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Myogenic Growth Factors Can Decrease Extraocular Muscle Force Generation: A Potential Biological Approach to the Treatment of Strabismus

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Abstract

PURPOSE—Future pharmacologic treatment of strabismus may be optimized if drugs that are less potentially toxic to patients can be developed. Prior studies have shown that direct injection of extraocular muscles (EOMs) with insulin growth factor or fibroblast growth factor results in significant increases in the generation of EOM force. The purpose of this study was to examine the morphometric and physiological effects of direct EOM injection with the growth factors BMP4, TGF β 1, Shh, and Wnt3A.

METHODS—One superior rectus muscle of normal adult rabbits was injected with BMP4, TGF β 1, Shh, or Wnt3A. The contralateral muscle was injected with an equal volume of saline to serve as a control. After 1 week, the animals were euthanatized, and both superior rectus muscles were removed and assayed physiologically. The muscles were stimulated at increasing frequencies to determine force generation. A separate group of treated and control superior rectus muscles were examined histologically for alterations in total muscle cross-sectional area and myosin heavy chain isoform (MyHC) composition.

RESULTS—One week after a single injection of BMP4, TGF β 1, Shh, or Wnt3A, all treated muscles showed significant decreases in generation of force compared with control muscles. BMP4, TGF β 1, Shh, and Wnt3A significantly decreased the mean myofiber cross-sectional area of fast MyHC-positive myofibers. BMP4 resulted in a conversion of fast-to-slow myofibers and a significant decrease in the percentage of developmental and neonatal MyHC-positive myofibers. Alterations in mean cross-sectional area and proportion of MyHCs were seen after injection with TGF β 1, Shh, and Wnt3A. TGF β 1 and BMP4 injections resulted in increased Pax7-positive satellite cells, whereas BMP4, TGF β 1, and Wnt3A resulted in a decrease in MyoD-positive satellite cells.

CONCLUSIONS—These results suggest that, rather than using toxins or immunotoxins, a more biological approach to decrease muscle strength is possible and demonstrate the potential utility of myogenic signaling factors for decreasing EOM strength. Ongoing drug-delivery studies will elucidate means of extending treatment effect to make such agents clinically useful.

Recently, there has been renewed interest in developing pharmacologic treatments for strabismus,¹⁻⁷ in part because, although traditional surgery for patients with strabismus can be quite successful, the reoperation rate is high, ranging from 25% to 69%, depending on the study.^{8,9} Several new agents are under study that have the ability to modulate extraocular muscle (EOM) force generation without incisional surgery.

For underacting EOMs, surgery has been the mainstay of treatment. However, new reports suggest that there may be pharmacologic alternatives.⁴⁻⁷ The EOMs are extremely plastic and have retained the ability to remodel continuously throughout life.¹⁰⁻¹² The use of myogenic growth factors takes advantage of this phenomenon. Injections of either insulin growth factor^{4,6,7} or fibroblast growth factor (McLoon LK, et al. *IOVS* 2006;47:ARVO E-Abstract 2930) result in significant increases in muscle force generation in treated EOMs. These factors are being studied as potential treatments for age-related cachexia^{13,14} and Duchenne muscular dystrophy.^{15,16} Collectively, these studies demonstrate that naturally occurring myogenic signaling factors can be used very effectively to modulate adult muscle size and force.

The biological agent botulinum toxin is currently the only pharmacologic approach in use clinically for treating overacting muscles. It was first put into general use in the 1980s.¹⁷ More recent animal studies have examined immunotoxin injections, which may be used to treat overacting EOMs.¹⁻³ These agents target a potent toxin specifically to mature skeletal muscle fibers by using a muscle-specific antibody and are effective in reducing muscle cross-sectional area and force with little evidence of spread outside the specific muscle injected.¹⁻³ These immunotoxins function by causing muscle necrosis, and normal regeneration results in slow recovery of normal muscle force, myofiber size, and number.

