Expression profiling reveals heightened apoptosis and supports fiber size economy in the murine muscles of mastication

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Evans M, Morine K, Kulkarni C, Barton ER. Expression profiling reveals heightened apoptosis and supports fiber size economy in the murine muscles of mastication. Physiol Genomics 35: 86–95, 2008. First published July 1, 2008; doi:10.1152/physiolgenomics.00232.2007.—Distinctions between craniofacial and axial muscles exist from the onset of development and throughout adulthood. The masticatory muscles are a specialized group of craniofacial muscles that retain embryonic fiber properties in the adult, suggesting that the developmental origin of these muscles may govern a pattern of expression that differs from limb muscles. To determine the extent of these differences, expression profiling of total RNA isolated from the masseter and temporalis anterior (TA) muscles of adult female mice was performed, which identified transcriptional changes in unanticipated functional classes of genes in addition to those attributable to fiber type. In particular, the masseters displayed a reduction of transcripts associated with contractile and cytoskeletal load-sensing and anabolic processes, and heightened expression of genes associated with stress. Associated with these observations was a significantly smaller fiber cross-sectional area in masseters, significantly elevated load-sensing signaling (phosphorylated focal adhesion kinase), and increased apoptotic index in masseters compared with TA muscles. Based on these results, we hypothesize that masticatory muscles may have a fundamentally different strategy for muscle design, compared with axial muscles. Specifically, there are small diameter fibers that have an attenuated ability to hypertrophy, but an increased propensity to undergo apoptosis. These results may provide insight into the molecular basis for specific muscle-related pathologies associated with masticatory muscles.

caspase-12; focal adhesion complex; endoplasmic reticulum-stress apoptosis; myosin heavy chain

DISTINCTIONS BETWEEN THE CRANIOFACIAL muscles and the axial muscles of the limb and trunk are evident at the inception of development. During embryogenesis, myogenic precursor cells arise from epithelial somites, which have been segmented from the paraxial mesoderm flanking the neural tube (30, 36). Myogenic precursor cells from these somites produce all of the muscles in the limb and trunk and a subset of cranial muscles (the tongue). The remaining craniofacial muscles, however, are derived from more anterior non-somitic cells of the prechordal head mesoderm (25). The factors required for early phases of development differ in these muscle groups. For example, Pax3 and Myf-5 are needed for the existence of limb, trunk, and tongue muscles, but the absence of these factors does not affect the development of craniofacial muscles (43). In contrast, the basic helix-loop-helix transcription factor, MyoR, is important for the progression of craniofacial muscle development, where its absence in combination with its homologous protein capsin by gene targeting renders mice lacking craniofacial muscles (24). Therefore, both cell lineage and developmental regulation of craniofacial and limb muscles are distinct.

The developmental patterns may contribute to distinguishing features in adult head muscles. In the postnatal period, fiber type differences persist, where adult craniofacial muscle fibers retain fetal myosin heavy chain (MHC) and in some species α-cardiac MHC, neither of which are present in limb muscles (22, 48, 51). Within the craniofacial muscles, functional categorization exists, which includes the masticatory, laryngeal, and extraocular muscles, and these groups also bear unique properties. For example, with age, most skeletal muscles in the limbs and trunk exhibit a fast to slow shift in the fiber type distribution. However, jaw muscles shift in the opposite direction, where there is an increase in the fast fiber population in aging masseter muscles (29). Furthermore, it has been demonstrated that the repair process after acute damage is impaired in masticatory muscles compared with limb muscles (37). In the same study, a decreased rate of proliferation was observed in myoblasts isolated from regenerating masseters, suggesting that the satellite cells were, at least in part, responsible for the inability to repair. These results imply that unlike limb muscles, masticatory muscles are slow to remodel or regenerate, a property that could exacerbate the damaged state or hinder the healing process from any oral/facial intervention.

