

Talin, Vinculin and Nestin Expression in Orofacial Muscles of Dystrophin Deficient mdx Mice

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Abstract The activity of cytoskeletal proteins like talin, vinculin and nestin increases in muscle that regenerates. Little is known about their role or at least their expression in the process of regeneration in masticatory muscles of mdx mice, a model of Duchenne muscular dystrophy. To determine a potential role of cytoskeletal proteins in the regeneration process of mdx masticatory muscles, we examined the expression of talin 1, talin 2, vinculin and nestin in 100-day-old control and mdx mice using quantitative RT-PCR, Western blot analyses and histochemistry. The protein expression of talin 1, talin 2, nestin and vinculin in mdx muscles remained unchanged as compared with normal mice. However, in mdx masseter it was found a relative increase of nestin compared to controls. The protein expression of talin 1 and vinculin tended to be increased in mdx tongue and talin 2 to diminish in mdx masseter and temporal muscle. In mdx mice, we found significantly lower percentage of transcripts coding for nestin, talin 1, talin 2 and vinculin in masseter ($p < 0.05$) and temporal muscle ($p < 0.001$). In contrast, the mRNA expression of nestin was found to be increased in mdx

tongue. Activated satellite cells, myoblasts and immature regenerated muscle fibres in mdx masseter and temporal revealed positive staining for nestin. The findings of the presented work suggest dystrophin-lack-associated changes in the expression of cytoskeletal proteins in mdx masticatory muscles could be compensatory for dystrophin absence. The expression of nestin may serve as an indicator for the regeneration in the orofacial muscles.

Keywords mdx · Nestin · Regeneration · Dystrophy · Masticatory muscles

Introduction

The limb muscles are first to be affected in the progression of Duchenne muscle dystrophy (DMD), and these muscles are the muscles which have been mostly investigated. Orofacial muscles, in contrast, have somewhat been neglected although they are responsible for vital functions such as chewing, swallowing, drinking and speech.

Increased life-expectancy of the DMD patients revealed that orofacial muscles are involved in the dystrophic process, progressively compromising orofacial function. These patients may suffer from severe malocclusions, feeding difficulties and weight loss (Botteron et al. 2009; Ghafari et al. 1988; Matsuyuki et al. 2006). It is becoming evident that more data about the muscle regeneration process in muscle dystrophinopathies are needed to develop strategies for improving life quality of the DMD patients.

Duchenne muscular dystrophy is characterized by progressive deterioration of skeletal muscle due to the absence of dystrophin, which is a structural protein of the dystrophin–glycoprotein complex (DGC). The DGC connects the underlying contractile elements to the membrane anchored

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glycoproteins that themselves are affixed to the extracellular matrix. The loss of dystrophin causes destabilization of the sarcolemma leading to contraction-induced micro tears and the influx of ions such as Ca^{2+} , as well as the loss of selected proteins from the myofibers (Hopf et al. 2007; Lapidos et al. 2004; Whitehead et al. 2006). The mdx mouse model of DMD also has X-linked myopathy due to dystrophin mutation (Hoffman et al. 1987).

However, unlike the progressive muscle wasting seen in DMD, mdx muscle undergoes only a transient period of degeneration at 3–4 weeks of age, followed by nearly complete muscle regeneration (Hoffman et al. 1987; Pastoret and Sebillé 1995; Tanabe et al. 1986). Therefore, it has been suggested that mdx mice may compensate for dystrophin's absence by upregulating the expression of structural proteins of the cytoskeleton that can function similarly to dystrophin (Law et al. 1994).

One of these protein assemblies comprises vinculin, talin, integrin and several integrin-binding extracellular matrix proteins (Burridge and Mangeat 1984; Hynes 1987; Law et al. 1994). The other assembly is the dystrophin-associated glycoprotein complex, which connects actin via dystrophin and a transmembrane glycoprotein complex to the extracellular matrix. Both assemblies form links between the actin cytoskeleton and extracellular structural proteins. A third assembly is formed by intermediate filaments, such as nestin and vimentin (Michalczyk and Ziman 2005). Nestin is of particular interest since it is expressed predominantly in regenerating tissues including muscle (Kachinsky et al. 1994). However, the role of nestin in cellular proliferation during development and regeneration remains to be conclusively defined (Michalczyk and Ziman 2005).