There are several concerns about the use of toxins and immunotoxins to weaken an overacting muscle that are primarily related to local and systemic toxicity. The use of naturally occurring substances that are part of normal muscle growth or repair for weakening an EOM has the theoretical advantage of potentially lower toxicity and the possibility of improved patient acceptance. Based on the existing literature that compares signaling factors controlling muscle growth and size in cranial and somitic mesodermal development, several molecules appear to be likely candidates for producing a weaker and/or smaller EOM: bone morphogenetic protein (BMP)-4, transforming growth factor (TGF)- β 1, sonic hedgehog (Shh), and Wnt3A, a member of the Wntless transcription factor family. There is strong evidence that both BMP4 and TGF β 1 can negatively regulate EOM size and force generation. Elevated levels of BMP4 have been shown to interfere with muscle development by inducing apoptosis.¹⁸ BMPs also decrease protein synthesis in muscle and have been characterized as negative regulators of muscle growth.^{19,20} TGF β 1 alters myogenesis and muscle regeneration in a variety of ways, changing rates of both proliferation and differentiation.^{21,22} Of note, TGF β 1 levels are increased in patients with muscular dystrophy, and it is thought to play a role in apoptosis and atrophy in this disease.²³ In addition, TGF β 1 exogenously applied to myoblasts in culture increases the expression of myostatin, which is a negative regulator of muscle size.²⁴ These studies suggest that direct application of either BMP4 or TGF β 1 would result in decreased EOM size, and concomitantly, decreased EOM force generation.

There is less information about the effects of Shh and Wnt3A on muscle size and growth, and the literature is somewhat contradictory. Shh, for example, has been shown to increase satellite cell proliferation and prevent differentiation,²⁵ yet other work shows that Shh promotes differentiation, even in the presence of factors known to stimulate proliferation.²⁶ The same is true of Wnt3A, which promotes myogenesis in developing muscle²⁷ but induces differentiation of reserve cells in vitro.^{28,29} The strongest support for these factors to

weaken EOM specifically, however, is that many of the signaling factors that control limb muscle growth often have significantly different effects on cranial mesodermal cells.^{19,30,31} For example, Wnt and BMP specifically and locally repress cranial muscle formation during development.¹⁹ Based on these studies, we examined several myogenic growth factors and muscle signaling molecules for their ability to alter the muscle force and size of treated EOMs. Specifically, the purpose of this study was to determine whether adult rabbit EOMs, could be effectively weakened in the short term by direct injection of BMP4, TGFβ1, Shh, or Wnt3A and whether these agents would decrease myofiber size.

MATERIALS AND METHODS

Adult New Zealand White rabbits were obtained from Bakkom Rabbitry and housed at the University of Minnesota. All procedures followed the NIH Guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Rabbits were anesthetized by an intramuscular injection of ketamine, 10 mg/kg, and xylazine, 2 mg/kg. The superior rectus muscles were exposed through a limbal peritomy. One of the muscles was randomly assigned for treatment by intramuscular injection. The needle was inserted into the exposed superior rectus with the insertional tendon slightly elevated on a muscle hook so that the most of the needle was within the muscle. During injection, the needle was slowly withdrawn so that there was maximum spread of the injection volume. In all cases, the epimysium was intact, and no leakage was seen. The needle was left in place for 30 seconds, to minimize leakage at the entry point. One of the four following drugs was used for treatment: BMP4 at a dose of 1 μg/100 μL ($n = 9$), TGFβ1 at 250 ng/100 μL ($n = 9$), Shh at 2.5 μg/100 μL ($n = 12$), or Wnt3A at 0.7 μg/100 μL ($n = 9$; all from R&D Systems, Minneapolis, MN). Injected doses were based on activity provided by the data sheets from R&D Systems. On-going studies are directed at determining dose-response curves for each of the four factors. The contralateral muscle of each rabbit received an injection of 100 μL sterile saline as a control. TGFβ1 caused some conjunctival swelling, and so tobramycin-dexamethasone ophthalmic ointment (Tobradex; Alcon, Fort Worth, TX) was placed in the conjunctival cul-de-sac immediately after each injection on both sides as a prophylactic measure. It should be noted that no change in eye position was seen in any of the treated animals; however, it is known that strabismus is very difficult to produce in an animal by manipulation of the EOMs.

