Effects of Denervation on Cell Cycle Control in Laryngeal Muscle

Vincent J. Caiozzo, PhD; Ya Zhen Wu, MD; Michael J. Baker, MS; Roger Crumley, MD

Denervation of skeletal muscle is thought to lead to an accelerated proliferation of myogenic stem cells known as satellite cells. The transition of these cells from a quiescent to a proliferative state is thought to require satellite cells to enter the cell cycle and replicate. Little is known about the expression of genes associated with cell cycle control, and so the objective of this study was to examine the effects of denervation and reinnervation of the posterior cricoarytenoid (PCA) muscle on key cell cycle genes. Female Sprague-Dawley rats were assigned to control, denervated, or reinnervated groups. Animals were killed at 7, 14, and 30 days after ligation of the recurrent laryngeal nerve. The PCA muscle was then analyzed for changes in the messenger RNA levels of key genes associated with cell cycle control, differentiation, and proliferation. Cyclin D1 is a key gene responsible for initiating progression of the cell cycle from G1 to S phase. Interestingly, neither denervation nor reinnervation affected the expression of this gene. In contrast, we found large increases in key cell cycle inhibitors (p21 and the growth arrest and DNA destruction 45 [GADD45] gene) in both the denervated and reinnervated groups. We interpret the increases in these cell cycle inhibitors to reflect (1) an inhibition of satellite cell proliferation and/or (2) a special form of apoptosis that results in the loss of myonuclei known to occur under atrophic conditions. To our knowledge, this is the first study to examine the effects of denervation on cell cycle regulation.

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The literature contains a plethora of information regarding the biology of skeletal muscle. As is well recognized, there is an explosion of information that spans the molecular-to-functional spectrum. Much of this information has been gathered by focusing on the limb musculature, and as a consequence, our understanding of the biology of muscles such as those associated with the larynx is relatively limited. One might argue that what is true for one muscle must also be true for all others. In many instances this is the case, but surely during the past 20 to 30 years, the scientific community has come to realize that the response of skeletal muscle to perturbed physiologic conditions can also be highly specific. Hence, there is a need to better understand the biology of more specialized muscles, particularly branchiomeric muscles such as those of the larynx.

One of the most important properties of skeletal muscle is muscle fiber cross-sectional area. The fundamental importance of this parameter is that it determines the maximal force that a muscle can produce under isometric conditions. In reality, however, it is a key property that determines the shape of the force-velocity relationship (one of the most important mechanical properties of skeletal muscle) and so determines the amount of force that can be produced across a broad spectrum of shortening and lengthening velocities.

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The cross-sectional area of a skeletal muscle fiber is regulated by a number of different pathways and/or mechanisms. Previous studies have shown a direct relationship between muscle fiber cross-sectional area and the number of myonuclei. In this context, conditions that produce muscle hypertrophy appear to be dependent on the activation of myogenic precursor cells (most of which are satellite cells). When activated, satellite cells undergo proliferation and produce daughter cells that fuse with existing muscle fibers and increase the number of myonuclei, DNA content, and potential for growth. In contrast, conditions that produce muscle fiber atrophy are associated with a loss of myonuclei. Collectively, the ability to gain or lose myonuclei has given rise to the concept of gene dosing, which stipulates that the regulation of skeletal muscle fiber size is dependent on myonuclear number.

In this issue of the ARCHIVES, our team has published a companion article that examines the effects of denervation and reinnervation on laryngeal muscle mass and myosin heavy chain (MyHC) isoform composition. As expected, in that study denervation produced a loss in the mass of the posterior cricoarytenoid (PCA) and thyroarytenoid muscles. The loss of muscle mass that accompanies denervation of skeletal muscle is associated with a loss of myonuclei. As a first approximation, it might be hypothesized that the atrophy associated with denervation also results in an inhibition of satellite cell activation. Somewhat paradoxically, it has been reported that denervation of skeletal muscle actually leads to an accelerated level of satellite cell activation, and some have hypothesized that long-term denervation actually leads to a “burn out” of satellite cell population and subsequent death of the muscle fibers owing to an inability to maintain an appropriate number of myonuclei. If such a phenomenon occurs in laryngeal muscle, then denervation of laryngeal muscle must be reversed as soon as possible to maintain the health of the muscle and the potential for recovery.

The mitotic activity of satellite cells is dependent on the regulation of a complex set of events involving so-called cell cycle genes (Fig 1). In this context, some genes play intimate roles in promoting the progression of the cell cycle. For instance, the up-regulation of cyclin D and its binding to cyclin-dependent kinase (CDK) 4 is one of the initial events responsible for promoting the cell cycle (Fig 1). There are also a number of genes that inhibit or arrest the cell cycle. Some of these include genes such as growth arrest and DNA destruction 45 (GADD45), p21, p27, retinoblastoma, and p53.