The fragility of dystrophic fibres may be linked to the changes in expression and organization of proteins, comprising the cytoskeleton and sarcolemma and it is hypothesized that the absence of dystrophin may lead to disorganization of other structural proteins in the neighbourhood as vinculin, talin and nestin (Ziegler et al. 2008). On the other hand, these proteins may play an important role during muscle regeneration stages in mdx and DMD (Spasov et al. 2010) or during functional changes of muscles. At 100 days of age regeneration processes in mdx overcome degeneration and currently there is a lack of data concerning the muscle fibre structure and properties within newly regenerated masticatory fibres. Results from the present study may give a more complete picture of how masticatory muscles adapt to dystrophic changes.

Objective of the study was therefore to investigate in completely regenerated fibres of 100-day-old mdx mice masticatory muscles the expression of nestin, talin and vinculin in order to determine whether there is a compensation for the lack of dystrophin. Our objective was to describe further the distribution pattern of nestin in regenerated

orofacial muscles and to examine whether nestin could serve as an indicator for muscle regeneration in these muscles.

Materials and Methods

Animals

Mice of the inbred strains C57Bl/10ScSn (control) and C57/Bl10ScSn-*Dmd*^y (mdx) were originally obtained from Harlan Winkelmann (Borchen, Germany) and Charles River (Sulzfeld, Germany). Both strains were bred in the Department of Orthodontics of the Medical Faculty at the University of Greifswald. Age-matched pairs of mdx and control animals (male, 100-day-old, with the same body weight) were killed using overdose of ether inhalation. Muscle tissue samples were taken from the superficial part of masseter, the middle of temporal muscle and the superior longitudinal tongue muscle, and either frozen in liquid nitrogen for quantitative reverse transcription PCR and Western blot analyses or fixed in phosphate-buffered formalin for immunohistochemistry. All surgical and experimental procedures were approved by the Animal Welfare Committee on the State Government (LALLF M-V/TSD/7221.3-2.3-001/09).

TaqMan RT-PCR

Total RNA was isolated, reverse transcribed and amplified using the StepOne Plus[®] sequence detection system (PE Applied Biosystems) as described previously (Spasov et al. 2011a). Gene specific primers and probes were purchased from PE Applied Biosystems (talin 1: Mm01351134_m1; talin 2: Mm01150488_m1; vinculin: Mm01269586_m1; nestin: Mm01223404_g1) with each probe having been synthesized with a fluorescent 5'-reporter dye (FAM: 6-carboxy-fluorescein) and a 3'-quencher dye (TAMRA: 6-carboxy-tetramethyl-rhodamine). All values are given in relation to the mRNA of 18S rRNA.

Western Blot Analysis

Muscle tissue samples were mechanically homogenized during thawing in lysis buffer (5% glycerol, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (Sigma, Taufkirchen, Germany) using the TissueLyser (Qiagen, Hilden, Germany). Membrane proteins extracts (30 µg) were separated on SDS gels, transferred to nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotting system (Biometra, Göttingen, Germany) and incubated with antibodies against vinculin (monoclonal, clone VIN-11-5, Sigma Aldrich, München, Germany, dilution 1:1,000 in PBS containing 5% dry milk and 0.025%