One week after injection, the rabbits were anesthetized with ketamine and xylazine, and the animals were euthanatized by thoracotomy followed by exsanguination. The superior rectus muscles of both orbits were removed from the scleral insertion to the origin in the orbital apex. The muscles were placed into oxygenated Krebs-Ringers solution (118 mM NaCl, 4.8 mM KCl, 1.2 mM KPO₄, 1.2 mM MgSO₄, 11 mM dextrose, 1.5 mM CaCl, and 25 mM sodium bicarbonate [pH 7.4]) at 30°C and carefully pinned to their in situ length. Each muscle end was tied with 4-0 silk and suspended in incubation chambers, with the upper loop attached to a lever arm and force transducer. The muscles were bathed continually in oxygenated Krebs-Ringers at 30°C for the duration of the experiment. Generated force in grams was recorded using the 1205 Intact Muscle Test System and Dynamic Muscle Control software (Aurora Scientific, Aurora, Ontario, Canada). Force divided by muscle cross-sectional area was calculated to account for variations in force generation due to differences in muscle size. Muscle length and mass were obtained, and muscle cross-sectional area was determined by dividing muscle mass (grams) by the product of muscle length (centimeter) times a muscle density of 1.056 g/cm³.³² This yielded muscle force in g/cm², which was then mathematically converted to mN/cm². In addition, the mass of the treated muscles was averaged for each of the drug treatments and compared with the contralateral control

muscles. We previously have validated this method, such that in normal animals, the variance in force produced by the right and left superior rectus muscles measured in this way is less than 5%.

Both control and signaling factor-treated superior rectus muscles were tested simultaneously. Supramaximum stimulation intensity was determined by increasing voltage until maximum contraction was achieved using square-wave pulses of 0.5-ms duration (701B biphasic current stimulator; Aurora Scientific) and delivered to the muscles via flanking platinum electrodes. All tests were performed with supramaximum stimulus intensities at optimal preload, as previously described.⁷ After two stabilizing tetanic stimulations (150 Hz, 500 mA, 0.5 ms) with 2 minutes' rest between stimuli and 5 minutes' rest after two consecutive stimulations, force development was determined for single, double, and triple pulses (0.5 ms pulse duration) with 2 minutes of rest between stimuli. Muscles were then stimulated at frequencies of 10, 20, 40, 100, 150, and 200 Hz with a train duration of 500 ms and a 2-minute rest between each stimulation. After 2 minutes of rest, the muscles were subjected to a fatigue protocol as follows: A tetanic stimulus was delivered every 2 seconds, consisting of a 1-second train at 150 Hz. The muscles were stimulated for 600 seconds or until there was a 50% reduction in generated muscle force. Data from treated and control muscles were pooled at each postinjection interval and compared with the paired *t*-test. $P \leq 0.05$ was considered statistically significant.

In a second set, 16 rabbits received injections into one superior rectus muscle of BMP4, TGF- β 1, Shh, or Wnt3A in a volume of 100 μ L isotonic saline at physiologic doses, and the contralateral muscle received injections of an equal volume of saline only. One week after injection, the rabbits were euthanatized with an overdose of barbiturate anesthesia. The superior rectus muscles were removed, embedded in tragacanth gum, and frozen in methylbutane that had been chilled to a slurry on liquid nitrogen. Muscles were sectioned serially at 12 μ m and processed for immunohistochemical visualization of the fast, slow, developmental, and neonatal myosin heavy chain isoforms (MyHC; Vector Laboratories., Burlingame, CA). The sections were blocked for nonspecific binding with horse serum and avidin-biotin blocking reagents (Vector Laboratories) and incubated for 1 hour with the primary antibody. For pan-fast and -slow MyHC, sections were incubated with antibody at a 1:40 dilution. For developmental and neonatal MyHC, the antibody was used at a 1:20 dilution. The sections were rinsed and incubated with ABC reagents (Vectastain Elite; Vector Laboratories) and processed using a heavy metal intensified diaminobenzidine procedure. Additional sections were processed for expression of MyoD (1:100, Abcam, Cambridge, MA) and Pax7 (1:20, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) using similar protocols.