With this in mind, we examined changes in the messenger RNA (mRNA) levels of key cell cycle genes (cyclin D, p53, p21, p27, GADD45, GADD153) following the denervation and reinnervation of the PCA muscle. We hypothesized that if denervation accelerates the activation of satellite cells, then it should produce a significant elevation in cyclin D1 mRNA levels and a corresponding decrease in the mRNA levels of cell cycle inhibitors. Alternatively, we hypothesized that if satellite cell activation is inhibited during denervation and there is a corresponding loss of myonuclei, then there should be a significant increase in the mRNA levels of cell cycle inhibitors and little change in cyclin D1 mRNA levels.

The findings of the present study demonstrate that denervation produces a significant increase in the mRNA levels of cell cycle inhibitors that is partially relieved by reinnervation. Interestingly, we observed little change in cyclin D1 levels, but there were significant elevations in markers of differentiation (MyoD) and proliferation (myogenin).

**METHODS**

**ANIMAL MODEL AND CARE**

Given the findings in our team’s companion article, we were interested in examining possible mechanisms that might mediate the effects of denervation and reinnervation on PCA muscle mass and MyHC isoform composition. Specifically, we were interested in characterizing the response of the PCA muscle to denervation and reinnervation at the early time points of 7, 14, and 30 days. Supplemental cell cycle analyses were also performed at 90 and 180 days on muscles that demonstrated significant changes at the early time points.

The animal model and care used in the present study were similar to those described in the companion article. Approval was obtained from our institutional review board prior to conducting this study. Briefly, female Sprague-Dawley rats (weighing approximately 250-300 g) were randomly assigned to 1 of 3 groups: control, denervated, or reinnervated. The animals were humanely killed 7, 14, 30, 90, and 180 days after denervation or reinnervation. Seven animals were assigned to each group and time point. Denervation and reinnervation of the PCA muscle were surgically produced as described in the companion article. At each of the time points, animals in the various groups were anesthetized, and the PCA muscle was extirpated. The muscle was quickly blotted dry, weighed, clamp frozen using tongs, cooled in liquid nitrogen, and stored at −80°C.

**TOTAL RNA ISOLATION**

Preweighed frozen muscle samples were placed into TRI reagent (50 mg/mL; Molecular Research Center, Cincinnati, Ohio) and homogenized. Samples were allowed to sit at room temperature for 5 minutes. 1-Bromo-3-chloropropane (0.1 mL per 1 mL of TRI reagent) was then added to the homogenate, and phase separation was performed via centrifugation (12000g; 15 minutes; 4°C). The aqueous phase was then removed, and total RNA was extracted by adding isopropanol (0.5 mL per 1 mL of TRI reagent used in the initial cocktail) and centrifuging the sample at 12000g (4°C). The supernatant was removed, and then the RNA pellet was washed 3 times using ethanol and subsequently centrifuged at 7500g for 5 minutes at 4°C. The pellet was then dried and suspended in a known volume of nuclease-free water. The RNA concentration of the sample was then determined by taking a known aliquot of the sample, placing it into TAE buffer, and then determining the absorbance at 260 and 280 nm. The concentration was then determined by taking the A260 and multiplying by 40 µg/mL.