NaN₃), talin (monoclonal, clone 8d4, Sigma Aldrich, München, Germany, dilution 1:500 in dry milk/PBS) or nestin (monoclonal, Millipore, Schwalbach, Germany, dilution 1:250 in PBS containing 3% bovine serum albumin) over night at 4°C. Secondary HRP-conjugated goat anti-mouse immunoglobulins (Dako, Hamburg, Germany) were used at a dilution of 1:5,000. Visualisation and detection of bound antibodies were carried out using an enhanced chemiluminescence system (Perbio Science). To assess equal loading of the gel, every membrane was stripped with RestoreTM Plus Western blot stripping buffer (Perbio Science) and incubated with a monoclonal anti- α -actinin antibody (clone AT6/172, Upstate, dilution 1:1,000; 2 h at room temperature). Quantitative analyses of protein bands from talin 1, talin 2, vinculin, nestin and α -actinin in masticatory muscle of 100-day-old C57Bl/10Sc and mdx mice were done using the GelScan 5.2 software (Serva, Germany). Mean optical density (mod) $\pm$ SEM are given in all cases for $n = 4$ animals and four independent Western blot analyses.

Immunohistochemistry

Staining of serial paraffin sections (4 μ m) from the middle of the muscle tissue specimen and the Corpus linguae ($n = 5$, each group, control and mdx) were performed following the instructions for the M.O.M. kit of Vectastain (Vector Laboratories Burlingame, CA 94010) and using a monoclonal anti-nestin antibody (Millipore, Schwalbach, Germany, dilution 1:100). Incubation with the primary antibody was done over night (12–16 h) at 4°C. Visualization of bound nestin antibodies was done using a New Fuchsin alkaline phosphatase substrate protocol. Sections were counter stained in hematoxylin and then cover-slipped. The level of antigen expression was determined in one run in a blinded manner with identical staff, equipment and chemicals.

Statistical Analysis

All statistical analyses were performed using the SigmaPlot Software (Systat Software, Inc.1735, Technology Drive, San Jose, CA 95110, USA). Statistical analyses were made using Student's unpaired t test. Data are given as mean $\pm$ SEM. Value of $p < 0.05$ was considered statistically significant.

Results

mRNA Expression

Gene-specific TaqMan RT-PCR was performed to quantify the expression of genes coding for talin 1 and talin 2, vinculin and nestin in the masseter, temporal and tongue muscle (Table 1).

Table 1 Gene specific transcript levels of talin 1, talin 2, vinculin and nestin in the tested masticatory muscles of control and mdx mice

	Control	mdx	<i>p</i>
Masseter			
Nestin	0.175 $\pm$ 0.0052	0.036 $\pm$ 0.006	0.032
Talin 1	0.0093 $\pm$ 0.0046	0.0059 $\pm$ 0.0018	NS
Talin 2	0.0019 $\pm$ 0.0007	0.00056 $\pm$ 0.00023	NS
Vinculin	0.0081 $\pm$ 0.0018	0.0073 $\pm$ 0.0025	NS
Temporal			
Nestin	0.35 $\pm$ 0.064	0.05 $\pm$ 0.0087	0.0008
Talin 1	0.038 $\pm$ 0.0064	0.012 $\pm$ 0.0019	0.00073
Talin 2	0.056 $\pm$ 0.015	0.0019 $\pm$ 0.00085	0.0017
Vinculin	0.084 $\pm$ 0.014	0.027 $\pm$ 0.0078	0.0021
Tongue			
Nestin	0.038 $\pm$ 0.0075	0.117 $\pm$ 0.017	0.00066
Talin 1	0.016 $\pm$ 0.0044	0.0199 $\pm$ 0.0035	NS
Talin 2	0.0019 $\pm$ 0.0006	0.0031 $\pm$ 0.00069	NS
Vinculin	0.037 $\pm$ 0.0085	0.049 $\pm$ 0.01	NS

The mRNA levels of all tested genes are given in relation to that of 18S rRNA. Mean $\pm$ SEM, n : 6–11 samples, p : significant differences between control and mdx mice, unpaired Student's t test

NS not significant

In mdx mice we found significantly lower percentage of transcripts coding for nestin in both, the masseter ($p = 0.0032$) and temporal ($p = 0.0008$) muscle, whereas in tongue ($p = 0.000066$) muscle the mRNA expression of nestin was found to be increased as compared with normal mice (Table 1). The mRNA expression of talin 1 ($p = 0.00073$), talin 2 ($p = 0.0017$) and vinculin ($p = 0.0021$) was found to be diminished in mdx temporal muscle. In mdx masseter and tongue muscles the mRNA expression of talin 1, talin 2 and vinculin was not significant when compared to control mice.

