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FUNCTIONAL EVALUATION OF NERVE–SKELETAL MUSCLE CONSTRUCTS ENGINEERED IN VITRO

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Summary

Previously, we have engineered three-dimensional (3-D) skeletal muscle constructs that generate force and display a myosin heavy-chain (MHC) composition of fetal muscle. The purpose of this study was to evaluate the functional characteristics of 3-D skeletal muscle constructs cocultured with fetal nerve explants. We hypothesized that coculture of muscle constructs with neural cells would produce constructs with increased force and adult MHC isoforms. Following introduction of embryonic spinal cord explants to a layer of confluent muscle cells, the neural tissue integrated with the cultured muscle cells to form 3-D muscle constructs with extensions. Immunohistochemical labeling indicated that the extensions were neural tissue and that the junctions between the nerve extensions and the muscle constructs contained clusters of acetylcholine receptors. Compared to muscles cultured without nerve explants, constructs formed from nerve–muscle coculture showed spontaneous contractions with an increase in frequency and force. Upon field stimulation, both twitch (2-fold) and tetanus (1.7-fold) were greater in the nerve–muscle coculture system. Contractions could be elicited by electrically stimulating the neural extensions, although smaller forces are produced than with field stimulation. Severing the extension eliminated the response to electrical stimulation, excluding field stimulation as a contributing factor. Nerve–muscle constructs showed a tendency to have higher contents of adult and lower contents of fetal MHC isoforms, but the differences were not significant. In conclusion, we have successfully engineered a 3-D nerve–muscle construct that displays functional neuromuscular junctions and can be electrically stimulated to contract via the neural extensions projecting from the construct.

Key words: myooid; coculture; neuromuscular junction.

Introduction

The production and use of engineered muscle from myogenic cells harvested from muscle biopsies is potentially a powerful tool for the restoration of muscle function following acute injury, surgery, or disease. Musculoskeletal tissues are highly integrated elements within the context of a living system, none of which are known to achieve adult phenotype without input from other components of the living system. Skeletal muscle has three principal tissue interfaces: vascular, myotendinous, and neuromotor. The inclusion of a functional nerve–muscle interface in vitro greatly expands the potential to control the phenotype of the muscle tissue in culture and thus expands the usefulness of engineered muscle for virtually all of its possible applications. Nerve–muscle cocultured monolayers have been used previously to study nerve–muscle interaction and the formation of neuromuscular junctions (Ecob, 1983; Ecob et al., 1983; Bryers and Ecob, 1984; Ecob, 1984; Whalen et al., 1984, 1985; Dennis and Kosnik, 2000; Wagner et al., 2003). Therefore, the goal of this experiment was to combine previously described methods for constructing three-dimensional (3-D) muscle constructs (Dennis and Kosnik, 2000; Dennis et al., 2001; Kosnik et al., 2001; Baker et al., 2003) and the methods for nerve–muscle coculture to construct and evaluate the contractile and structural characteristics of 3-D skeletal muscle constructs cocultured with fetal nerve explants.

Our laboratory has developed (Dennis and Kosnik, 2000; Dennis et al., 2001; Kosnik et al., 2001) a repeatable technique for engineering scaffold-free 3-D skeletal muscle tissue for the study of the functional development of muscle that is based largely on the work of Strohman (Strohman et al., 1990). The 3-D muscle tissues produced in this manner, termed "myooids," display many important functional similarities with skeletal muscle, including positive force frequency, normal length–tension relationships, and a normal metabolic profile (Dennis and Kosnik, 2000; Dennis et al., 2001; Kosnik et al., 2001). We have expanded this muscle model and developed a new model for a 3-D nerve–muscle construct using the coculture of a confluent monolayer of myotubes and embryonic day 15 (E-15) rat spinal cord with dorsal root ganglia attached. The use of the intact embryonic spinal cord has been previously shown to maintain the cytoarchitecture of the dorsal root ganglion and promote myocyte innervation (Kobayashi et al., 1987). Using this model of nerve–muscle coculture, Wagner et al. (2003) demonstrated that mouse myotubes were innervated and that further differentiation

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from myotube to mature muscle fibers occurred. Further investigation of this nerve–muscle coculture system demonstrated sensitivity to d-tubocurarine, an adult-like metabolic enzyme profile, and organized sarcomeric structure (Dorchies et al., 2001).

In vivo, as the nervous system develops and innervation increases, fetal muscles shift toward the adult phenotype. Just as motor neurons control the expression of myosin heavy chain in adult muscle (Close, 1965, 1969), the motor neurons also exert this control during development of muscle cells (Schiaffino et al., 1986). The control over fiber type expression is by two distinct mechanisms (Schiaffino et al., 1999). The first is a local mechanism involving the release of neural regulatory factors at the level of the neuromuscular junction and a second mechanism that is mediated by the activation pattern generated by the nerve (Schiaffino et al., 1999).

Acetylcholine receptors are among the first proteins expressed during myogenesis (Burden, 1998; Sanes and Lichtman, 1999). Prior to innervation, acetylcholine receptors are randomly dispersed along the surface of the developing myoblast. During the formation of a neuromuscular junction, the chemotrophic, electrical, and mechanical signals between the nerve and muscle induces the aggregation of nicotinic acetylcholine receptors in the plasma membrane of the developing muscle fiber. The maintenance of the neuromuscular junction depends on continual cross talk between the nerve and muscle at the site of nerve–muscle contact.