At least four cross sections each from both the mid belly and tendon end of each muscle were analyzed for individual myofiber cross-sectional area by manual tracing under bright-field microscopy. Between 200 and 400 myofibers from a minimum of four fields were analyzed. Mean cross-sectional areas for the myofibers were determined using an image-analysis system (Nova Prime; Bioquant Inc., Nashville, TN). The percentage of myofibers positive for the neonatal and developmental MyHC was determined in muscles treated with Shh, TGF β 1, BMP4, and Wnt3A. Care was taken to analyze sections from the mid-belly region and at the tendon ends, since the percentage of myofibers positive for neonatal and developmental MyHC changes significantly along the tendon-to-tendon muscle length.³³ In addition, the number of MyoD-positive cells, which identifies myogenic precursor cells that are activated to divide, and Pax7-positive cells, which identifies all satellite cells, was determined based on total number of myofibers in each field counted.¹¹ All data were analyzed for statistical significance using either Students' paired *t*-test or analysis of variance (ANOVA) and the Dunn multiple comparison test (Prism and Statmate software;

GraphPad, San Diego, CA; and SigmaStat ver. 2.03; SPSS Science, Chicago, IL). An *F*-test was used to verify that the variances were not significantly different. Data were considered significantly different at $P \leq 0.05$.

RESULTS

Single injections of any of the four factors, BMP4, TGF β 1, Shh, and Wnt3A, resulted in significant decreases in force generation at all stimulation frequencies examined after 1 week (Figs. 1–4, respectively). The average decrease in force as measured in grams was 25% for BMP (range, 20%–30%), 18% for Shh (range, 13%–24%), 28% for TGF β 1 (range, 24%–33%), and 29% for Wnt3A (range, 14%–29%). When force was calculated as millinewtons per square centimeter (mN/cm²), the average decrease in force was 47% for BMP4 (range, 43%–50%), 27% for Shh (range, 24%–33%), 29% for TGF β 1 (range, 24%–30%), and 32% for Wnt3A (range, 28%–34%). Time to fatigue was determined for control superior rectus muscles and muscles treated with BMP4, TGF β 1, Shh, or Wnt3A. Only the BMP4-treated muscles showed a significant difference in fatigability compared with the control muscles, with a twofold increase in their time to fatigue (Fig. 5). The mass of the BMP4, Shh, and Wnt3A-treated muscles was significantly reduced (35%, 20%, and 20%, respectively) compared with the contralateral control (Fig. 6). There was no significant difference between the mass of the TGF β 1-treated muscles and the contralateral control; however, some evidence of edema was seen in the epimysium of some of the muscles treated with TGF β 1 before our use of tobramycin-dexamethasone (Tobradex; Alcon). The edema would have affected the measurements of total muscle mass, but would have no effect on measurements of individual myofiber cross-sectional areas.

Mean myofiber cross-sectional areas for fast MyHC-positive myofibers in the orbital and global layers in the middle of the muscle belly (Figs. 7, 8A) and at the tendon end (Figs. 7, 8B) 1 week after a single injection of either Shh, TGF β 1, BMP4, or Wnt3A were all significantly reduced compared with the contralateral control muscles (Figs. 7, 8). The reduction in mean cross-sectional area ranged from 32% to 44% in Shh-treated muscles, 24% to 31% in TGF β 1-treated muscles, 26% to 52% in BMP treated muscles, and 25% to 50% in Wnt3A-treated muscles compared with control muscles. A single injection of BMP4 resulted in a decrease in percentage of fast MyHC-positive myofibers, decreasing 25% to 30% in the orbital layer and approximately 10% in the global layer (Figs. 8C, 8D). A concomitant increase in slow MyHC-positive myofibers (not shown) was seen in these muscles. Wnt3A decreased the percentage of fast MyHC-positive fibers in the orbital layer, with a reduction of 22% (Fig. 8). After TGF β 1 treatment, there was a 25% decrease in the percentage of fast MyHC-positive myofibers in the orbital tendon layer; the other regions showed no change from control. No significant alteration in overall percentages of fast MyHC-positive fibers was seen after treatment with Shh.