**REVERSE TRANSCRIPTION**

Following the isolation of total RNA, reverse transcription was performed by taking 1 µg of total RNA and mixing it with a cocktail of SuperScript II reverse transcriptase (Gibco-BRL, Carlsbad, Calif), oligo dTs (100 ng/reaction), and random primers (200 ng/reaction). The total volume of the cocktail including total RNA was 20 µL, and reverse transcription was performed...
Figure 1. Schematic of satellite cell proliferation and cell cycle control in denervated laryngeal muscle. A, Based on previous studies, it is hypothesized that denervation of laryngeal muscle leads to degenerative events that activate satellite cell proliferation. This requires satellite cells to undergo cell division and replication, producing daughter cells that will fuse with the muscle fiber. B, Cell division and replication involves a complex set of genes and events. Mitogens normally cause cyclins (eg, cyclin D and cyclin E) to bind to cyclin-dependent kinases (CDKs). (CAK indicates CDK-activating kinase.) These complexes can then lead to the phosphorylation of the retinoblastoma protein (rb). Phosphorylation of rb causes it to dissociate from E2F (transcription factor), and this leads to progression of the cell cycle from the G1 to S phase. This transition from G1 to S phase can be inhibited by 2 families of proteins known as inhibitors of CDK (INKs, not shown) and CDK inhibitory proteins (CIPs). Both p21 and p27 belong to the family of CIPs. The progression of the cell cycle from the G2 phase to the M phase is regulated, in part, by the combined activity of cyclin B and CDK1. This action, however, can be inhibited by the growth arrest and DNA destruction gene (GADD) 45. The actions of p21 and GADD45 are both under the control of p53, which is regulated by various factors, some of which include DNA damage and excessive mitogenic activity. In contrast, p27 is regulated via another pathway that involves transforming growth factor-β (TGF-β). (The color coding of the arrows at the bottom of panel B is consistent with that shown for the cell cycle in panel A.)
scribed by Wright et al.22 The utility of this approach is that an
form, we used a modified version of the technique first de-
To determine the relative proportion of each MyHC mRNA iso-
step consisted of 3 minutes at 72°C. The PCR conditions for
sisting of 1 minute at 96°C; 1 minute at 55°C (this varied be-
initial denaturing step was performed at 96°C for 3 minutes.
and 0.75 U of Taq polymerase. Amplification was performed
that contained 1mM to 3mM magnesium chloride, 0.2mM
dNTP, 1µM specific primer set, 0.5µM primer-competimer mix,
verse transcriptase sample and mixing it with a PCR cocktail
Wisc). The PCR was performed by taking 1 µL of a given re-
lected using LaserGene 5 software (DNA Star Inc, Madison,
ers for determination of the various mRNA levels were se-
 sequences used for the primer sets are listed in the Table.
Abbreviations: GADD, growth arrest DNA destruction; MyHC, myosin heavy chain isoform; mRNA, messenger RNA; NA, not applicable; reg, regeneration; RT-PCR, reverse-transcriptase polymerase chain reaction.

t for 50 minutes at 42°C. Reverse transcription was terminated by
samples at a temperature of 90°C for 5 minutes. The
step were then stored at −20°C until used for polymer-
PCR FOR DETECTION OF CELL CYCLE MARKERS
The primer sets used in this study are listed in the Table. Prin-
ers for determination of the various mRNA levels were se-
Table. Prim-

tions and Antisense Sequences Used for RT-PCR Analyses

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<th>mRNA Target</th>
<th>Sense</th>
<th>Anti-Sense</th>
<th>Gene Function</th>
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Embryonic MyHC
Neonatal MyHC
Type I MyHC
Type IIa MyHC
Type IIX MyHC
Type IIB MyHC

<table>
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<th>Target</th>
<th>Sense</th>
<th>Anti-Sense</th>
<th>Gene Function</th>
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| Speci-

The muscle weights for the different groups and time points
The muscle weights for the different groups and time points

for 50 minutes at 42°C. Reverse transcription was terminated by heating the samples at a temperature of 90°C for 5 minutes. The samples were then stored at −20°C until used for polymerase chain reaction (PCR).

PCR FOR DETECTION OF CELL CYCLE MARKERS
The primer sets used in this study are listed in the Table. Primers for determination of the various mRNA levels were selected using LaserGene 5 software (DNA Star Inc, Madison, Wisc). The PCR was performed by taking 1 µL of a given reverse transcriptase sample and mixing it with a PCR cocktail that contained 1mM to 3mM magnesium chloride, 0.2mM dNTP, 1pM specific primer set, 0.5pM primer-competimer mix, and 0.75 U of Taq polymerase. Amplification was performed using a Stratagene Robocycler (Stratagene, La Jolla, Calif). The initial denaturing step was performed at 96°C for 3 minutes. This step was then followed by 25 cycles with each cycle consisting of 1 minute at 96°C; 1 minute at 55°C (this varied between 55°C and 60°C, depending on the primer set); and 1 minute at 72°C. After completion of these 25 cycles, the final step consisted of 3 minutes at 72°C. The PCR conditions for each primer set and 18s competitor were optimized such that the target mRNA and 18s products were in the linear range.

PCR ANALYSIS FOR MyHC mRNA ISOFORMS
To determine the relative proportion of each MyHC mRNA isoform, we used a modified version of the technique first described by Wright et al.22 The utility of this approach is that an internal control fragment is amplified along with each MyHC isoform primer product, which provides a method for correcting for variations in PCR efficiency. The MyHC isoform primer product is then normalized relative to the internal control fragment. This approach provides a method for determining the relative proportion of each MyHC mRNA isoform.

The 5’ oligonucleotide (5’ AGA AGG AGC AGG ACA CCA GC 3’) that was used in all PCR reactions was from a highly conserved region found in all MyHC genes. Myosin heavy chain mRNA isoform–specific antisense primers were designed from the 3’ untranslated region of the different MyHC isoform genes. The sequences used for the primer sets are listed in the Table. A correction factor was calculated for each band to normalize the intensity of the Sybr Green staining (Molecular Probes, Eugene, Ore) to account for the different sizes of the control synthetic fragment (Table).