Western Blot Analysis

Representative western blots showed presence of talin 1, talin 2, vinculin and nestin (Fig. 1) in masticatory muscles of both mdx and control mice. Their protein expression in mdx muscles remained unchanged as compared with normal mice (Fig. 2). However, the densitometry revealed some relative, insignificant differences in the protein expression of talin 1, talin 2, vinculin and nestin which we have expressed in sense of tendencies.

The protein concentrations of talin 1 were found to be relatively increased in mdx tongue, whereas in mdx masseter and temporal the concentrations were equal between control and mdx mice (Fig. 2). Concentrations of talin 2 were found to be diminished in mdx masseter and

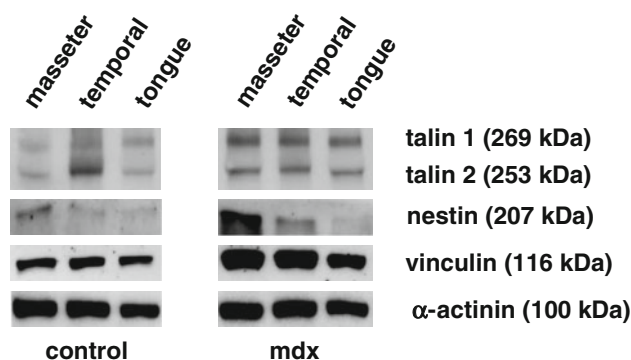


Fig. 1 Talin 1, talin 2, vinculin and nestin in masseter and temporal muscle as well as tongue from control and mdx mice (representative Western blots). The membranes were probed with monoclonal antibodies against talin isoforms, vinculin and nestin. A monoclonal antibody was used to detect α -actinin serving as an internal control

temporal, whereas in mdx tongue muscle concentrations of talin 2 did not differ relative to controls.

The protein amounts of vinculin in mdx tongue were found to be increased in comparison with normal tongue muscle (Fig. 2b). For the mdx masseter and temporal the quantity of vinculin proteins remained unchanged as compared with controls.

In mdx masseter and temporal muscles the protein expression of nestin was increased, whereas in mdx tongue the expression was diminished relative to controls.

Localisation of Nestin

The nestin distribution was investigated using monoclonal anti-nestin antibodies. Staining with the antibody against nestin in the masticatory muscles of control mice was almost negative (Fig. 3). In contrast, the cytoplasm of activated satellite cells, myoblasts and immature regenerated muscle fibres in mdx masseter and temporal muscle revealed positive staining for nestin, which however, was heterogeneous. Matured regenerated muscle fibres with centrally placed nuclei did not contain nestin. Muscle fibres in mdx tongue muscles revealed only faint staining for nestin.

Discussion

It is suggested that in mdx muscle contractions cause transient mechanically-induced defects in the membrane. Dystrophin, which is a part of a mechanical link between the contractile machinery and the extracellular matrix, is thought to contribute to membrane strength so that in its absence mechanically-induced defects are worse. Therefore proteins, co-responsible for the stability of the sarcolemma and cytoskeleton may experience changes in