The effect of Shh, TGF β 1, BMP4, and Wnt3A on expression of neonatal and developmental MyHC-positive myofibers was more complex; different effects of the signaling factors were seen in the orbital and global myofibers in the mid belly and tendon regions of the treated muscles compared with the controls. There were significant reductions in the mean myofiber cross-sectional areas of neonatal MyHC-positive fibers in all regions and layers after each treatment except in the orbital layer in the mid region of the Shh-treated muscle (Fig. 9A). BMP4 and Wnt3A significantly reduced the overall percentage of myofibers expressing neonatal MyHC in all regions compared with control (range, 84%–96% for BMP4; 12%–92% for Wnt3A; Fig. 9B). There were significant reductions in the percentage of neonatal MyHC-positive myofibers in both the orbital and global layers in the mid region after Shh or TGF β 1 treatment (decrease of 58% and 27%, respectively, after Shh injection and of 66%

and 46% after TGF β 1), but no change in the percentage of this isoform was seen in the tendon end for either of these two factors (Fig. 9).

Changes in mean cross-sectional areas of developmental MyHC-positive fibers were more complex. Treatment with Shh resulted in a reduction in the mean cross-sectional area of developmental MyHC-positive myofibers in all layers and regions of the muscle with decreases from 10% to 48% except in the global layer in the tendon region where no change was seen. Treatment with TGF β 1 resulted in significant reductions in mean cross-sectional area only in fibers within the middle of the muscle belly, with a reduction of 19% in the orbital layer and 40% in the global layer. Treatment with BMP4 resulted in a 19% decrease in mean cross-sectional area in the orbital tendon region, 32% in the global layer of the tendon region, and 53% decreases in the global layer of the mid region of the treated muscles (Fig. 10A). Wnt3A resulted in significantly decreased mean cross-sectional area in the orbital layer of the mid region of the muscle of 12%, and decreases of 26% and 36% in the global layers of the tendon and mid belly region of the treated muscles (Fig. 10A). When the percentage of developmental MyHC-positive myofibers was determined, there were decreased numbers of developmental positive myofibers only in the global layer, with decreases of 17% in the tendon end and 47% in the mid region of the muscle after Shh treatment, a decrease of 51% in the global layer in the mid-belly region after TGF β 1 treatment, reduction of 90% and 96% after BMP4 treatment, and reductions of 38% and 74% after Wnt3A treatment. In general, changes in the neonatal MyHC isoform expression pattern were fairly widespread in the treated muscles, whereas only the global layer appeared to be sensitive to alteration of the developmental MyHC isoform expression pattern after intramuscular injection of these signaling factors.

The percentage of Pax7 satellite cells per myofiber cross section was elevated above the control level in the TGF β 1- and BMP4-treated muscles, with increases of 1.5- and 2-fold respectively (Fig. 11A). The total number was similar to control levels after Shh treatment or Wnt3A treatment. A single injection of TGF β 1, BMP4, or Wnt3A resulted in a significant 70% to 73% decrease in the percentage of MyoD-positive satellite cells per myofiber cross section (Fig. 11B). No change in percentage of MyoD-positive satellite cells was seen after Shh treatment.

DISCUSSION

This study demonstrates that treatment of adult EOM with Shh, TGF β 1, BMP4, or Wnt-3A results in significantly decreased force generation and decreased mean myofiber cross-sectional areas 1 week after a single injection. This supports the general concept that the signaling factors that are involved in muscle development and regeneration can modulate force and myofiber cross-sectional areas of adult EOM.

There are many studies examining the use of myogenic growth factors and signaling factors to increase myofiber size and force generation in animal models of muscular dystrophy or aging.^{13–16} Myogenic growth factors, either by single injection or sustained release, have demonstrated efficacy in increasing EOM mass and force generation.^{4–7} This study, however, is the first demonstration that signaling factors that are a normal part of muscle development may be useful in decreasing myofiber cross-sectional area and muscle force generation in EOM.

