3 \pm 0.4** ^{a,b,e,f}	0.2 \pm 0.1*** ^{a,b,f}

$n = 5$ –6; values are mean \pm SD

EDL extensor digitorum longus, TA tibialis anterior

* $P < 0.05$, ** $P < 0.01$

^a Statistical significances with atrium

^b Statistical significances with ventricle

^c Statistical significances with masseter

^d Statistical significances with tongue

^e Statistical significances with diaphragm

^f Statistical significances with soleus

muscle. The mean α -tubulin content ranged from 0.2 ng/ μ g in psoas muscle to 2.7 ng/ μ g in atrial muscle. Thus α B-crystallin content varied 10–30 times, while α -tubulin content varied <13.5 times among the studied muscles.

MHC isoforms

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the muscle samples showed five MHC isoforms (Fig. 1c). The relative content of each MHC isoform to that of total MHC content is shown in Table 2. Muscles were divided into three groups according to the percentage of each MHC isoform. A dominant MHC isoform was defined when its content was >1.5 times as large as that of other MHC isoforms. Atrium, ventricle, and soleus muscles were almost exclusively MHC-I or MHC- α (MHC-I/ α) dominant. MHC-II_d was dominant in the diaphragm, tongue, and masseter muscles. However, the plantaris, gastrocnemius, extensor digitorum longus, tibialis anterior, and psoas muscles were classified as MHC-II_b dominant muscles.

Correlations between α B-crystallin, α -tubulin, and MHC isoforms

There is a high correlation between the quantity of α B-crystallin and that of α -tubulin in various muscles

($n = 11$; $r = 0.837$, $P < 0.01$, Fig. 2). MHC-I/ α dominant muscles, including atrium, ventricle, and soleus muscles, showed higher contents of α B-crystallin and α -tubulin than the MHC-II dominant muscles, e.g., plantaris, gastrocnemius, extensor digitorum longus, tibialis anterior, and psoas muscles. Among MHC-II dominant groups, MHC-II_d dominant muscles, such as diaphragm and tongue muscles, showed a relatively higher amount of α B-crystallin and α -tubulin than MHC-II_b dominant muscles (Fig. 2).

Discussion

In the present study, tubulin content in the variety of striated muscles correlated well with α B-crystallin content (Fig. 2); their content is higher in MHC-I/ α dominant muscles, intermediate in MHC-II_d dominant muscles, and lower in MHC-II_b dominant muscles. Together with so-far reported evidence described in the Sect. "Introduction," this strongly supports our hypothesis that dynamics between microtubules and their constituent tubulin transduce mechanical stress to adaptive responses in mature muscles.

Persistently alternating passive lengthening and active shortening are the common features of MHC-I/ α dominant muscles. Soleus muscles (MHC-I dominant) are stretched and shortened during walking and running, and heart muscles (MHC- α dominant) are extended and contracted during the pumping cycles of the heart. The alternating stress would elicit dynamic remodeling of microtubules, which in turn may induce adaptive protein turnover in muscles as in general cells.

It is reported that microtubules in differentiated cultured myotube are stabilized by the replacement of tyrosine at the tip with glutamate [16]. A considerable fraction of microtubules in fast muscles may be stabilized with the tip replacement. However, we infer that, at least in continuously stressed slow skeletal and heart muscles, a considerable fraction of microtubules are dynamically remodeling.

A closer inspection of the correlation suggests more details of the adaptive process in striated muscles. The muscles of relatively higher tubulin/ α B-crystalline ratio include heart, tongue, and masseter muscles. These muscles work at higher alternating frequency of passive lengthening and active shortening with relatively low loads. On the other hand, the muscles of lower tubulin/ α B-crystalline ratio include soleus muscle and diaphragm. They work at lower alternating frequency of passive stretching and active shortening with relatively high loads. Excessively frequent switching of passive lengthening and active shortening likely destabilize microtubule networks, perturbing their

Table 2 Myosin heavy chain content

Number	Striated muscles	I/α (%)	IIa (%)	IId (%)	IIb (%)
1	Atrium	100.0** ^{a,b,c}	0.0	0.0	0.0
2	Ventricle	100.0** ^{a,b,c}	0.0	0.0	0.0
3	Masseter	0.0** ^b	8.2 ± 9.6** ^d	64.3 ± 15.5* ^{b,d,f}	27.4 ± 22.2* ^f
4	Tongue	0.0** ^{b,c}	4.3 ± 7.1** ^{d,e}	71.7 ± 6.9** ^{b,d,f}	24.0 ± 3.7** ^{c,e,f}
5	Diaphragm	23.3 ± 4.2** ^{b,c}	21.0 ± 4.7** ^{d,e}	55.7 ± 5.0** ^{b,d,f}	0.0** ^{c,e,f}
6	Soleus muscle	92.7 ± 3.4** ^{a,b,c}	7.3 ± 3.4** ^{a,d,e}	0.0** ^{b,d}	0.0** ^{c,e}
7	Plantaris muscle	3.8 ± 3.1** ^{b,c}	0.0** ^{d,e}	37.1 ± 8.2** ^{b,d,f}	59.1 ± 10.2** ^{c,e,f}
8	Gastrocnemius muscle	5.1 ± 1.0** ^{b,c}	0.0** ^{d,e}	21.7 ± 6.0** ^{b,d,f}	73.2 ± 6.4** ^{c,e,f}
9	EDL muscle	1.7 ± 1.7** ^{b,c}	0.0** ^{d,e}	25.3 ± 6.1** ^{b,d,f}	73.0 ± 6.6** ^{c,e,f}
10	TA muscle	1.5 ± 1.7** ^c	0.0* ^{d,**,e}	8.2 ± 5.1** ^{d,f}	90.3 ± 3.6** ^{c,e,f}
11	Psoas muscle	1.1 ± 2.3** ^c	0.0** ^e	9.1 ± 7.1** ^f	89.8 ± 6.5** ^{c,e,f}