QUANTIFICATION AND ANALYSIS OF REVERSE-TRANSKRITASE PCR PRODUCTS
Amplified products were separated on a 2% agarose gel containing Sybr Green, visualized under UV illumination and photographed using a Nikon Coolpix digital camera (Nikon, Tokyo, Japan). Digital images were scanned using a laser densitometer (Molecular Dynamics, Sunnyvale, Calif) and analyzed in ImageQuant software (Molecular Dynamics). Data for the cell cycle markers were expressed as the ratio of signal intensity of the gene of interest to the signal intensity of the corresponding 18s subunit. As noted above, the signal intensity for each MyHC mRNA isoform was normalized to the signal intensity of the coamplified internal control. This value was then normalized to the values for each of the other MyHC mRNA isoforms. All PCR samples were run in duplicate, and the mean value was used for data analysis.

STATISTICAL ANALYSIS
All data are reported as mean ± SE. The overall data for any given parameter (eg, muscle weight or p21 mRNA levels) were analyzed using a 2-way analysis of variance with treatment and time representing the grouping variables. If a significant effect was observed, supplemental analyses were performed using a Student t test. The main effects are reported in the figure legends. In all statistical analyses, statistical significance was defined as P ≤ 0.05. All statistical analyses were performed using Systat 10.2 (Systat, Richmond, Calif).

RESULTS
MUSCLE WEIGHT
The muscle weights for the different groups and time points are shown in Figure 2. Note that the muscle weights for the denervated and reinnervated groups were significantly lower than those for the control group at all time points.

Abbreviations: GADD, growth arrest DNA destruction; MyHC, myosin heavy chain isoform; mRNA, messenger RNA; NA, not applicable; reg, regeneration; RT-PCR, reverse-transcriptase polymerase chain reaction.
The mRNA levels of cell cycle inhibitors

The mRNA levels for p53 are shown in Figure 4. As illustrated, the p53 mRNA levels of the denervated and reinnervated groups were similar to those of the control group at all time points. Analyses were limited to the early time points given the lack of change observed at these initial time points.

The p21 mRNA levels for the different groups and time points are shown in Figure 4. Denervation produced a rapid and large increase in p21 mRNA levels that was evident at the 7-day time point. The p21 mRNA levels subsequently remained elevated at both 14 and 30 days, and although the p21 mRNA levels declined, they remained significantly elevated at both 90 and 180 days. The reinnervated group exhibited a similar trend in p21 mRNA levels, except that eventually these mRNA levels returned to baseline.

The p27 mRNA levels in the denervated and reinnervated groups were similar to those of the control group at all time points.

The mRNA levels of the 2 different GADD45 isoforms are shown in Figure 5. As shown, there was a large increase in the mRNA levels for GADD45α 7 days after denervation. A similar elevation was observed for the reinnervated group at this same time point. Subsequently, the GADD45α mRNA levels of the denervated and reinnervated groups declined, while those of the reinnervated group returned to baseline 30 days after denervation. Interestingly, however, the GADD45α mRNA levels of the denervated group remained elevated even at the 180-day time point. The changes in mRNA levels of GADD45β mirrored those seen for GADD45α.

CORRELATIONS BETWEEN MyoD, MYOGENIN, AND CELL CYCLE mRNA LEVELS

As noted, the patterns of change in MyoD, myogenin, GADD45α, and GADD45β appeared to be similar. Hence, we examined the robustness of these similarities by performing correlations. As shown in Figure 6, the best coefficient of determination was 0.444, which was found for the relationship between MyoD and myogenin mRNA levels. Interestingly, the slope approximated 1.0. The worst coefficient of determination was 0.260, which occurred between GADD45 and myogenin mRNA levels. Additionally, denervation produced a rapid downregulation (7-14 days after denervation) of the fast type IIX and IIB MyHC isoforms. Importantly, the expression of the embryonic MyHC isoform in adult skeletal muscle has been interpreted to reflect a degenerative or regenerative phenomenon and may indicate the activation of satellite cells.29 We did not attempt to analyze changes in the type III MyHC mRNA isoform because its expression was found to be very low at the protein level.31

As shown in Figure 7, the embryonic MyHC isoform mRNA levels on the denervated and reinnervated
groups were elevated above control values as early as the 7-day time point. A similar phenomenon was observed for the neonatal MyHC mRNA isoform. It should be noted, however, that for both developmental MyHC isoforms, the increases were relatively modest.