This study will investigate whether the introduction of fetal neural tissue to developing engineered muscle constructs will lead to functional motor innervation of the muscle constructs and a shift toward the adult phenotype. We hypothesize that the innervation of muscle constructs will shift the phenotype of the nerve–muscle from fetal toward adult and that nerve–muscle constructs will exhibit an increase in force production and adult myosin heavy-chain expression.

Materials and Methods

Animal model and animal care. Tissue engineering studies were carried out using muscle tissue from female Fischer 344 pregnant rats obtained from the Charles River Laboratories, Inc. (Wilmington, MA). All animals were acclimated to our colony conditions (i.e., light cycle and temperature) for 1 wk before any procedure. Rats were housed in pairs in hanging plastic cages (28 × 56 cm) and kept on a 12:12 light:dark light cycle at a temperature of 20–22° C. The animals were fed Purina Rodent Chow 5001 laboratory chow and water ad libitum. At E15 gestation day, surgical procedures were performed to remove both soleus muscles and tail tendon from the pregnant mom. E15 fetuses were cesarean derived and spinal cord explants obtained. All surgical procedures were performed in an aseptic environment with animals in a deep plane of anesthesia induced by i.p. injections of sodium pentobarbital (65 mg/kg). Supplemental doses of pentobarbital were administered as required to maintain an adequate depth of anesthesia. All animal care and animal surgery were in accordance with the Guide for Care and Use of Laboratory Animals (Public Health Service, 1996, NIH Publication No. 85-23); the experimental protocol was approved by the University Committee for the Use and Care of Animals.

Preparation of solutions and media. Unless otherwise indicated, all solutions and media were prepared and stored at 4° C before isolation and culture of muscle cells and warmed to 37° C in a heated water bath immediately before use. The media, with slight modifications from Dennis and Kosnik (2000; Baker et al., 2003) were as follows: growth medium (GMA) consisted of 400 ml of HAM F-12 Nutrient Mixture (Gibco BRL Cat# 11765-054), 100 ml fetal bovine serum (Gibco BRL Cat# 10437-028), and 5 ml A9909 (Sigma A9909). Differentiation medium (DMA) consisted of 465 ml Dulbecco modified Eagle medium (DMEM; Gibco BRL Cat# 11995-065), 35 ml 100% horse serum albumin (Gibco BRL Cat# 16050-122), and 5 ml A9909. The tissue was dissociated in a dispase and collagenase solution (D&C) that was pre-

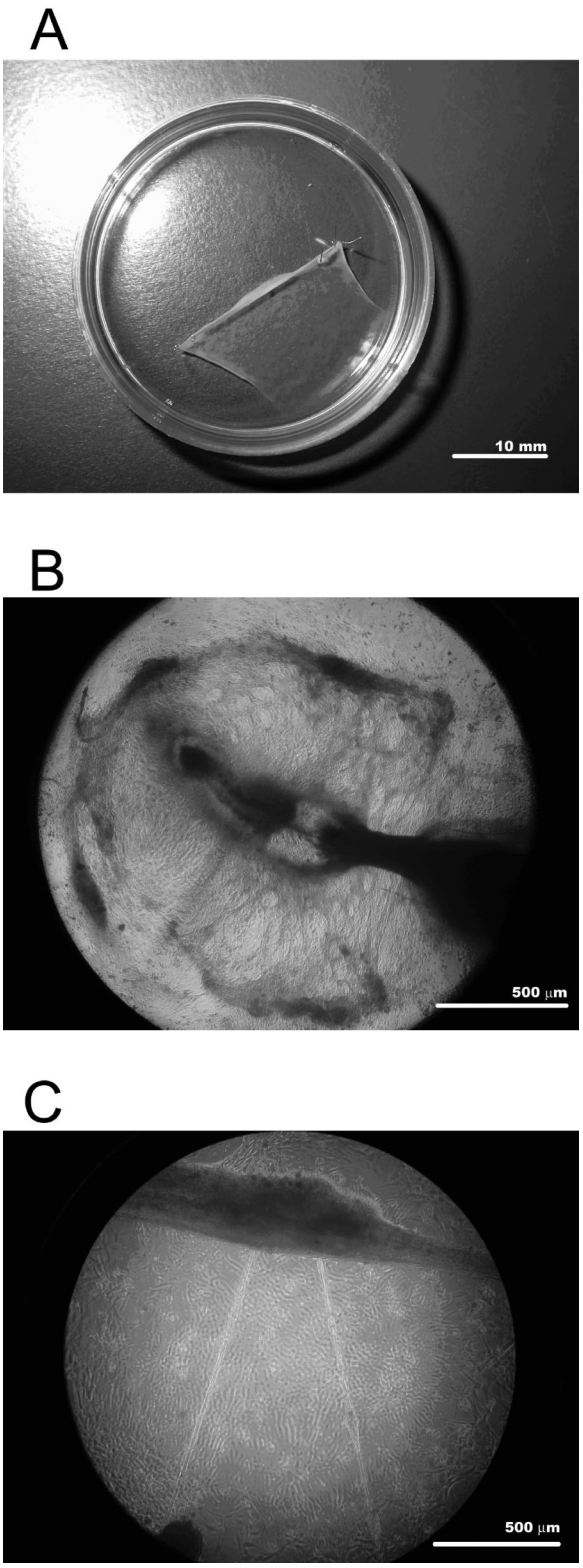


Fig. 1. Two weeks after the initial plating of the muscle stem cells, two spinal cord explants from E-15 fetal rats with tail still attached were pinned onto the muscle cell monolayer 12 mm apart with the tail portion oriented toward the edge of the dish (*A*). E-15 neural tissue integrates with muscle monolayer (*B*). Approximately 1 wk later, monolayers will roll up around the tail anchors and form a cylindrical construct. Following formation of a three-dimensional construct, some of the neural tissue forms extensions projecting from the construct (*C*).