n = 4, Values are mean ± SD

EDL extensor digitorum longus, TA tibialis anterior

* *P* < 0.05, ** *P* < 0.01

- ^a Statistical significances between I/α and IIa
- ^b Statistical significances between I/α and IId
- ^c Statistical significances between I/α and IIb
- ^d Statistical significances between IIa and IId
- ^e Statistical significances between IIa and IIb
- ^f Statistical significances between IId and IIb

protein-turnover accelerating effect. Therefore, there would be an optimal switching frequency for the adaptive induction of αB-crystallin. The frequency of walking and breathing that governs the switching frequency of soleus muscle and diaphragm, respectively, seems to be optimal for the adaptive function of microtubule networks.

Two other cytoskeletons are found in striated muscles. One is the intermediate filament system, including desmin, which maintains the integrity of sarcomere structure and organizes cell shape [2]. Its mechanical character is similar to connectin, a gigantic protein filament that anchors thick filament lattice to Z-disk transmitting passive force in sarcomere [23]. The other is actin filaments. β-actin filaments line plasma membrane of, at least, cultured cells to form stress-fiber transmitting active force as well as passive force [10]. α-Actin filaments constitute I-bands that transmit active force of the thick filament lattice to Z-disks [17, 21]. Z-disks include γ-actin [25]. The intermediate filaments for passive force balance in relaxed muscle and the actin filaments for active force transmission in contracting muscle are both specialized to sustain the stability of functioning muscle. However, our hypothesis suggested that microtubules are specialized in transforming muscles in accordance with the mechanical demand from environment [19]. In cultured cells as well as striated muscles, intermediate filament and microtubule coaligns frequently [9, 15], and αB-crystallin also works as a chaperone for the free forms of intermediate filament [26].

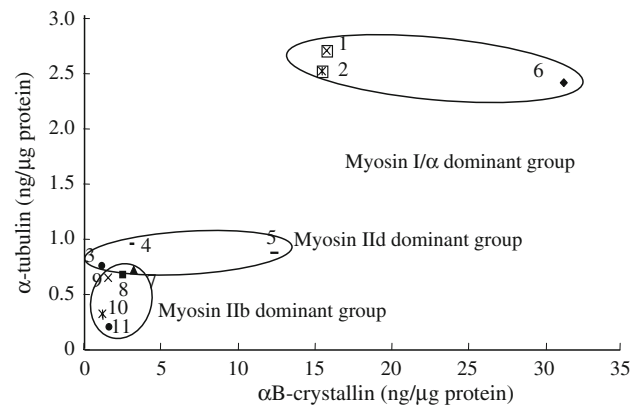


Fig. 2 Correlation between the contents of αB-crystallin and α-tubulin. Muscles are grouped by dominant MHC-isoform. The attached numbers are the same as in Fig. 1. There is a significant correlation (*P* < 0.01) between the quantity of αB-crystallin and that of α-tubulin (*n* = 11, Pearson correlation coefficient 0.837)

The “dynamic instability” [24] would be a necessary feature of microtubules to realize the adaptive transforming function. The heterodimers of α- and β-tubulin self-assemble to rigid microtubules at an expense of free energy derived from GTP, not only transmitting passive tensile force, but also producing active pushing force to create cell polarity [22]. In general cells, thus formed microtubules are reported to function as a railroad for the shuttling of newly synthesized and denatured proteins, with the help of motor proteins, and for transmitting signals to regulate

metabolism [15]. We infer that microtubules in mature striated muscle cells bear similar functions to general cells.

In our hypothesis, we consider α B-crystalline is induced by a microtubule network under the optimal dynamic instability due to the switching of passive and active tensile stress. As a general rule in biochemical regulation, there is a possibility that equilibrium between microtubules and tubulin dimers receives feedback from α B-crystalline. Several recent reports support the feedback. For instance, α B-crystallin in striated muscles is reported to interact with both tubulin dimers [27] and MAPs (microtubule-associated proteins) microtubules [14]. In oligodendroglia, Bauer and Richter-Landsberg suggested that the stress-induced binding of α B-crystallin prevents microtubules from aggregation [8].

Our hypothesis supported by the present correlation analysis thus further suggests that microtubules and α B-crystallin constitute a conducting system that links mechanical environmental demands to morphological and biochemical processes, which eventually evolve adaptive transformation in the function and metabolism of mature striated muscle cells. Studies to test the hypothesis are awaited.

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