The properties of the craniofacial muscle groups challenge the dogma that there is a generalized unified tissue called skeletal muscle in the entire body. To date, this has been illustrated most clearly in the extraocular muscles, where expression profiling has helped to define the components that support rapid, fatigue-resistant activity for eye movements (15), and careful monitoring of proliferation has revealed constant remodeling of the extraocular muscles by active satellite cells (26). However, this analysis may not be relevant for the craniofacial muscles involved in mastication or speech. Because limb and masticatory muscles have been shown to differ at the developmental, physiological, and pathological levels, we sought to define a molecular signature for the muscles of mastication, which could help to identify the factors contributing to these distinctions.

METHODS

Animals. All studies were approved by the university’s animal care committee. Adult C57BL/6 female mice, aged 6 mo, were utilized for all experiments to eliminate potential effects of growth, aging, or sex on the measurements. For each experiment, mice were killed by CO2...
isopentane. Muscle samples for RNA and protein were stored in liquid nitrogen covered in mounting medium (OCT) prior to freezing in melting asphyxiation, after which skeletal muscles were rapidly dissected and analyzed.

**Expression analysis.** To identify differences between craniofacial and axial muscles at the molecular level, expression profiling was performed on total RNA isolated from the masseter and tibialis anterior (TA) muscles of 3 mice. Total RNA was isolated from the frozen muscle samples (TRIzol; Invitrogen, Carlsbad, CA) and further purified using RNeasy columns (Qiagen, Valencia, CA). RNA integrity was confirmed by gel electrophoresis. Profiled measurements were completed at the Penn Microarray Core Facility utilizing Affymetrix Mouse 430 2A chips. All protocols were conducted as described in the Affymetrix GeneChip Expression Analysis Technical Manual. Briefly, total RNA was converted to first-strand cDNA using Superscript II reverse transcriptase primed by a poly(T) oligomer that incorporated the T7 promoter. Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript and incorporation of biotinylated UTP. The cRNA products were fragmented to 200 nucleotides or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to six microarrays. The microarrays were fragmented to 200 nucleotides or less, heated at 99°C for 5 min, and hybridized for 16 h at 45°C to six microarrays. The microarrays were then washed at low (6×) SSPE and high (100 mM MES, 0.1 M NaCl) stringency and stained with streptavidin-phycocerythrin. Fluorescence was amplified by adding biotinylated anti-streptavidin and an additional aliquot of streptavidin-phycocerythrin stain. A confocal scanner was used to collect fluorescence signal after excitation at 570 nm.

**Initial data analysis.** The Affymetrix Microarray Suite 5.0 algorithm in GeneChip Operating Software was used to quantitate expression levels for targeted genes; default values provided by Affymetrix were applied to all analysis parameters. Border pixels were removed, and the average intensity of pixels within the 75th percentile was computed for each probe. The average of the lowest 2% of probe intensities occurring in each of 16 microarray sectors was set as background and subtracted from all features in that sector. Pairwise comparisons were made and normalized for detection of the targeted sequence by comparing signals from the perfect match and mismatch probe features. The number of probe pairs meeting the default discrimination threshold (τ = 0.015) was used to assign a call of absent, present, or marginal for each assayed gene, and a P value was calculated to reflect confidence in the detection call. A weighted mean of probe fluorescence (corrected for nonspecific signal by subtracting the mismatch probe value) was calculated using the one-step Tukey’s biweight estimate. This Signal value, a relative measure of the expression level, was computed for each assayed gene. Global scaling was applied to allow comparison of gene Signals across multiple microarrays: after exclusion of the highest and lowest 2%, the average total chip Signal was calculated and used to determine what scaling factor was required to adjust the chip average to an arbitrary target of 150. All Signal values from one microarray were then multiplied by the appropriate scaling factor.

Transcripts that received an absent call in all TA and masseter samples were eliminated from subsequent analysis. Pairwise comparisons of the probe set signals were performed between the TA and masseter for each animal using significance analysis of microarrays (SAM) (46) to detect >1.5-fold changes between samples with a false discovery rate <7%. This included transcripts present or marginal in TA, masseter, or all samples. Gene ontology of the differentially expressed transcripts was performed using Affymetrix analysis tools (www.affymetrix.com/analysis/netaffx). In addition, literature searches were performed to determine functions of transcripts without gene ontology assignments or to find specific functions with muscle. Expression profiling results were submitted to the NCBI-GEO (GSE11114) and the EMBL-EBI ArrayExpress (E-MEXP-1288) repositories.