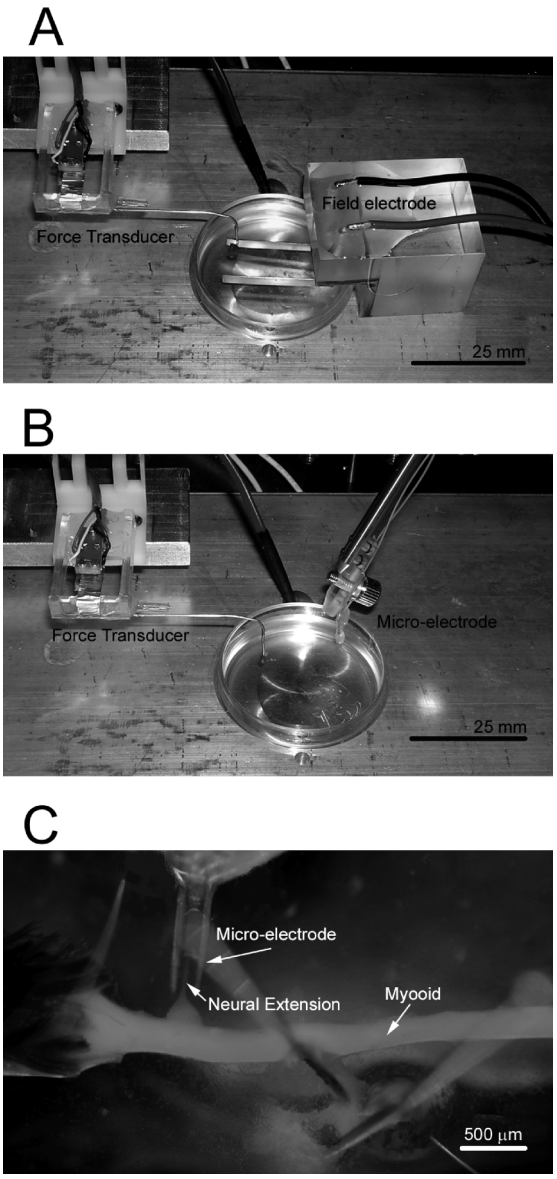


FIG. 2. Equipment setup for measuring contractile properties of a three-dimensional construct. For field stimulation of the entire construct, platinum wire electrodes were positioned on either side of the construct (*A*). Following the direct field stimulation of the entire construct, a microelectrode was used to electrically stimulate the neural extensions projecting from the construct (*B*). Nerve–myooid with neural extension (*C*).

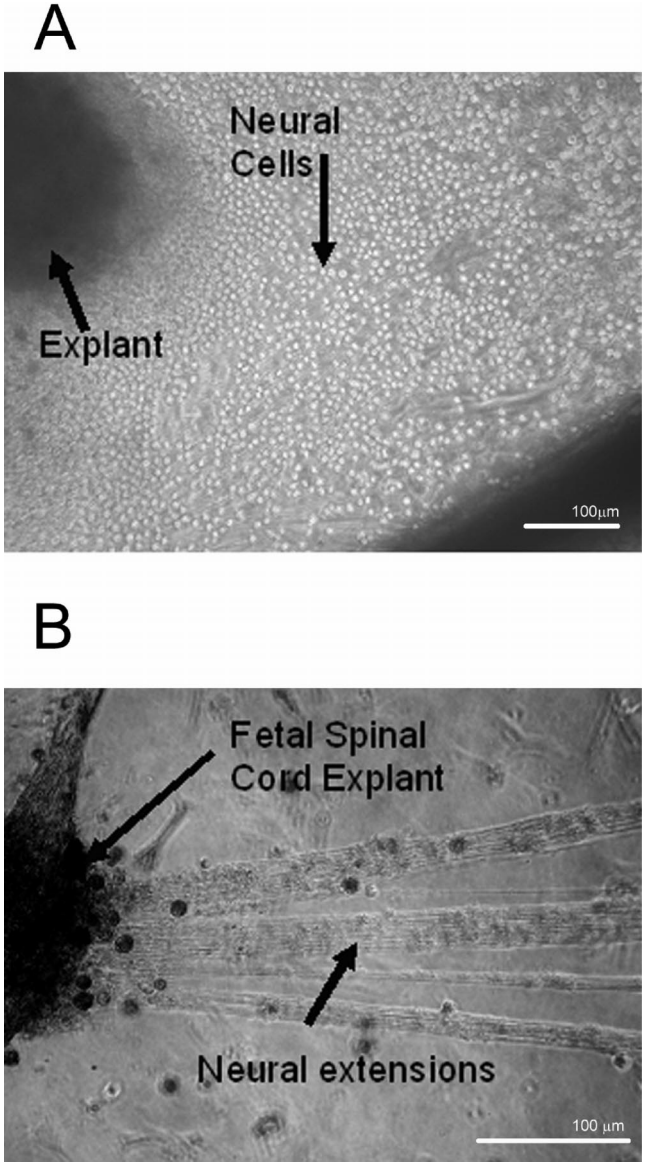


FIG. 3. Light microscopy of E-15 fetal spinal cord explants following 3 d of coculture with muscle cell monolayers. (*A*) When fetal spinal cord explants are placed in a dish with a myooid, neural cells first migrate away from the site of explant (*B*) then aggregate to form larger neural extensions.