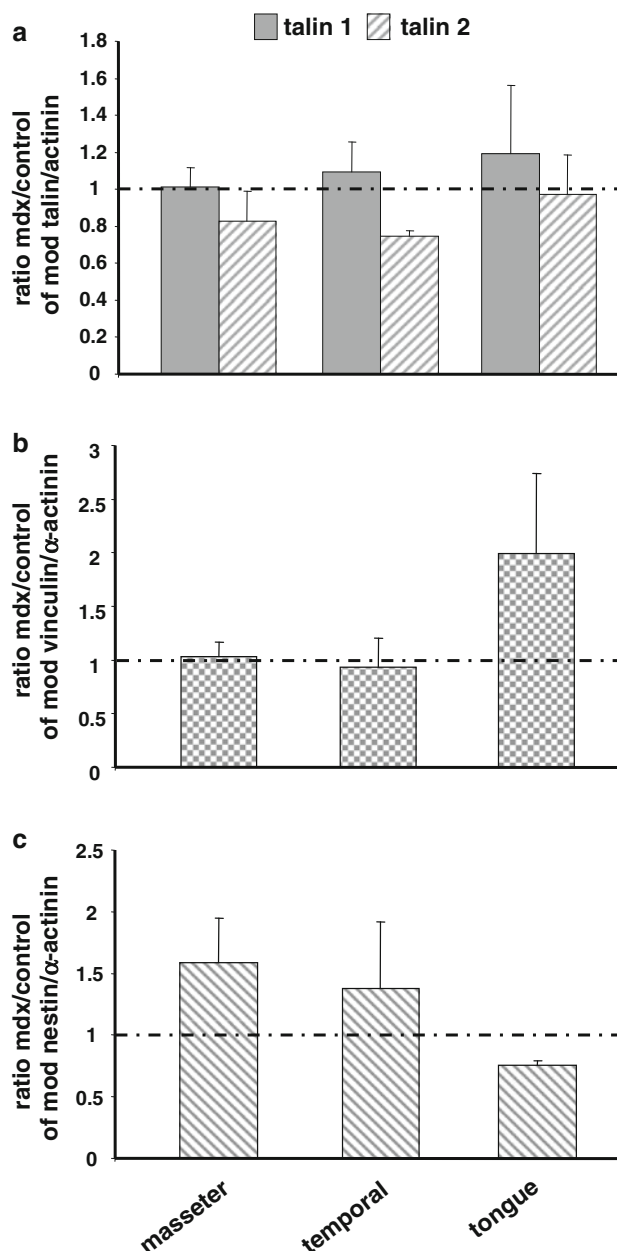


Fig. 2 The ratios, mdx versus control mice, of the mean optical densities (mod) following quantitative analyses of talin 1, talin 2, vinculin and nestin by Western blot, as shown in Fig. 1. Protein bands attributed to talin 1, talin 2, vinculin, nestin and α -actinin were evaluated using the GelScan 5.2 software (Serva, Germany). Mean $\pm$ SEM from four independent experiments

their organization and expression in dystrophinopathies as DMD and its animal model, mdx mice. In this respect most studies concentrated their attention on mdx hindlimb muscles. In contrast, our research group has focused primarily on mdx orofacial muscles showing that they not only have similarities but also differences in the response to dystrophin deficiency when compared to hindlimb muscles (Spasov et al. 2010, 2011b).