**Quantitative RT-PCR.** Total RNA was isolated as described above from the TA and masseter muscles of an additional seven adult female mice. Quantification of RNA was performed with Ribogreen quantitation kit (Molecular Probes, Eugene, OR), and RNA integrity was confirmed by gel electrophoresis. Equal amounts of total RNA from each sample was subjected to single strand reverse transcription (Applied Biosystems, Foster City, CA). The resultant cDNA was utilized for quantitative RT-PCR (qRT-PCR) with oligonucleotides specific for genes listed in Table 2 using the Roche Lightcycler system, and reagents (LightCycler FastStart DNA MasterSYBR Green I; Roche Applied Science, Indianapolis, IN) as previously described (4). Expression levels were assessed by calculating the crossing point (Cp), where measured fluorescence rises above background, as the second derivative maximum of the reaction curve. Each sample was analyzed in duplicate and the resulting data averaged. The mean Cp values for the TA and masseters were calculated from the respective muscle samples. The relative change in expression between muscle groups was based on the assumption that a difference of 1 Cp resulted from a twofold change in expression. Melting point analysis of experimental samples confirmed that all primers were specific for their respective transcripts, where there was only one melting point observed for each primer pair. Controls included RNA not subjected to reverse transcription, and water only.

**Immunoblotting.** Muscles were removed from liquid nitrogen and homogenized in 10 vol/muscle wt of modified lysis buffer [50 mM Tris-HCl pH 7.4, 1% wt/vol Triton X-100, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, protease inhibitor and phosphatase inhibitors (Sigma, St. Louis, MO)]. Homogenates were centrifuged to pellet debris, and the total protein was measured in the supernatant (Bio-Rad, Hercules, CA). Equal amounts of protein from each muscle lystate were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated in a blocking buffer [5% nonfat dry milk in Tris-buffered saline plus 0.1% Tween 20 (5% milk-TTBS)], and then incubated with primary antibody diluted in 5% milk-TTBS overnight at 4°C. Primary antibodies included those for caspase-12 (1:1,000, #2202; Cell Signaling, Beverly, MA); phosphorylated and total focal adhesion kinase (FAK, 1:1,000 for each; #MAB1144, #06543; Upstate, Waltham, MA); integrin-β1D (1:1,000, ab8991; Abcam, Cambridge, MA); spectrin (1:500, ab11182; Abcam) and talin (1:2,000, T3287; Sigma). Antibodies for myosin 1 (chMy2, 1:10,000) and myosin 2 (AA269, 1:100) were a kind gift from Irina Agarkova (Zurich, Switzerland). Antibodies for a muscle-ankyrin repeat protein (MARPs) family members (diluted to 2 μg/mL) were a kind gift from Siegfried Labeit (Mannheim, Germany). Membranes were then washed in 5% milk-TTBS, and incubated with horseradish peroxidase-conjugated secondary antibody. After a series of washes in 5% milk-TTBS, and TTBS, detection was performed using enhanced chemiluminescence scanned immediately by Kodak mr4000 detection system (Eastman Kodak, Rochester, NY). Membranes were stained with Coomassie brilliant blue R-250 after immunoblotting to confirm equal protein loading.

**Muscle morphology and fiber typing.** We subjected 10 μm frozen cross sections taken from the midbelly of each muscle to immuno-histochemistry for laminin (rabbit anti-laminin Ab-1; Neomarkers, Fremont, CA) to outline the muscle fibers. Fiber typing was performed with antibodies recognizing MHC 2a (SC-71), MHC 2b (BF-F3), MHC 1 (BAF-8), and fibers lacking MHC 2X (BF-35) as previously described (3, 40). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Stained sections were visualized on a Leica DMR microscope, captured with a digital camera using image analysis software (OpenLab, Improvion, UK). Cross-sectional areas...
were assessed in at least 500 fibers from four nonoverlapping fields at ×100 magnification from n = 3 muscle pairs.