The control of muscle cell proliferation, differentiation, and growth is complex. The roles played by various signaling molecules during development are different when they act alone or in concert with other signaling molecules, and they have different effects on somitic muscles compared with paraxial mesoderm during cranial development.^{34–37} Many of the

signaling molecules that increase myogenesis and differentiation in somitic muscle actually inhibit myogenesis and differentiation in cranial mesoderm.¹⁹ It is known that the EOMs undergo continuous myofiber remodeling throughout life,^{10,12} and even aged human EOM contains activated satellite cells.¹¹ The results of these studies suggest that the EOM has the capacity to respond to injected signaling factors in adult animals by up- or downregulating satellite cell activation and myofiber remodeling. In addition, many myogenic signaling factors can alter myosin heavy chain expression and other characteristics of mature muscle,^{26,38} which in turn would alter force generation.

BMP4 proved to be a very effective signaling factor, significantly decreasing EOM myofiber cross-sectional area and force generation. BMP4 has complex effects on populations of proliferating cells. At low concentrations, it stimulates myogenic precursor cell proliferation^{17,39,40} yet at high concentrations, it upregulates apoptotic pathways.¹⁸ In cultures derived from limb muscle taken from patients with Duchenne muscular dystrophy, myoblast differentiation was impaired, and this correlated with significant upregulation of BMP4 in these cultures compared with normal controls.⁴¹ Increased levels of BMPs correlate with decreased protein synthesis and are considered negative regulators of muscle growth.²⁰ When cranial mesoderm was specifically examined, BMP4 resulted in the downregulation of myogenic pathways, in direct contrast to its role in the upregulation of myogenesis and differentiation in somitic muscle precursor cells.¹⁹ In light of this study, it was not surprising to see that BMP4, when added exogenously to a superior rectus muscle undergoing continuous myofiber remodeling, significantly decreased satellite cell activation, as evidenced by the decrease in MyoD-positive cells and myofiber size. BMP4 has been shown to result in apoptosis of myogenic precursor cells.¹⁸ The increase in Pax7 satellite cells is interesting in light of potential apoptotic effects; if myogenic precursor cell apoptosis occurs, it is unlikely to be in the Pax7 satellite cell pool. Future studies will determine whether apoptosis occurs after BMP4 injection in the EOM. In addition, BMP4 treatment resulted in a decreased percentage of myofibers expressing fast and immature MyHC isoforms, compared with the control EOM, and changes in MyHC isoform composition play a significant role in determination of muscle force.⁴² Clearly, the decrease in force generation is due to a combination of complex changes that occur in these muscles.

TGF β 1 also was very effective in reducing EOM mass and force generation. Studies have demonstrated that both TGF β 1 and BMP reduce myotube formation, apparently by reducing the competency of myoblast fusion.⁴³ In addition, TGF β 1 has been shown to decrease proliferation in embryonic maxillary mesenchymal cells, both alone and in combination with Wnt.⁴⁴ In patients with Duchenne muscular dystrophy, TGF β is low in infant muscle, but increases when muscle wasting occurs in these patients.²³ This observation is particularly interesting, relative to the function of TGF β , because other muscle wasting pathways such as myostatin and atrogin-1 are not turned on in these muscles. In the present study, TGF β 1 resulted in a decreased number of activated satellite cells in the treated muscles. This would result in less myonuclear addition, presumably playing a role in the decreased myofiber size in the TGF β 1-treated SR muscles. This factor also significantly altered neonatal MyHC isoform composition of the treated muscles, which would also play a role in determining muscle force generation. No change in fast MyHC isoform expression was seen in the treated SR muscles, which agrees with a previous study in soleus muscle where TGF β 1 drove fast fiber type expression in regeneration.⁴⁵