pared in the amount of 20 ml per four soleus muscles and consisted of 8 units Dispase (Sigma Cat# P-3417; 0.4 units/mg) and 200 units of type 4 collagenase (Gibco BRL Cat# 17104-019; 239 units/mg) per ml DMEM. Transport medium (TM) was prepared in the amount of 5 ml per muscle dissected at the concentration of 2% A9909 in DPBS. Preincubation medium (PIM) was prepared in the amount of 3 ml per plate and consisted of 2.5 ml of 0.05% sodium azide (NaN_3; Sigma Cat# S-8032) in DPBS solution, 22.5 ml DMA, and 0.25 ml A9909 per muscle dissected.

Preparation of culture dishes and anchors. Myooids were engineered in individual 35-mm plates as described earlier (Dennis and Kosnik, 2000). Briefly, individual plates allowed for functional evaluation of individual myooids and facilitated removal of any contaminated plates. Each 35-mm plate was coated with 1.5 ml of Sylgard (Dow Chemical Corporation, Midland, MI; type 184 silicon elastomer) and allowed to cure for 3 wk before use. One week before use, Sylgard-coated plates were then coated with laminin at 1.0 μg/cm² per plate (10 μg of natural mouse laminin [Gibco BRL Cat# 23017-015] and 3 ml of Dulbecco phosphate-buffered saline [DPBS] pH 7.2 [Gibco BRL Cat# 14190-144] per plate) and left to dry for 48 h. Salt crystals were dissolved and removed by rinsing the plates with 3 ml DPBS. The plates were then filled with 2 ml of previously described GMA and decontaminated with UV light (wavelength 253.7 nm) for 90 min and placed in a 37° C 5% CO_2 incubator for 1 wk before plating muscle cells.

Preparation of muscle and isolation of satellite cells. Both soleus muscles were surgically removed under aseptic conditions, weighed, sterilized in 70% ETOH, and incubated for 5 min in TM. A single-edged razor blade and a pair of number 5 forceps (Fine Science Tools) were then used to slice the soleus muscle longitudinally into three strips. Next, 35-mm Sylgard-treated plates were sterilized in 70% ETOH, and muscle slices were pinned to length, two muscle strips per plate. Then 3 ml PIM were added to each plate, and the plates were UV treated for 90 min. The plates were then placed in a 37° C 5% CO_2 incubator for 50 h.

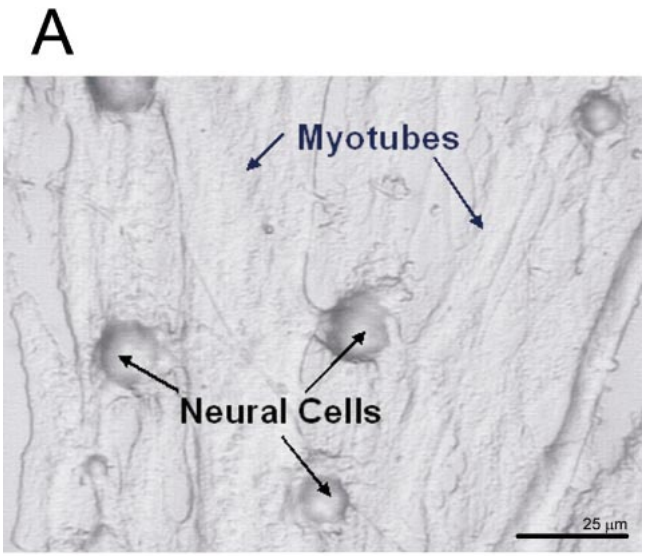


FIG. 4. Light microscopy of myotubes and neural cells following 1 wk of coculture. Images were taken at ×400. (*A*) Light microscope, (*B*) fluorescent image of neural cells stained with CY3-labeled neurofilament. When placed on a monolayer of muscle cells, neural cells migrate off the fetal spinal cord explant and fuse with the existing myotubes.

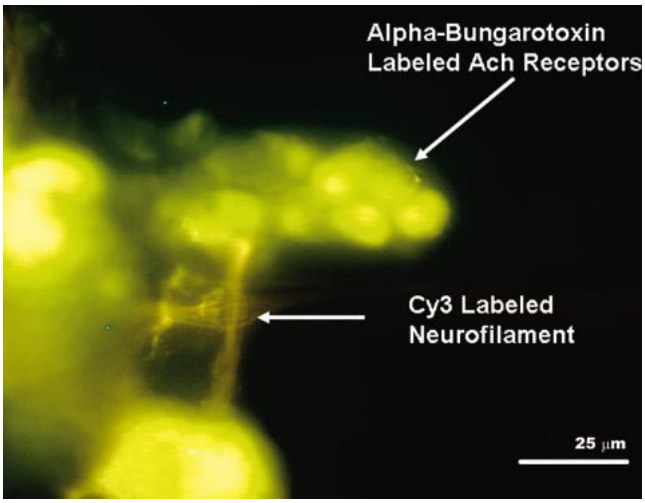


FIG. 5. Fluorescent microscopy images of neuromuscular junctions formed between rat fetal spinal cord explants and three-dimensional muscle constructs. Immunohistochemistry indicates that the muscle constructs contain the Ach receptors necessary for innervation and that these receptors are clustered in a structure resembling a neuromuscular junction.