**MHC composition.** Frozen muscles taken from n = 2 adult female mice were homogenized in Laemmli buffer (23). Total protein in each homogenate was determined by a Bradford assay (Bio-Rad) and normalized to total tissue wet weight, and equal protein amounts were then separated using SDS-PAGE (44). Resolved gels were stained with Coomassie blue R-250 for 45 min followed by destaining for 2 h in 50% methanol-7% acetic acid. Images of the gels were captured with a high-performance digital camera (Eastman Kodak, Rochester, NY).

**Apoptosis measurements.** TUNEL staining was performed on 7 μm paraffin sections of masseter and TA muscles (n = 5 muscle pairs) using Tdt-FragEL DNA Fragmentation Detection kit (Calbiochem, EMD, Gibbstown, NJ) to determine the apoptotic index of the muscles. Subsequent immunohistochemistry was performed with anti-laminin (as above) and anti-dystrophin (DYS2, 1:100; Novacastra) to determine the proportion of TUNEL positive nuclei within the muscle fibers. TUNEL-positive myonuclei were calculated for four nonoverlapping fields at ×200 magnification from each muscle, averaged, and presented as percent TUNEL-positive myonuclei per sample. Negative control samples were incubated without TdT enzyme.

**Statistics.** Data are presented as means ± SE. Paired t-tests were utilized for comparisons between masseter and TA from the same animal for qRT-PCR validation. Unpaired t-tests were utilized for comparisons between groups for immunoblotting and TUNEL analysis. Fiber size distributions were not normal and required the Mann-Whitney test for comparisons. Statistical significance was accepted for P < 0.05. Statistical analysis for the expression profiling data utilized SAM (46) and is described above.

**RESULTS**

To identify differences between masticatory and axial muscles at the molecular level, expression profiling was performed on total RNA isolated from the masseter and TA muscles of three adult female mice. The quality of the microarray data is summarized in Table 1. All cRNA samples had a 260:280 ratio between 1.8 and 2.0. The percentage of transcripts expressed in all samples utilized for analysis exceeded 40% of the total muscle RNA content. Changes were also observed in genes that could regulate muscle fiber size. Interleukin-15 (IL-15) is an anabolic cytokine (39) that was downregulated fivefold in masseters. DNA-damage-inducible transcript 4-like (Ddit4L, also known as Snsr1, RTP801L) had sixfold higher expression in masseters compared to TA tissues. This is a negative regulator of the mammalian target of rapamycin (mTOR) pathway (8), and expression increases in rat skeletal muscle subjected to hindlimb suspension (9). Fiber cross-sectional area was determined in masseters and TAs by immunohistochemistry with antibodies recognizing the MHC isoforms. This resulted in two classes of genes: the contraction/cytoskeleton transcripts and those associated with stress and apoptosis.

Oligonucleotides were designed for seven of the identified transcripts and GAPDH (Table 2), to validate the profiling results by qRT-PCR on additional RNA samples (n = 7 muscle pairs). These transcripts represented a range of differences in expression level. The mean Cp were significantly lower in the masseters for Myh8, Ddit4l, Casp12, Egln3, and Mst4, whereas Cp values for Myl3 and Il15 were significantly higher (Table 3). Using the mean difference between Cp values for each transcript, we calculated the fold change in expression level between masseter and TA muscles. In all cases, the trends observed by qRT-PCR were similar to those found by expression profiling (Table 3), thereby validating the results for this group of transcripts.

**Table 1. Quality of RNA samples for expression profiling**

<table>
<thead>
<tr>
<th>Muscle</th>
<th>cRNA 260:280</th>
<th>Present Probe Sets (out of 22,690)</th>
<th>β-Actin</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>Signal</td>
<td>3′:5′</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Signal</td>
<td>3′:5′</td>
</tr>
<tr>
<td>Masseter</td>
<td>1.92±0.02</td>
<td>10,338±862</td>
<td>45.6±2.2</td>
<td>3,114±148</td>
</tr>
<tr>
<td>TA</td>
<td>1.91±0.02</td>
<td>10,018±293</td>
<td>44.1±1.3</td>
<td>3,029±100</td>
</tr>
</tbody>
</table>

Data are represented as means ± SE for n = 3 samples per muscle.