A single injection of Shh was effective in decreasing muscle mass and force generation. The role of Shh during somitic muscle development is not well characterized; however, studies show that elevated levels result in myogenic induction in somites,⁴⁶ and in vitro it stimulates proliferation and myotube formation.^{26,47} Of interest, Shh, alone or together with IGF1, has been used in limb skeletal muscle to reduce muscle atrophy in several models of disuse

atrophy.⁴⁸ However, in contrast to its myogenic induction in somitic muscle derivatives, it acts to suppress myogenic pathways in cranial mesoderm.¹⁹ Based on our results on adult SR muscle, Shh did not alter the number of activated satellite cells in the treated SR muscles, nor did it alter the overall number of satellite cells in the muscles. Nevertheless, a single injection of Shh resulted in significant decreases in overall muscle cross-sectional area and force generation. In developing limb muscle, Shh increases slow MyHC isoform expression.^{26,38} In the present study, Shh injection significantly decreased slow and neonatal MyHC isoform expression; these changes would have significant effects on muscle force generation.⁴⁹ The specific mechanism responsible for the reduction in muscle force and mass after Shh injection is unclear, and further studies are needed to determine the basis for its effect in the present study. It is most likely multifactorial and could be due to isoform switching, alterations in overall rates of protein synthesis, and/or decreases in the efficacy of the neuromuscular junctions.

In normal somitic development and in studies of mesenchymal stem cells, Wnt3A plays a role in stimulating cell proliferation^{50,51}; however, in maxillary mesenchymal cells it has an inhibitory effect.⁴⁴ Ectopic Wnt3A specifically blocks myogenesis in cranial paraxial mesoderm.¹⁹ Although its effect on mature myogenic precursor cells in EOM has not been directly established, the findings in the present study suggest that, as it reduces the number of activated satellite cells present in the treated muscles, it inhibits this process in adult EOM satellite cells as well. In addition, Wnt3A treatment altered myosin isoform expression patterns, which also would play a role in decreasing myofiber cross-sectional area and muscle force characteristics.

The control of myogenesis and differentiation in craniofacial musculature is not well understood. However, several things are clear from the literature. First, the craniofacial musculature, particularly the EOM, is extremely heterogeneous in the embryo and in adults. The EOMs develop from unsegmented prechordal and paraxial mesoderm. Several studies have shown that distinct regulatory sequences control myogenesis in the head compared with limb skeletal muscle, and mice lacking both *myf-5* and *pax3* are devoid of body and limb skeletal muscle, yet they develop normal head musculature.³⁴ Other signaling factors known to upregulate myogenic pathways in limb skeletal muscle have an inhibitory effect on vertebrate head muscle, including Wnt, BMP, Shh, and β -catenin.¹⁹ Myogenesis is promoted in head mesoderm by antagonists to these normally myogenic signaling factors. In this study of adult EOM, a single injection of TGF β 1, BMP4, or Wnt3A resulted in fewer activated satellite cells, as identified by immunostaining for MyoD. Thus, mature EOM retains sensitivity to these antagonists to myogenesis. Although many different processes are involved, it appears that for at least three of these factors, reduction in activated satellite cells could be causative of the decrease in myofiber mass and force, due to interference in the normal process of myofiber remodeling. The use of biologically active reagents to work on processes that are a normal part of cell operation is appealing, in that it results in decreased muscle mass and strength without using biological toxins¹⁷ or other agents that kill or cause inflammatory reactions locally within muscle tissue.

It appears that using myogenic signaling factors to reduce muscle size and force generation is a viable approach to weakening an overacting EOM. Future studies will be directed at administering these factors in sustained-release form, to ensure long-term safety and efficacy. Titratable, naturally occurring myogenic signaling factors used to control the antagonist–agonist pairs of EOMs would be important additions to the treatment options available for patients with strabismus.

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A **B****FIGURE 1.**

Decreased force generation in grams or as force/cross-sectional area in millinewtons per square centimeter of superior rectus muscles 1 week after a single injection of 1 μ g BMP4 at twitch and 10-, 20-, 40-, 100-, 150-, and 200-Hz stimulation frequencies. Control muscles were injected with saline only. *Significant difference from the control.