In our team’s companion article,\(^1\) it was observed that neither denervation nor reinnervation significantly influenced the slow type I and fast type IIA MyHC isoforms at the protein level. Consistent with these observations, there were no consistent changes in the mRNA levels for either of these MyHC isoforms.

At the protein level, the present results confirm those of earlier studies\(^8,11\) that there was a large increase in the relative proportion of the fast type IIX MyHC isoform in both the denervated and reinnervated groups. Similarly, the mRNA levels of this MyHC isoform were significantly elevated at all time points for both the denervated and reinnervated groups (Figure 7).

Finally, again confirming earlier studies,\(^6,11\) we observed that denervation and reinnervation produced large decreases in the relative proportion of the fast type IIB MyHC protein isoform. A corresponding large decrease was observed at the mRNA level for both the denervated and reinnervated groups (Figure 7). Note, however, that the mRNA levels were altered earlier than was observed at the protein level.

**COMMENT**

Very little is known about the biology of laryngeal muscle, especially as it applies to denervation. To date, a large proportion of studies related to denervation of laryngeal muscle have addressed issues specific to surgical reinnervation, electromyography, and the neuromuscular junction.\(^25-29\) Recently, however, there has been an interest in examining other properties of denervated and atrophied laryngeal muscle.\(^11,23,30-35\) In this context, we were intrigued with observations regarding the effects of denervation on satellite cell proliferation in hindlimb musculature,\(^14-17,36\) and this prompted our interest in exploring the effects of denervation on cell cycle control in laryngeal muscle.

Herein, we report a number of unique findings. First, to our knowledge, this study is the first (not only in laryngeal muscle but in any muscle) to examine the effects of denervation and reinnervation on genes associated with cell cycle control. Second, we observed that neither denervation nor reinnervation produced an increase in cyclin D1 mRNA levels. This finding refutes the
Figure 5. Growth arrest and DNA destruction gene (GADD) 45α (A) and
GADD45β (B) messenger RNA (mRNA) levels for both the denervated (DEN) and reinnervated (REIN) groups underwent rapid increases, as evidenced by the large elevations seen at the 7-day time point. Subsequently, the GADD45α and GADD45β mRNA levels of both the DEN and REIN groups declined at 14 days. However, there was a divergence: the DEN GADD45α mRNA levels remained elevated, while those of the REIN group moved toward the control (CON) value at 30 days. The inserts represent actual reverse-transcriptase polymerase chain reaction data. For GADD45α and GADD45β, main effects were found for group (P<.001), time (P<.001), and interaction (P<.002). a Indicates significant difference between the CON and DEN groups; b, significant difference between the CON and REIN groups; and c, significant difference between the DEN and REIN groups. All values are means±SEs.

Figure 4. Levels of p53, p21, and p27 messenger RNA (mRNA). A, No changes were noted for p53 mRNA levels. B, At 7 days, there was a large increase in the p21 mRNA levels for both the denervated (DEN) and reinnervated (REIN) groups. The p21 mRNA levels for the DEN group were highly elevated at each time point. In contrast, p21 mRNA levels of the REIN group began to return toward those of the control (CON) group at the 30-day time point. C, The level of p27 was unaffected in the DEN and REIN groups. The insert in panel B illustrates actual reverse-transcriptase polymerase chain reaction data. These representative samples illustrate the large initial increase in p21 mRNA levels and the eventual reduction in the REIN group. There were no main effects for p53 and p27. Main effects for p21 were group (P<.001), time (P<.001), and interaction (P<.002). a Indicates significant difference between the CON and DEN groups; b, significant difference between the CON and REIN groups; and c, significant difference between the DEN and REIN groups. All values are means±SEs.
Figure 6. Regression analyses for MyoD, myogenin, growth arrest and DNA destruction gene (GADD) 45α, and GADD45β messenger RNA (mRNA) levels. Analyses were performed among these markers because they all exhibited similar patterns of change. Y Int indicates intercept.
Relative Expression of Embryonic mRNA