**Table 2. Differential expression of selected transcripts**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Present Probe Sets (out of 22,690)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myl3</td>
<td>158</td>
<td>1.50-fold</td>
</tr>
<tr>
<td>Il15</td>
<td>158</td>
<td>1.50-fold</td>
</tr>
<tr>
<td>Casp12</td>
<td>158</td>
<td>1.50-fold</td>
</tr>
<tr>
<td>Egln3</td>
<td>158</td>
<td>1.50-fold</td>
</tr>
<tr>
<td>Mst4</td>
<td>158</td>
<td>1.50-fold</td>
</tr>
<tr>
<td>Myh8</td>
<td>158</td>
<td>1.50-fold</td>
</tr>
</tbody>
</table>

These transcripts represented a range of differences in expression level. The mean Cp were significantly lower in the masseters for Myh8, Ddit4L, Casp12, Egln3, and Mst4, whereas Cp values for Myl3 and Il15 were significantly higher (Table 3). Using the mean difference between Cps for each transcript, we calculated the fold change in expression level between masseter and TA muscles. In all cases, the trends observed by qRT-PCR were similar to those found by expression profiling (Table 3), thereby validating the results for this group of transcripts.
To determine whether increased expression of stress and proapoptotic genes is associated with apoptosis in this tissue, we performed TUNEL staining on paraffin sections of masseter and TA muscles (n = 5 muscle pairs). Masseter muscles had more TUNEL-positive nuclei than TA muscles, which were found at the periphery of the muscle fibers (Fig. 4A). To determine whether the TUNEL-positive nuclei are myonuclei, we performed subsequent immunohistochemistry with antibodies for laminin and dystrophin (Fig. 4B). The masseters exhibited significantly more TUNEL-positive myonuclei than the TA muscles (Fig. 4C). Nuclei between the dystrophin and laminin staining (presumably satellite cells)
had no detectable TUNEL staining. The identity of TUNEL-positive nuclei outside of the basal lamina was not determined or quantified.

Immunoblotting for caspase-12 was performed on masseters and TA muscles (Fig. 5A, n = 3 muscle pairs). The caspase-12 antibody epitope was immediately COOH-terminal to K158 (CS in Fig. 5B), and so the protease involved in caspase-12 cleavage could be identified (32). In masseters, cleavage and activation of caspase-12 occurred at K158, which is an m-calpain site, and at D318, to result in an 18 kDa band. Cleavage and activation in the TA muscles occurred at the recognition site for caspase-7 (DEDD 94) resulting in the 40 kDa band.

Cytoskeletal genes were also differentially expressed. Expression of talin, a component of the focal adhesion complex, was reduced by 3.6-fold in masseters. Immunoblotting for focal adhesion complex proteins was performed to determine whether the lower expression of talin found in masseters was indicative of a decrease of the entire membrane complex. Because the masseter muscle fibers are smaller than those in TA muscles (Fig. 3) and the fiber surface area-to-volume ratio in masseter muscles is higher than in TA muscles, protein levels were normalized to spectrin, which has similar sarcolemmal localization. Spectrin expression did not differ between masseter and TA muscles. There was no significant difference in talin or β1D-integrin protein levels between masseter and TA muscles (Fig. 6A, n = 3 muscle pairs), counter to the expression profiling results. To test if masseters were sensing load through the focal adhesion complex, immunoblotting for phosphorylated and total FAK was performed. Proportional phosphorylation of FAK was significantly higher in masseters (Fig. 6B).

Genes expressing components of the M-line (myomesins 1 and 2) and I-band (Ankrd2) were lower in masseters than TA muscles. Components associated with the cytoskeleton that respond to and report mechanical stress (myomesin 1, 2, and Ankrd2) were lower in masseters than TA muscles. Components associated with the cytoskeleton that respond to and report mechanical stress (myomesin 1, 2, and Ankrd2) were lower in masseters than TA muscles (Fig. 3) and the fiber surface area-to-volume ratio in masseter muscles is higher than in TA muscles, protein levels were normalized to spectrin, which has similar sarcolemmal localization. Spectrin expression did not differ between masseter and TA muscles. There was no significant difference in talin or β1D-integrin protein levels between masseter and TA muscles (Fig. 6A, n = 3 muscle pairs), counter to the expression profiling results. To test if masseters were sensing load through the focal adhesion complex, immunoblotting for phosphorylated and total FAK was performed. Proportional phosphorylation of FAK was significantly higher in masseters (Fig. 6B).