Following the 50-h incubation period, muscle slices were inspected for contamination, and any infected plates were discarded. The remaining muscle strips were then removed from the plates and incubated in D&C (two soleus muscles per 20 ml) in a 37° C shaking water bath for 4 h. The dissociation was aided by occasionally shaking each vial slightly by hand. Once the muscle was fully digested 4 h, the dissociated cells were filtered through a 100-micron filter and centrifuged at 700 × *g* for 10 min at 25° C. Finally, the supernatant was aspirated from the vials, and the pellet was resuspended in GMA to obtain a concentration of 10 mg of dissociated muscle per 2 ml GMA.

Cell culture and myooid formation. The previously prepared laminin-coated plates were examined for contamination after storage in an incubator for 1 wk, and the GMA was aspirated from the plates. Two milliliters of the cell suspension were plated in each culture dish and placed in a 37° C 5% CO_2 incubator for 5 d. Culture plates were not disturbed for at least 72 h to allow cell adherence to the plates. After 5 d, the cells were fed with GMA every 48 h until the cells became confluent (approximately 7 d). Once the cells achieved confluence, they were fed with DMA every 48 h until the myocytes fused to form multinucleated myotubes that began to contract spontaneously. Two weeks after the initial plating of the muscle stem cells, two spinal cord explants from E-15 fetal rats with tail still attached were pinned onto the muscle cell monolayer 12 mm apart with the tail portion oriented toward the edge of the dish (Fig. 1). Approximately 1 wk later, monolayers will roll up around the tail anchors and form a cylindrical construct. Sixteen to 18 d post construct formation, the nerve–muscle constructs were tested for contractile function. At the conclusion of functional tests, each specimen was snap frozen between dry ice and stored for subsequent analysis of myosin heavy-chain profile. Muscle constructs consisting of primary muscle cells and adult rat tail tendon served as control for the nerve–muscle constructs.

Functional testing of constructs. Contractile properties were initially measured 16–18 d after the formation of a three-dimensional construct. The protocol for measuring contractility of engineered muscle constructs was adapted from Dennis and colleagues (Dennis and Kosnik, 2000; Dennis et al., 2001; Kosnik et al., 2001) and Irintchev et al., (1998). Briefly, the pin on one end of the construct was freed from the Sylgard and attached to a force transducer with canning wax. For field stimulation of the entire construct, platinum wire electrodes were positioned on either side of the construct (Fig. 2). The temperature of the construct was maintained at 37 ± 1° C using a heated aluminum platform. The diameter of the construct was determined and used to calculated cross-sectional area, assuming a circular cross section. Passive baseline force was measured as the average baseline passive force preceding the onset of stimulation. Twitches were elicited using a single 1.2-ms pulse at 2.5, 5, 10, and 20 V, whereas maximum tetanic force was determined using a 1-s train of 1.2-ms pulses at 10 V and 10, 20, 40, 60, and 80 Hz. Data files for each peak twitch force and peak tetanic force trace were recorded at 1000 samples/s and stored for subsequent analysis using LabVIEW data acquisition software. Peak tetanic force was normalized for cross-sectional area to determine maximum specific force. Following the direct field stimulation of the entire construct, a microelectrode was used to electrically stimulate the neural extensions projecting from the construct using the same stimulation parameters as described previously for the field stimulation (Fig. 2).

Myosin heavy-chain Western blotting. Western analysis of myosin heavy chains was conducted as previously described (Talmadge and Roy, 1993). Briefly, muscle-only and nerve–muscle constructs (n = 5) were homogenized in phosphate buffer in a 1:100 (wt/vol) dilution. Twenty microliters of homogenate was used to determine proteins via Bradford protein assay (Biorad, Richmond, CA). Western analysis was performed by using a vertical SDS page gel electrophoresis in the presence of sodium dodecyl sulfate and aliquots of muscle membranes (2 μg protein per lane). Several internal control

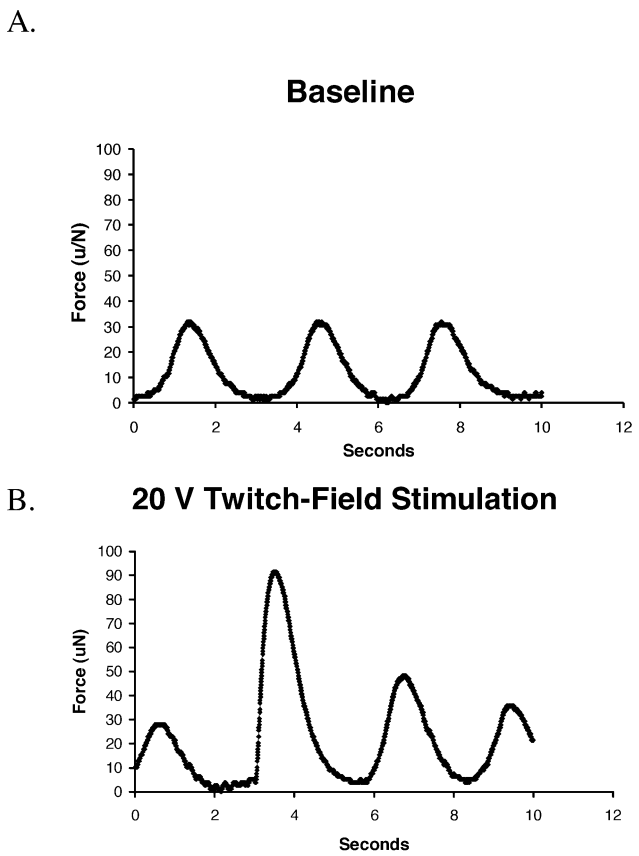


FIG. 6. Representative force traces for baseline (*A*), twitch (*B*), and tetanus (*C*) in response to field stimulation. The field stimulation was across the nerve–muscle construct and not the neural extensions.