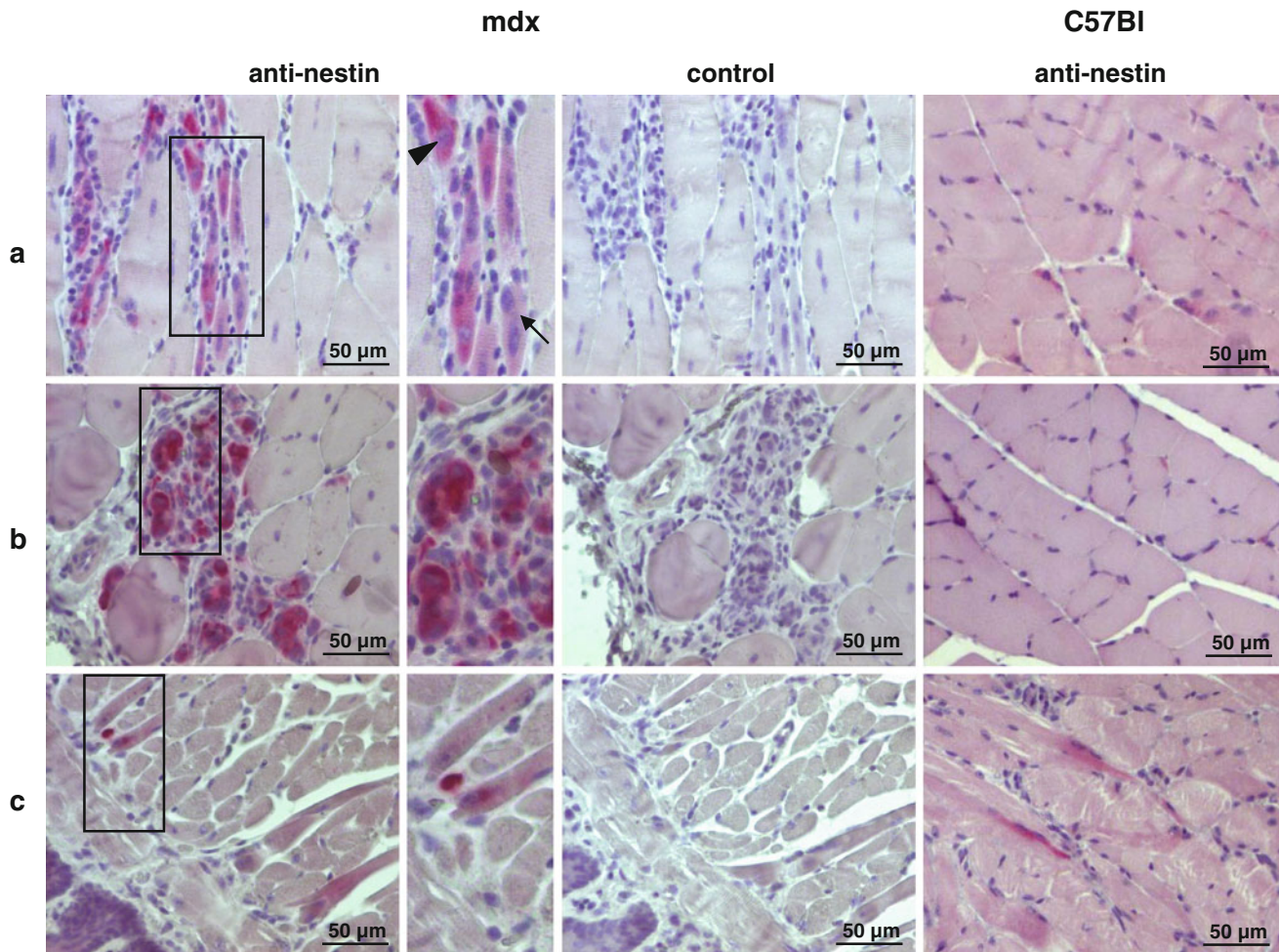


Fig. 3 Representative immunohistochemical staining of nestin in paraffin sections of masticatory muscle samples of mdx mice at two different magnifications. Nestin staining is in *red*; counterstaining of the nuclei with hemalaun (*blue*). The *arrow* indicates an immature

regenerated myofiber, the *triangle* shows a regenerated myoblast. *Bars* represent 50 μ m. **a** Masseter, **b** temporal muscle, **c** tongue muscle

The main findings of the present study are: (1) protein expression of nestin tended to increase in mdx masseter; (2) protein concentrations of talin 1 and vinculin increased relatively in mdx tongue and these of talin 2 decreased in mdx masseter and temporal, (3) the corresponding mRNA expression of talin1 and 2, vinculin and nestin was down regulated and did not coincide with the expression of the corresponding proteins, and (4) nestin is localized only in activated satellite cells, myoblasts and immature regenerated muscle fibres.