Genes expressing components of the M-line (myomesins 1 and 2) and I-band (Ankrd2) were at even lower levels than the other contractile genes in the microarray experiment. Myomesin expression was 1.7- and 5.3-fold lower in masseters for myomesins 1 and 2, respectively. Ankrd2 expression was between 4- and 30-fold lower in masseters. Immunoblotting for these components (normalized to tubulin) confirmed that myomesin 1 and 2 and Ankrd2 were lower in masseters than TA (Fig. 7). Furthermore, blotting for other family members of Ankrd2 revealed that other proteins did not compensate for lower expression of Ankrd2 in masseters.  

**DISCUSSION**

Past studies have shown that masticatory muscle is morphologically and physiologically distinct from axial muscles. A contributing factor may be the embryonic origin of these muscles, where axial muscles are derived from somites, and masticatory muscles arise from the neural crest. As established by several previous studies, fiber size is smaller, and there is increased expression of neonatal MHC. Our study has revealed additional and potentially novel features of masticatory muscle. In particular, there was an upregulation of stress-induced genes. Consistent with these changes, masseter muscle exhibited increased TUNEL-positive myonuclear staining, as well as a change in the cleavage/activation pattern of caspase-12, which is the canonical caspase for endoplasmic reticulum (ER) stress. The focal adhesion complex showed elevated activation as indicated by FAK phosphorylation, suggesting that in the normal state, murine masseters are loaded to a greater extent than TA muscles. Components associated with the cytoskeleton that respond to and report mechanical stress (myomesin 1, 2, and Ankrd2) were expressed at significantly lower levels, suggesting that mechanical loading in masseters may not be efficiently transmitted to the myonuclei. In contrast to cardiac and limb skeletal muscle, where sustained increased load drives muscle hypertrophy (16, 38), the apparent load on masseters is associated with small fibers and high apoptotic index, supporting a model in which load exacerbates masseter muscle damage and apoptosis. The masseter shares some but not all features of expression profiling with extraocular, aging, regenerating, and unloaded muscles. Therefore, to our knowledge, the masseter has a unique molecular signature within the spectrum of muscle properties, which may provide insight into novel load-sensing and stress mechanisms in skeletal muscle.

The stress-associated genes found in the masseters represent many different stress pathways. Ddit4l expression rises by 10.220.33.5 on April 14, 2017 http://physiolgenomics.physiology.org/downloaded from http://physiolgenomics.physiology.org by 10.220.33.5 on April 14, 2017
is the member of the cysteine protease family associated with ER stress-mediated apoptosis (33). The serine/threonine protein kinase, Mst4 (MASK), had 10-fold higher expression in masseters. Overexpression of Mst4 can induce apoptosis in mammalian cells, and the protein is also a caspase-3 substrate (10). Egln3 (SM-20) is a prolyl hydroxylase with fivefold higher expression in masseters. In response to anoxia, Egln3 modifies hypoxia-inducible factor alpha (HIF-α), to target this transcription factor subunit for proteasomal degradation, thus inhibiting the ability of HIF to transactivate gene targets (17). Egln3 has also been shown to mediate cytochrome c release and caspase-dependent cell death in a neuronal cell line (42). At this point, it is not clear which of these multiple pathways contribute to the heightened apoptotic index in masseter muscles.

If myonuclear apoptosis were not counterbalanced by satellite cell proliferation and fusion to the fibers, muscle size would dwindle to nothing. Because this is not the case for murine masseters, it suggests that satellite cells are actively replacing these nuclei. Alternatively, it may be the satellite cell pool that is dividing and undergoing apoptosis without disturbing the nuclei within the fibers. The TUNEL-positive nuclei in the masseters appeared to be within the fibers, indicated by their location within the dystrophin border (Fig. 4B), and there was no evidence of apoptotic satellite cells between the laminin and dystrophin staining. The remaining TUNEL-positive nuclei appear to be interstitial, but their identity was not determined. Thus, it appears that the apoptotic myonuclei are being replaced by activated satellite cells, but this has not been confirmed. A similar phenomenon has been observed in extraocular muscles, where continued remodeling and increased apoptosis exist simultaneously (26). The lack of central nuclei in the masseters and in the extraocular muscles, which is a hallmark of muscle remodeling, suggests that the mechanism of satellite cell fusion and muscle turnover may be very different in the craniofacial muscles. Furthermore, the expression profiling results support divergent regulation of proliferation and differentiation in masseters and TA muscles (Fig. 1). It will require further investigation to determine whether a link exists between proliferation and apoptosis in these muscles.