samples of adult rat soleus, rat E-15 fetal limb buds, and rat 3-d-old neonatal limb muscle were run on all gels, and duplicate samples from each myooid were separated on an 8% polyacrylamide-glycerol resolving gel and then eletrophoretically transferred onto Immobilon polyvinyldifluoride membrane (Millipore, Milford, MA). Immunoblotting was performed by using antibodies against rat myosin heavy-chain (MHC) slow (Cat #-NCL-MHCs), MHC fast (Cat #-NCL-MHCf), MHC neonatal (Cat #-NCL-MHCn), and MHC developmental (Cat #-NCL-MHCd) obtained from (Novocastra Laboratories Ltd, Newcastle, United Kingdom), followed by chemiluminescent labeled IgG (Jackson Laboratories, Bar Harbor, ME), and the chemiluminescent signal was monitored, digitized, and analyzed using autoradiography. Myooid signals were

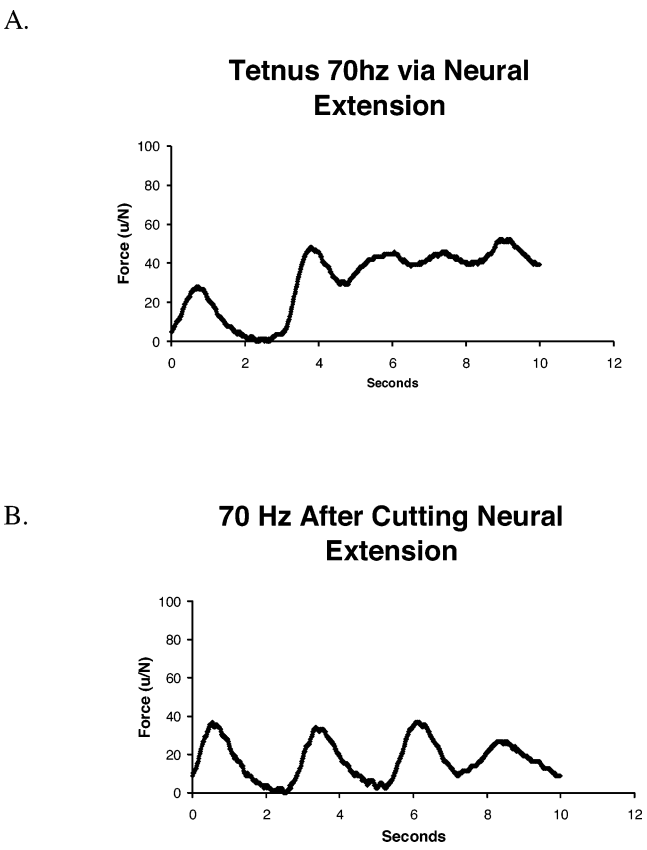


FIG. 7. Representative tetanus recordings of nerve–muscle constructs during the direct stimulation of an attached neural extension (*A*) before and (*B*) following the severing of the neural extension.

corrected for background and myosin heavy-chain signals were compared to the adult soleus, fetal, and neonatal muscle signals.

Statistics. Values are presented as means ± SE. Statistical analysis was performed by using Jump In 5.1 (SAS Institute Inc., Cary, NC). A one-way analysis of variance was conducted to compare the differences between myooids and nerve–muscle constructs. Differences were considered significant at $P < 0.05$.

RESULTS

Morphology of nerve–muscle construct. Approximately 1 wk after the introduction of the neural explant, neuronal cells from the fetal explants migrate away from the explant and move across the monolayers of myotubes (Fig. 3). Many of the neural cells fuse with the myotubes (Fig. 4), and others form neuromuscular-like junctions with clustering of acetylcholine receptors surrounded by neurofilament-stained neural extensions (Fig. 5).

Contractile properties. A representative tracing from a nerve–muscle constructs is shown in Figure 6. The nerve–muscle constructs show the same response to field stimulation as previously described for muscle-only constructs. The nerve–muscle constructs exhibit spontaneous baseline activity and can elicit both a twitch and a tetanus response to a field stimulation (Fig. 6). Following the field stimulation of the nerve–muscle construct, microelectrodes were used to electrically stimulate the neural extensions radiating from the construct. The maximum tetanus generated from the neural extension was approximately 25% of the total tension elicited with field stimulation, suggesting that the neural extension was recruiting

approximately 25% of the myotubes in the construct during the contraction (Fig. 7). Following the severing of the extension without the removal of the microelectrode, the nerve–muscle construct returned to spontaneous baseline contractile activity (Fig. 7). The construct would no longer respond to the stimulation of the microelectrode.

The average diameters of the myooid and nerve–muscle constructs were not significantly different (Fig. 8). The average maximum twitch and tetanus generated by field stimulation was significantly greater in the nerve–muscle versus myooid ($P = 0.005$ and $P = 0.005$, respectively).