(a) Embryonic Control Fragment
(b) Day 7
(c) Day 30

Relative Expression of Neonatal mRNA

(d) Neonatal Control Fragment
(e) Day 7
(f) Day 30

Relative Expression of Type IIX mRNA

(g) Type IIX Control Fragment
(h) Day 7
(i) Day 30

Relative Expression of Type IIB mRNA

(j) Type IIB Control Fragment
(k) Day 7
(l) Day 30
Figure 7. Effects of denervation and reinnervation on developmental (embryonic and neonatal) and adult (slow type I, fast type IIA, fast type IIX, and fast type IIB) myonin heavy chain (MyHC) messenger RNA (mRNA) isoforms. A, Denervation and reinnervation produced small but significant increases in the embryonic MyHC mRNA isoform. These changes reflect degenerative/regenerative events that involve satellite cell activation. B, Similarly, there were small significant elevations in the neonatal MyHC mRNA isoform levels. The relative expressions of MyHC isoforms type I (C) and type IIA (D) are also shown. As reported in the companion article, the largest changes in protein MyHC isoform expression occurred with the fast types IIB and IIX MyHC isoforms. E and F, Similarly, in the present study, we observed large corresponding changes in the mRNA levels of these isoforms that were manifested at the earliest time point. The inserts in panels A, B, E, and F show actual reverse-transcriptase polymerase chain reaction data. Note that the analysis of the expression of MyHC mRNA data for any given muscle sample used an internal control for normalization across all MyHC mRNA isoforms (see Wright et al for a detailed rationale for this approach). This is much different than the normalization scheme used for the other sets of mRNA species examined in the present study. Main effects for the embryonic MyHC isoform occurred for group (P < .001) and treatment (P < .001). The main effect observed for the neonatal MyHC isoform occurred between groups (P < .001). There were no main effects for the slow type I and fast type IIA MyHC isoforms. The fast type IIX MyHC mRNA demonstrated main effects for group only (P < .001), whereas group (P < .001), time (P < .0001), and interaction (P < .0001) main effects were observed for the fast type IIB MyHC mRNA isoform. A significant difference was observed between the control (CON) and denervated (DEN) groups; b, significant difference between the CON and reinnervated (REIN) groups; and c, significant difference between the DEN and REIN groups. All values are means ± SEs.

concept that denervation accelerates satellite cell proliferation. Third, we found that denervation produced a dramatic increase in the mRNA levels of key cell cycle inhibitors (ie, GADD45α, GADD45β, and p21). Fourth, consistent with the findings in our team’s companion study in which reinnervation of the PCA muscle became evident about 30 days after ligation and surgical repair of the recurrent laryngeal nerve, the present results show that mRNA levels of key cell cycle inhibitors in the reinnervated group had returned to baseline at the 30-day time point. Finally, we observed that denervation led to the expression of the embryonic MyHC mRNA isoform, and this finding may reflect degenerative or regenerative events involving satellite cells. The following discussion addresses each of these points and also includes comments regarding important technical limitations of this study.

**SATELLITE CELL ACTIVATION, DENERVATION, AND CYCLIN D1**

Satellite cells are myogenic precursor cells that are thought to play key roles in regulating muscle fiber size during development, repair of injury, and adaptation to altered loading states. Interestingly, denervation has also been shown to cause satellite cell proliferation. This effect of denervation on satellite cell proliferation might be in response to several phenomena. First, atrophy of skeletal muscle is associated with a loss of myonuclei, and some have suggested that cell size is dependent on myonuclei number. In this context, satellite cell activation initiated by denervation may reflect a negative feedback loop that attempts to maintain muscle size by maintaining a normal number of myonuclei via satellite cell activation. Alternatively, denervation may initiate a set of degenerative events that can only be mitigated via satellite cell activation and regeneration. Clearly, other less obvious events may also be responsible for the accelerated level of satellite cell activation that accompanies denervation. Regardless of the mechanism, it is known that prolonged denervation leads to a loss of muscle fibers (ie, death of muscle fibers), and it has been suggested that this occurs because the accelerated activation of satellite cells eventually depletes the pool of myogenic stem cells.

If such events occur in laryngeal muscle, this has major clinical implications regarding laryngeal muscles that have been denervated for prolonged periods. It is well known within the clinical laryngologic community that laryngeal muscle has a remarkable capacity for reinnervation, and this may have evolved as a consequence of the vital role that these muscles play in phonation, swallowing, and breathing. Given the hypothetical role that satellite cells might play in mitigating the effects of denervation, it should be noted that no studies to date have explored the response of satellite cells in denervated laryngeal muscle. This is an important area of research within the otolaryngology community given the importance of satellite cells in maintaining the health of skeletal muscle.

The cell cycle data of the present study are unique and provide interesting insights into the potential responses of satellite cells and myonuclei. As shown in Figure 1, cyclin D1 is thought to play a key role in initiating the cell cycle via its influence on E2F. Under nonproliferative conditions, the retinoblastoma protein is hypophosphorylated and is bound to E2F thereby inhibiting E2F. When cyclin D1 binds to cyclin-dependent kinases, this complex acts to phorphorylate retinoblastoma releasing its inhibition of E2F. This then leads to progression of the cell cycle from the G1 to the S phase and synthesis of DNA. Hence, cyclin D1 plays an initial and central role in controlling the cell cycle.