FIGURE 2.

Decreased force generation in grams or as force/cross-sectional area in millinewtons per square centimeter of superior rectus muscles 1 week after a single injection of 250 ng TGF β 1 at twitch and 10-, 20-, 40-, 100-, 150-, and 200-Hz stimulation frequencies. Control muscles were injected with saline only. *Significant difference from the control.

FIGURE 3.

Decreased force generation in grams or as force/cross-sectional area in millinewtons per square centimeter of superior rectus muscles 1 week after a single injection of 2.5 µg Shh at twitch and 10-, 20-, 40-, 100-, 150-, and 200-Hz stimulation frequencies. Control muscles were injected with saline only. *Significant difference from the control.

FIGURE 4.

Decreased force generation in grams or as force/cross-sectional area in millinewtons per square centimeter of superior rectus muscles 1 week after a single injection of 2 μ g Wnt-3A at twitch and millinewtons per square centimeters stimulation frequencies. Control muscles were injected with saline only. *Significant difference from the control.

FIGURE 5.

Fatigability was determined in superior rectus muscles after single injections of BMP4, TGF β 1, Shh, or Wnt3A and in control muscles by stimulation at 150 Hz every 2 seconds for 600 seconds or until there was a 50% reduction in generated muscle force. *Significant increase in fatigability in the BMP4-treated muscles compared with control muscles.

FIGURE 6.

The average muscle mass 1 week after a single injection of BMP4, TGF β 1, Shh, or Wnt3A and in control muscles was determined immediately after the muscles were removed. Shh, BMP4, and Wnt3A all significantly reduce muscle mass 1 week after a single injection.

*Significant difference from the control.

FIGURE 7.

Cross-sections of myofibers immunostained for fast MyHC isoform in the global region of the mid region of superior rectus muscles 1 week after a single injection with (A) Shh, (B) TGFβ1, (C) BMP4, or (D) Wnt3A compared to (E) control muscle. Muscles fiber heterogeneity is increased, and increased numbers of fibers of smaller cross-sectional area are apparent. Bar, 50 μm.

FIGURE 8.

Morphometric analysis of expression patterns of fast MyHC in the orbital and global layers of SR muscles 1 week after Shh, BMP4, TGF β 1, or Wnt3A treatment showing decreased myofiber cross-sectional areas and decreased overall percentage of fast-positive myofibers. (A, B) Mean cross-sectional area 1 week after treatment examined in the middle of the muscle (A) or at the tendon end (B). (C, D) The percentage of fast MyHC myofibers per total number of myofibers in the middle of the muscle (C) or at the tendon end (D).

*Significant difference from the control.

FIGURE 9.

Morphometric analysis of the expression pattern of the neonatal MyHC isoform in the orbital and global layers of SR in both the middle of the muscle belly and the tendon end 1 week after Shh, BMP4, TGFβ1, or Wnt3A treatment showing decreases in numbers of myofibers positive for this MyHC isoform. (A) Mean cross-sectional area of myofibers positive for neonatal MyHC expression 1 week after treatment (B) The percentage of neonatal MyHC-positive myofibers per 100 myofibers in cross-section in the orbital and global layers in the middle of the muscle and the tendon end. *Significant difference from the control.

FIGURE 10.

Morphometric analysis of the expression pattern of the developmental MyHC isoform in the orbital and global layers of SR in both the middle of the muscle belly and the tendon end 1 week after Shh, BMP4, TGFβ1, or Wnt3A treatment showing decreases in size and numbers of myofibers positive for this MyHC isoform. **(A)** Mean cross-sectional area of myofibers positive for this isoform 1 week after treatment. **(B)** The percentage of developmental MyHC myofibers per 100 myofibers in cross section in the orbital and global layers in the middle of the muscle and the tendon end. *Significant difference from the control.

FIGURE 11.

Morphometric analysis of (A) all satellite cells as identified with Pax7- and (B) MyoD-positive satellite cells 1 week after a single injection of Shh, BMP4, TGF β 1, or Wnt3A.