Myosin heavy-chain profile. Western analysis of the myosin heavy-chain content in the nerve–muscle versus myooid constructs is shown in Figure 9. The sample from 3-d-old neonatal limb muscle showed the greatest expression of myosin per gram of protein than any other sample with a greater signal with the developmental antibody and gave a moderate signal with the neonatal antibody. The fetal E-15 limb bud sample only gave a signal with the neonatal antibody. The expression of developmental myosin heavy chains tended to be greater, while the neonatal myosin heavy chains tended to be less in the nerve–muscle versus myooid samples, suggesting that the myosin content per total gram of protein is greater in the nerve–muscle constructs and that the myosin heavy chains present are more neonatal-like than fetal.

Discussion

We have constructed three-dimensional nerve–muscle constructs from cocultures of myogenic cells from adult soleus muscle with E-15 fetal spinal cord explants of rats. We have extended our muscle-only "myooid" model to a new model with functional neural muscular junctions and neural muscular projections that respond to electrical stimulation by contraction. The introduction of the neural cells to the muscle-only construct resulted in nerve–muscle constructs with functional neuromuscular junctions that generate more force per gram of tissue than muscle-only constructs. In addition, we have altered the myosin heavy-chain profile from that of a fetal to a more neonatal phenotype as indicated by the increase in the neonatal form of myosin heavy chain compared to muscle-only constructs.

The introduction of the neural cells to a monolayer of myotubes resulted in the formation of neuromuscular-like junctions with clustering of acetylcholine receptors surrounded by neurofilament stained neural extensions. The costaining of neuronal derived tissues with neurofilament and acetylcholine receptors with alpha-bungaro toxin allowed us to identify neuromuscular junctions. During development, acetylcholine receptors are dispersed randomly along the plasma membrane of myotubes (Burden, 1998; Sanes and Lichtman, 1999). Introduction of the E-15 fetal rat spinal cord resulted in the clustering of the acetylcholine receptors. In addition, neuronal clefts and projections form around and away from the cluster of acetylcholine receptors. Morphologically, the introduction of the E-15 spinal cord resulted in what resembles architecturally a neuromuscular junction.

Western analysis of the myosin heavy-chain content in the nerve–muscle versus myooid constructs indicates that the expression of both developmental myosin heavy chains tended to be greater, while the neonatal myosin heavy chains tended to be less in the nerve–muscle versus myooid samples, and that there was a greater content

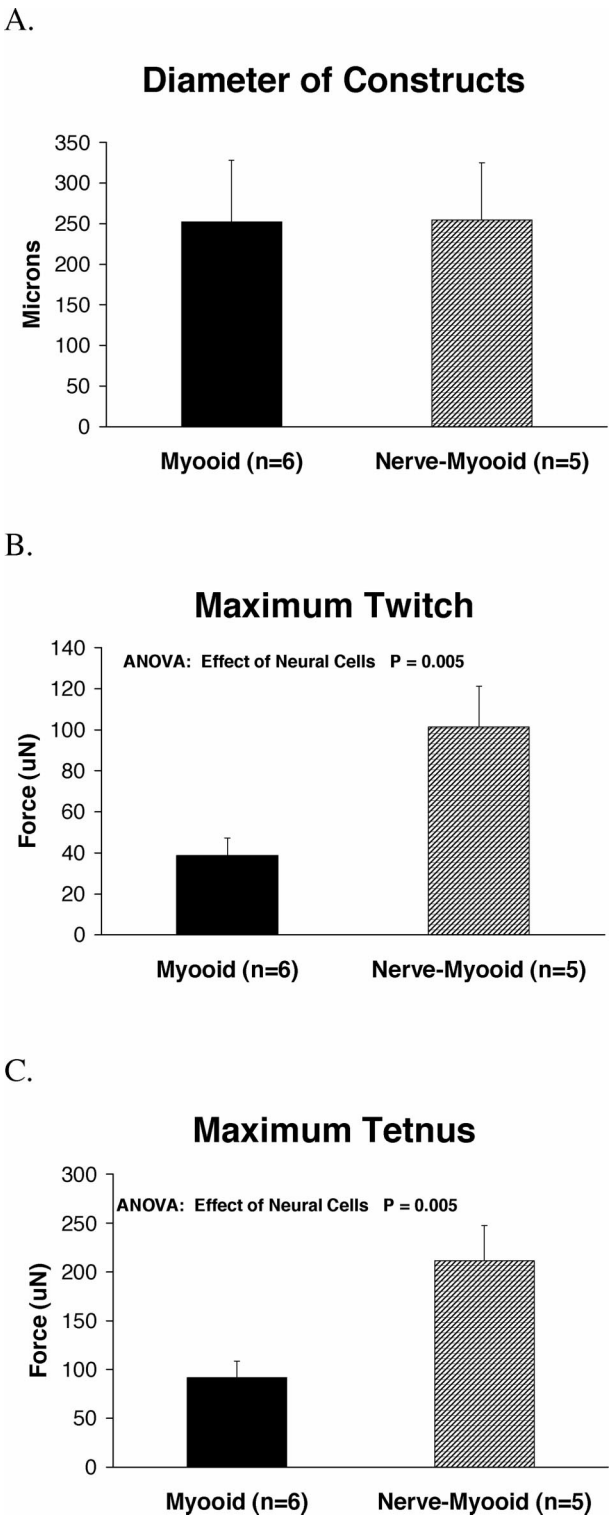
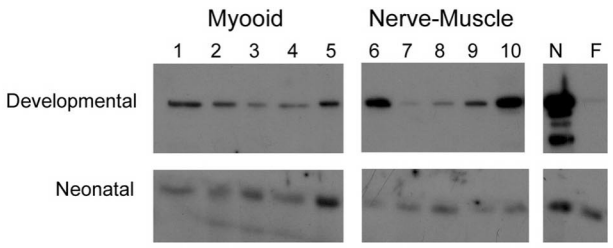
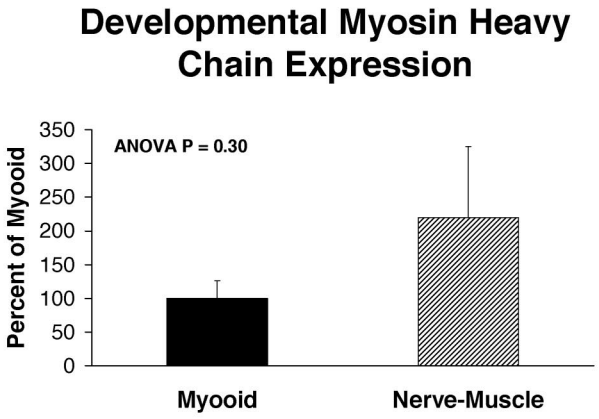