On this basis, we used cyclin D1 as a marker of cell proliferation, and it was surprising to find that denervation of the PCA muscle did not produce an increase in cyclin D1 mRNA levels. It should be noted that there are isoforms of cyclin D (D1, D2, and D3), and these are thought to be expressed in a tissue-specific manner. Some might suggest that the absence of change in cyclin D1 simply reflects a lack of its expression in skeletal muscle. However, previous investigators have shown that cyclin D1 is expressed in skeletal muscle, and its up-regulation occurs in parallel with satellite cell activation. Hence, the lack of change in cyclin D1 mRNA levels might reflect 1 of 2 possibilities. First, satellite cells may have been activated by denervation but not to a detectable level. This seems unlikely given the large increases in satellite cell activation noted in previous studies. Second, the lack of change in cyclin D1 mRNA levels may simply reflect the absence of satellite cell activation (at least within the window of our observations).

**EFFECTS OF DENERVATION ON MUSCLE REGULATORY FACTORS**

Muscle regulatory factors (MRFs) consist of MyoD, Myf5, myogenin, and MRF4, so-called basic helix-loop-helix DNA binding proteins. Under developmental conditions, it is believed that MyoD and Myf5 play key roles in regulating determination of myogenic precursor cells,
whereas myogenin and MRF4 have been implicated in controlling differentiation of myogenic precursor cells. In adult animals, the specific functions of these MRFs remain to be completely elucidated. In general, however, their expression in adult muscle has been hypothesized to be associated with the activation and proliferation of satellite cells. While it is tempting to conclude that the elevations in MyoD and myogenin levels seen in the present study reflect the activation of satellite cells, such a conclusion would be contrary to the lack of change observed in the MyoD and myogenin mRNA levels (Figure 3). These data must be interpreted cautiously, and future studies are required to localize satellite cell expression in denervated and reinnervated laryngeal muscle.

As noted, the developmental importance of the MRFs might be interpreted to suggest that their expression in adult skeletal muscle reflects the activation of satellite cell proliferation. However, Hyatt et al. recently examined the effects of 3, 14, and 28 days of denervation and spinal isolation and observed that most of the increases in MyoD and myogenin occurred in myonuclei and not satellite cells. Maier et al. isolated satellite cells from soleus and tibialis anterior muscles denervated for 2 to 28 days. These investigators observed a small increase in myogenin and no change in MyoD mRNA levels. Collectively, these data suggest that the increases observed in MyoD and myogenin occurred primarily in myonuclei and not in satellite cells.

As noted by other investigators, MyoD and myogenin also have been implicated in controlling the transcription of a number of key proteins such as the fast type IIB MyHC isoform, insulinlike growth factor 1, and myostatin. With respect to a possible relationship between the presence of MyoD and the fast type IIB MyHC isoform, it has been suggested that MyoD modulates the relative proportion of the fast type IIB MyHC isoform in a direct fashion. In other words, it has been proposed that increases in MyoD will lead to a relative increase in the fast type IIB MyHC isoform. In the present study, we observed that denervation produced a large increase in MyoD mRNA levels and a concomitant decrease in the relative proportion of the fast type IIB MyHC mRNA isoform. This finding is clearly contrary to the regulation of the fast type IIB MyHC by MyoD. Furthermore, we also observed that there was a significant decrease in MyoD mRNA levels in the reinnervated group at 30 days without any corresponding change in fast type IIB MyHC mRNA levels. Collectively, these findings strongly suggest that MyoD does not regulate the expression of the fast type IIB MyHC mRNA levels under conditions of denervation. It is possible, however, that other factors may dominate the regulation of the fast type IIB MyHC gene obscuring any potential regulatory effect of MyoD.

### DENERVATION UPREGULATES mRNA LEVELS OF KEY CELL CYCLE INHIBITORS

A schematic of the cell cycle is shown in Figure 1. Denervation has been shown to activate the proliferation of satellite cells, so one might hypothesize that this proliferation would be associated with the up-regulation of key genes associated with promoting progression of the cell cycle and not those inhibiting the cell cycle. One of the key findings to emerge from the present study is that denervation produces a significant increase in the mRNA levels of key cell cycle inhibitors. Specifically, we observed large increases in the mRNA levels of p21, GADD45α, and GADD45β. These genes are often referred to as checkpoint genes and can be upregulated by a host of factors, many of which are associated with DNA damage and repair.

With respect to denervation of laryngeal muscle, there are several possible roles that these cell cycle inhibitors might play. The first is that they may directly affect the ability of satellite cells to proliferate. In other words, they may put the “brakes” on satellite cell proliferation, and perhaps this explains the lack of change in cyclin D1 mRNA levels. As a consequence, this may impair the ability of satellite cells to prevent the degeneration of muscle fibers that occurs during denervation. Alternatively, both p21 and GADD45 may play key roles in mediating apoptotic events specific to myonuclei, producing a loss of myonuclei that is known to occur during atrophy. Importantly, the data from the denervated and reinnervated groups demonstrate that key genes associated with the cell cycle are very sensitive to innervation state.