Caspase-12 is highly expressed in skeletal muscle, and levels increase with age (7, 12). In the current study, there was more than threefold increase in caspase-12 expression in masseters. ER stress-mediated activation of caspase-12 by accumulation of unfolded/misfolded proteins or loss of calcium homeostasis can result in apoptosis (reviewed in Ref. 28). This

Fig. 2. Myosin heavy chain (MHC) analysis shows that both murine masseter and TA muscles are composed of fast fiber types. A: MHC isoforms from profiling results show that MHC 2x has the highest expression level in masseters. There was no apparent expression of embryonic (emb), α-cardiac (card), or 1/β-MHC in masseter or TA muscles. neo, Neonatal MHC (Myh8). B: SDS-PAGE MHC separation shows the predominant band for masseters in MHC 2a/x and for TA muscles in MHC 2b. Dia, diaphragm; Mas, masseter; Ht, heart. C: fiber typing of adjacent sections of a masseter shows that there is coexpression of MHC 2x and 2a in a subset of fibers (*) and that most fibers contain MHC 2x by the lack of positive staining by the BF-35 antibody (~2x panel). Scale bar, 50 μm.

Fig. 3. A: fibers appear smaller in the masseters compared with TA muscles, as shown by immunohistochemistry for laminin Scale bar, 100 μm. B: fiber size distribution of masseter and TA muscles. In females, masseters have significantly smaller fibers relative to TA muscles by Mann-Whitney test.
activation occurs by m-calpain, triggered by high intracellular calcium. Alternatively, mitochondrial disturbances can also lead to caspase-12 activation via caspase-7. The cleavage sites for each protease are distinct (shown in Fig. 5), but both enable the activation of caspase-12 (32). Our results demonstrate that m-calpain is the primary activator of caspase-12 in masseters, suggesting the presence of high intracellular calcium, or an inability to properly handle calcium. Genes expressing calcium-handling proteins were not differentially expressed between the two muscle groups. Indeed, because the flux of ER/sarcoplasmic reticular calcium is high during activation of any skeletal muscle, it is not clear why the TA and masseter cleavage patterns differed. A recent study clearly demonstrated that ER stress was high in limb skeletal muscle and increased with age (19), but the level of ER stress in masticatory muscles was not determined. Comparisons of ER stress or other muscle groups should be addressed in future studies.

Caspase activation is also involved in nonapoptotic processes, which are necessary for proper muscle development. Differentiation of satellite cells and myoblasts requires caspase activation to proceed. For example, primary myoblasts from caspase-3-deficient mice were defective in myotube formation (14), demonstrating that cleavage of caspase targets is necessary for muscle differentiation. More recently, ER stress was high in limb skeletal muscle and increased with age (19), but the level of ER stress in masticatory muscles was not determined. Comparisons of ER stress or other muscle groups should be addressed in future studies.

Because the focal adhesion complex is one of the primary and well-characterized load sensors in muscle, our initial impression was low talin expression in masseters indicated a deficit in load sensing via this complex. However, no differences at the protein level were detected. The complex seems intact and, in fact, had increased signaling contrary to our initial hypothesis (Fig. 6). Furthermore, genes encoding the dystrophin glycoprotein complex, which are also involved in load sensing, were present at levels similar to the TA (data not shown). Therefore, other differences in the expression profile data may indicate increased load-induced cell stress or changes in the cellular response to load, perhaps within the contractile apparatus. These may include the reduction in myomesin expression, a protein that is found at the M-line and has been proposed to be part of the intracellular load-sensing apparatus (1). Studies of extraocular muscles have shown the M-line in the outer orbital layer and inner global displays a lack of organization and diminished myomesin expression (1, 49), suggesting this may be a common feature of the craniofacial musculature. Second, deficits were also observed in Ankrd2, which is found at the I band. Ankrd2, a MARP family member, senses dynamic load and translocates to the nucleus from the I band after stretch, contractile activity, or injury, where it interacts with the tumor suppressor p53 to potentially regulate gene expression (21, 27, 45). Whether these cytoskeletal proteins contribute to integration of load sensing bears further investigation.