Fig. 8. (*A*) Diameter, (*B*) twitch, and (*C*) tetanus of muscle and nerve–muscle constructs. Values are means ± SE.



B.



C.

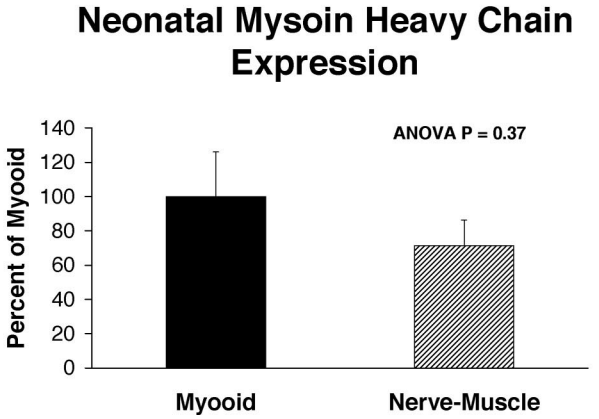


FIG. 9. (*A*) Western blot of muscle and nerve–muscle constructs probed for (*A*) neonatal and (*B*) developmental myosin heavy-chain proteins. "N" is a sample obtained from neonatal rat muscle, and "F" is a sample obtained from limb buds of E-15 fetal rats. Average expression of developmental (*B*) and neonatal (*C*) myosin heavy chain expressed as a percentage of the myooid expression. Values are means ± SE.

of neonatal versus fetal myosin heavy-chain isoform in the nerve–muscle versus the muscle-only constructs.

The functionality of the nerve–muscle constructs, that is, the ability to convey an action potential from the nerve across the junction to elicit a muscular contraction, was verified by stimulation with specially designed microelectrodes. Using field stimulation, which elicits an electrical stimulation along the entire length of the muscle construct, we observed the same mechanical response as previously described for muscle-only constructs. The nerve–muscle constructs exhibited spontaneous baseline activity and both a twitch and a tetanus response to field stimulation. The contractility of the nerve–muscle constructs was greater than that of muscle-only constructs. We can therefore conclude either that the ratio of muscle tissue to connective tissue is greater in the nerve–muscle constructs or that the contractile machinery present is capable of producing more specific force. The cumulative signal from the myosin heavy-chain Western suggests that there is more muscle tissue per gram of total tissue in the nerve–muscle constructs than the muscle-only constructs. The diameters of the nerve–muscle versus muscle-only constructs were not different, and the addition of the neural tissue to the monolayer of muscle would suggest a decrease in muscle per diameter of the construct. This would imply that the muscle present in the nerve–muscle construct has a more adult-like phenotype because it is capable of producing more force per gram of muscle tissue. These data are in agreement with a study by Wagner et al. (2003) that studied the electrical current patterns generated by acetylcholine receptors in a nerve–muscle coculture system. Following 3 wk of nerve–muscle coculture, Wagner showed a clustering of acetylcholine receptors in the plasma membranes of the myotubes and a shift to a more developed pattern of electrical currents. Using patch clamp techniques for monitoring fetal versus adult acetylcholine activated currents in noninnervated and innervated myotubes from nerve–muscle cocultures, Wagner showed a shift from a fetal current in the noninnervated myotubes to an adult current in the innervated myotubes (Wagner et al., 2003).

Following the field stimulation of the nerve–muscle construct, microelectrodes were used to electrically stimulate the neural extensions radiating from the construct. The maximum tetanus generated from the neural extension was approximately 25% of the total tension elicited with field stimulation, suggesting that the neural extension was recruiting approximately 25% of the myotubes in the construct during the contraction. We then mechanically severed the neural extension without altering the position of the microelectrode, and the nerve–muscle construct returned to spontaneous baseline contractile activity. The muscle construct no longer responded to stimulation of the nerve via the microelectrode, indicating that the stimulation via the microelectrode was not due to the proximity of the electrode to the construct but actually elicited the stimulation along the neural projection. The increase in both twitch and tetanus force elicited by field stimulation in the nerve–muscle versus the muscle-only constructs further suggests that the sarcomeres present in the nerve–muscle constructs are capable of producing more force compared to muscle-only constructs. The inclusion of a functional nerve–muscle interface in vitro greatly expands the potential to control the phenotype of the muscle tissue in culture and thus expands the usefulness of engineered muscle for virtually all its possible applications.

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