Our results of increase of nestin in mdx masseter are in accordance with previous studies describing re-expression of nestin in situations that reproduce developmental phases such as regeneration of adult muscles after injury or diseases (Cizkova et al. 2009a, b). Muscle regeneration after necrosis in mdx mice and DMD patients represent such phases. Interestingly, to our knowledge, there is only one study investigating the role of nestin in dystrophin-related

muscle dystrophinopathies as DMD and Becker dystrophy and no studies are available on nestin in mdx mice. Nestin immunoreactivity was observed in a subset of muscle fibres in all analyzed biopsies from DMD/BMD (Duchenne and Becker muscular dystrophies; lower limbs) whereby most muscle fibres staining positive for nestin were small, with centrally located nuclei and displayed morphological hallmarks of regeneration, whereas biopsies from normal muscle did not (Sjöberg et al. 1994). These findings are in accordance with our results where small myoblasts and immature regenerated muscle fibres revealed strong positive staining for nestin in the mdx muscles compared to controls, where nestin could not be detected. Thus, our results confirm previous findings (Cizkova et al. 2009b) that presence of nestin immunoreactivity and expression is correlated with muscle regeneration and extends this event on orofacial muscles. However, the role of nestin in cellular proliferation during development and regeneration

remains to be conclusively defined (Michalczyk and Ziman 2005).

Concomitantly to increased nestin protein expression, we found a tendency for increased protein expression of talin 1, talin 2 and vinculin in mdx tongue which may indicate some protective mechanism in mdx mice, may be in order to maintain muscle integrity. Talin and vinculin frame a complex that connects muscle fibre actin to the sarcolemma via integrins (Ziegler et al. 2006). Thus, we suppose that in mdx muscle fibres talin and vinculin are over expressed to compensate the lack of dystrophin and to stabilize the cytoskeleton. The findings of our work are supported by investigations from 11-week-old adult mice mdx tibialis muscles which showed increased concentrations of vinculin and talin whereas no significant differences in concentrations of any of the proteins from the 2-week-old mice were found (Law et al. 1994).

However, in our study the protein amounts of vinculin and talin 1 were nearly equal in masseter and temporal muscle of both groups and those of talin 2 were found to have a tendency to decrease, which is in contrast to the above cited study (Law et al. 1994). The findings of our study revealed differences in protein amounts between masticatory and hindlimb muscles. The disparate expression of cytoskeletal protein in masseter and temporal compared to tibialis anterior (Law et al. 1994) indicates differences in the response of orofacial muscles to dystrophy deficiency as compared to hindlimb muscles. On the other hand, it seems that a tendency to an increase of talin 1, talin 2 and vinculin in mdx tongue is not a predetermined event as a result of dystrophin deficiency, but rather a process linked to the mdx muscle pathology. The latter may explain why the above cited study did not find any increase in the levels of talin and vinculin in 2-week-old mice, which is a period preceding onset of dystrophy.

In this study, the use of a loading control (α -actinin) on Western blots was performed to check whether the lanes in the gel have been evenly loaded. It is essential to use a loading control in this case, because we have compared expression of proteins between different samples. However, we recognize that the stripping of the membrane before applying the loading control might produce some changes on the proteins of interest and bias the results obtained. Comparative blot analysis was therefore performed to minimize biases due to stripping of the membrane.

Interestingly, our results reveal discrepancies between what was found on the protein level and what was found on the transcriptional level (Figs. 1, 2; Table 1). This might be explained by the fact that changes in gene expression of nestin, vinculin and talin 1 and talin 2 during transcription may not be immediately translated into proteins. Thus, gene regulation modulates different steps in gene

expression and changes in mRNA expression may occur time-shifted on the protein level. There may also exist some form of negative translational control or other form of feedback resulting in inadequacies between mRNA levels and expressed protein amounts. However, the limitations of the method of protein quantification (densitometry) used allow at most a relative quantification and may therefore contribute to the discrepancies to the mRNA levels (Gassmann et al. 2009). In fact, we evaluated the quotient or index between the mod of the proteins and the respective mod of α -actinin (Fig. 2).

We conclude that mdx masticatory muscles, comparable to hindlimb muscles, may compensate for some structural defects associated with dystrophin deficiency by apparently increasing the concentration of structural proteins such as nestin, vinculin and talin that serve analogous functions in connecting cytoskeletal-membrane associations in order to enable muscle regeneration. Nestin expression may also serve as a muscle regeneration indicator in mdx masticatory muscles.

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