Comparison of the masseter profile to expression profiling of regenerating muscle shows similarities to TA and gastrocnemius at 2 wk after cardiotoxin injection (50, 53). Neonatal myosin expression remains high (5–10× increase) from 3 to 14 days of regeneration. In addition, expression of myomesins 1 and 2 is significantly reduced during this phase of muscle repair. These patterns may be indicative of muscle undergoing...
turnover. However, increased expression of the basic helix-loop-helix factors, which coordinate myogenesis and are transiently upregulated during muscle regeneration and reloading regardless of muscle fiber type (11, 50, 53), was not evident in the masseter profile. Furthermore, no changes in any of the stress-associated genes identified in the current study were found in either muscle regeneration profile. These comparisons counter a model in which masticatory muscle is continuously remodeling.

From a pathological perspective, masticatory muscles are the site for myogenic pain in the largest proportion of sufferers of temporomandibular joint disorder (TMD), for which there is no established cause (18). Previous studies have proposed that inefficient repair of masticatory muscle could lead to the prolonged pain associated with TMD (37). Our observations suggest that repair and muscle turnover may be occurring, but that nuclei also undergo cell death, thereby preventing resolution of damage. If the native state of masticatory muscle is one of heightened cell stress, this could provide a pathological environment for the onset of disease or myogenic pain.

Genetic diseases including both Duchenne muscular dystrophy and myotonic dystrophy (DM) also affect the muscles of mastication (20, 52). The genetic basis of each disease has been identified, but how the disease manifests in masticatory muscles compared with limb and trunk muscles differs in timing and severity. In DM patients, for example, the masseter and anterior temporalis are more severely affected than other masticatory muscles, including significant muscle atrophy and weakness, and fatty infiltration. In contrast, jaw muscles such as the anterior digastric, display milder pathohistological changes (2, 35).

Differences in susceptibility and symptoms could be dependent on the development of these muscle groups or their physiological state. Alternatively, masticatory muscles may simply adapt to the patterns of activity, as in other examples of muscle plasticity, resulting in a muscle with very different fiber properties than limb muscles. For instance, a correlation between fiber type and the stress gene profile may exist but has not been directly addressed in this study. Part of the adaptation may include muscle turnover or compensatory hypertrophy, which would give rise to activated satellite cells and expression of embryonic and neonatal myosin. Indeed, the association of masseter hypertrophy with TMD pathology has been proposed (41) and could be part of a spectrum of responses within the masticatory muscles.

Ultimately, the emerging story is that there may be a fundamentally different strategy for muscle design in the cranio-
Facial muscles in general compared with axial muscles. Specifically, there are small diameter fibers that have an attenuated ability to hypertrophy, but an increased propensity to undergo apoptosis. This strategy seems evident on several levels. For example, masticatory muscles have increased expression of Ddit4L, a known inhibitor of the primary cell survival and hypertrophic pathway in muscle: the Akt/mTOR pathway (5, 8). Ddit4L may limit a normal drive for growth or survival in the muscles of mastication. Second, lower expression of IL-15, an anabolic cytokine, may prevent progrowth signals from the muscles of mastication. Third, muscle fibers are undergoing continuous nuclear turnover (26), suggesting this feature is apparent not only in the masseters but also in the extraocular muscles, where small muscle fibers are undergoing continuous nuclear turnover (26), suggesting this adaptation positions masticatory muscles at a set point that is ideal for their functions, or if these strategies lead to heightened susceptibilities to specific genetic or use-dependent pathologies is unknown. Further study is needed to determine if and how these characteristics are linked to the physiology and pathology associated with this muscle group.

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REFERENCES