As illustrated in Figure 1, p53 has been shown to control both p21 and GADD45. Hence, it is interesting to note that p53 mRNA levels were unaffected by innervation state. p53 plays a wide array of roles in cell cycle control and apoptosis, and in some instances the action of p53 is mediated by site-specific phosphorylation and not transcriptional control. Given that both p21 and GADD45 are regulated by p53, it seems reasonable to suggest that the phosphorylation state of p53 was altered by innervation state, and this should be a target for future research.

### DENERVATION PRODUCES RAPID CHANGES IN DEVELOPMENTAL AND ADULT MyHC mRNA ISOFORM EXPRESSION

As noted, denervation of the PCA muscle produces a small but statistically significant elevation in the mRNA levels for both the embryonic and neonatal MyHC mRNA isoforms (Figure 7). In adult skeletal muscles, elevation especially of the embryonic MyHC isoform is typically associated with degenerative or regenerative events that typically involve proliferation of satellite cells. The activation of satellite cells is believed to be associated with a recapitulation of the myogenic developmental program thereby elevating the relative proportion of the embryonic MyHC isoform.

With respect to the adult MyHC isoforms, our team’s companion study found that, at the protein level, denervation of the PCA muscle produced a significant reduction in the fast type IIB MyHC isoform. This was accompanied by a large increase in the relative proportion of the fast type IIX MyHC isoform. In the present study, we incorporated MyHC mRNA data for both of these isoforms to determine if parallel changes occurred at the mRNA level and also to identify how soon after denerv-
viation such changes were manifested. Clearly, there is a
good degree of concordance between the protein and
mRNA data. Hence, the mRNA data suggest that MyHC
isoform expression at the protein level is probably me-
diated largely by changes in transcription. With respect
to the time course of altered MyHC isoform expression,
the data shown in Figure 7 demonstrate that most of the
change occurred by 7 days after denervation. Clearly, these
data demonstrate that changes in contractile protein iso-
form expression occur very soon after denervation.

IMPORTANT TECHNICAL LIMITATIONS
OF THIS STUDY

As with most studies, there are some important limita-
tions of this study that should be addressed. First, the
analyses performed in this study were done on whole
muscle homogenates (albeit of small mass). Therefore,
while it is presumed that the changes noted occurred ex-
clusively in skeletal muscle fibers, it is entirely possible
that some of the changes in cell cycle markers occurred
in other cell types (eg, endothelial cells). In this con-
text, we have performed single-fiber analyses of cell cycle
markers in hindlimb muscles and observed muscle fiber–
specific changes in GADD45α and GADD45β mRNA lev-
els (data not shown). Hence, we have a high degree of
confidence that most, if not all, changes in the cell cycle
markers occurred in the muscle fibers of the PCA muscle.
Further work is required in this area, however, to make
more definitive comments.

A second limitation of this study is that no attempt
was made to determine if the changes in cell cycle mark-
ers were specific to satellite cells, myonuclei, or both.
Clearly, this represents an exciting area for further study.

Finally, the focus of this study was to characterize
the effects of denervation and reinnervation on the mRNA
levels of key genes associated with cell cycle, determi-
nation, and differentiation. It was not our intent to
determine if such changes were manifested at the protein
level, but we recognize the importance of such analyses.
We would argue that the present findings are meritori-
ous independent of analyses at the protein level.

SUMMARY

To our knowledge, this is the first study to examine
changes of cell cycle regulation in response to denerva-
tion. Denervation has been shown to produce an in-
crease in the mitotic activity of satellite cells. Hence,
it was surprising to find that denervation did not affect
mRNA levels for cyclin D1, a key gene responsible for
initiating progression of the cell cycle. Instead, we ob-
served large increases in cell cycle inhibitor genes that
are believed to act on at least 2 checkpoints (G_{1} → S and
G_{2} → M). From a basic science perspective, we interpret
these findings to suggest that in laryngeal muscle, (1) de-
nervation acts to inhibit satellite cell activation via cell
cycle inhibitors and/or (2) the cell cycle inhibitors may
reflect apoptotic events that lead to a loss of myonuclei
and ultimately loss of muscle fiber. From a clinical per-
spective, the findings of this study illustrate the impor-
tance of innervation in maintaining the health of laryn-
geal muscle. Future studies are needed to better
understand these paradoxical observations and their im-
portance in mediating the reinnervation and recovery of
function in laryngeal muscle.

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