

Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation

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Pedrotty, Dawn M., Jennifer Koh, Bryce H. Davis, Doris A. Taylor, Patrick Wolf, and Laura E. Niklason. Engineering skeletal myoblasts: roles of three-dimensional culture and electrical stimulation. *Am J Physiol Heart Circ Physiol* 288: H1620–H1626, 2005. First published November 18, 2004; doi:10.1152/ajpheart.00610.2003.—Immature skeletal muscle cells, or myoblasts, have been used in cellular cardiomyoplasty in attempts to regenerate cardiac muscle tissue by injection of cells into damaged myocardium. In some studies, muscle tissue within myoblast implant sites may be morphologically similar to cardiac muscle. We hypothesized that identifiable aspects of the cardiac milieu may contribute to growth and development of implanted myoblasts in vivo. To test this hypothesis, we designed a novel in vitro system to mimic some aspects of the electrical and biochemical environment of native myocardium. This system enabled us to separate the three-dimensional (3-D) electrical and biochemical signals that may be involved in myoblast proliferation and plasticity. Myoblasts were grown on 3-D polyglycolic acid mesh scaffolds under control conditions, in the presence of cardiac-like electrical current fluxes, or in the presence of culture medium that had been conditioned by mature cardiomyocytes. Cardiac-like electrical current fluxes caused increased myoblast number in 3-D culture, as determined by DNA assay. The increase in cell number was due to increased cellular proliferation and not differences in apoptosis, as determined by proliferating cell nuclear antigen and TdT-mediated dUTP nick-end labeling. Cardiomyocyte-conditioned medium also significantly increased myoblast proliferation. Expression of transcription factors governing differentiation along skeletal or cardiac lineages was evaluated by immunoblotting. Although these assays are qualitative, no changes in differentiation state along skeletal or cardiac lineages were observed in response to electrical current fluxes. Furthermore, from these experiments, conditioned medium did not appear to alter the differentiation state of skeletal myoblasts. Hence, cardiac milieu appears to stimulate proliferation but does not affect differentiation of skeletal myoblasts.

cardiomyoplasty; myoblast; electrical current; differentiation

WHEN THE MYOCARDIUM IS INJURED after an acute myocardial infarction (heart attack), a fibrous, noncontractile scar develops, because mature myocardial tissue cannot effectively regenerate. In patients with extensive atherosclerosis and myocardial infarction, this often results in congestive heart failure, which occurs in more than 400,000 patients in the United States each year (4).

In efforts to provide a long-term augmentation for damaged myocardium, investigators have utilized mechanical and tissue-based support, including whole skeletal muscle, in a wrap

around the ventricle. The most widely studied wrap is the pedicled latissimus dorsi flap, utilized for dynamic cardiomyoplasty (21). Latissimus dorsi muscle must be subjected to a chronic, low-frequency electrical stimulation to transform its morphological, ultrastructural, physiological, and metabolic properties from those of fast-twitch skeletal muscle fibers to those of fatigue-resistant, slow-twitch type I fibers (1, 28, 29). Unfortunately, long-term cardiac support with electrically stimulated intact skeletal muscle has not proven efficacious, presumably because of skeletal muscle fatigue after it assumes the demands of the myocardium (36). Nonetheless, these studies demonstrate that the phenotype of skeletal muscle, which contains mature myocytes and immature myoblasts, can be altered by electrical stimulation (2, 21).

As an alternative to whole muscle, several investigators are utilizing isolated muscle cells for cardiac repair (8, 24, 32). Immature skeletal muscle cells, or myoblasts, have been used in cellular cardiomyoplasty, which is an approach to regenerate lost cardiac muscle tissue by injecting cells into the damaged myocardium (5, 6, 32). In contrast to mature cardiomyocytes, skeletal myoblasts are morphologically undifferentiated, can undergo mitosis, and are relatively resistant to ischemic insult (35). When injected into injured myocardium, myoblasts can engraft, survive, and potentially augment the contractile performance of the heart.

In some studies, there may be evidence that muscle tissue within myoblast implant sites is morphologically similar to cardiac muscle (8). Several investigators have reported that transplanted myoblasts become connected by intercalated disks and/or have single, centrally located nuclei (6, 32), which are characteristic of mature cardiomyocytes. This implies that the implanted myoblasts may undergo phenotypic changes in vivo, such that they begin to resemble cardiomyocytes. However, any potential triggers for myoblast differentiation into a cardiac-like phenotype, if this indeed occurs, remain unclear.

Cultured myoblasts that are transplanted into injured myocardium are exposed to multiple new environmental cues. They are transferred from a two-dimensional (2-D) culture environment to a 3-dimensional (3-D) in vivo environment. Transplanted myoblasts are also exposed to electrical, chemical, and physical contractile stimuli that are unique to the myocardium. We hypothesized that identifiable aspects of the cardiac milieu may contribute to the growth and development of implanted myoblasts in vivo. To test this hypothesis, we designed a novel in vitro system to mimic some aspects of the electrical and

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biochemical environment of native myocardium. This culture system was designed to enable separation of the 3-D, electrical and biochemical signals that may be involved in myoblast proliferation and plasticity. By growing skeletal myoblasts in this environment, we demonstrate that myocardium-like electrical activity triggers myoblast proliferation *in vitro* and that this response is mediated by L-type calcium channels, because blockade of these channels with verapamil abolishes the effect. In the future, such a culture system may prove useful for the directed differentiation of precursor cells into cardiac-like cells for cell transplantation and cardiomyoplasty. Furthermore, this system may provide an environment in which we can begin to examine the role of specific agents in myoblast proliferation and plasticity.

MATERIALS AND METHODS

Myocardial culture device. A device was fabricated to grow skeletal myoblasts in a cardiac-like milieu (Figs. 1 and 2). Chambers ($4.0 \times 1.5 \times 2.0$ cm; Nunc) were fitted with biocompatible polyglycolic acid (PGA) mesh scaffolding (Albany International, Mansfield, MA) to allow myoblast culture in 3-D (Fig. 1).

The culture chambers were designed to provide electrical current fluxes that mimic the current fluxes that are experienced by native myocardial cells. Each culture chamber was fitted with rectangular platinum electrodes at either end ($2.0 \times 1.5 \times 0.01$ cm; Alfa Aesar, Ward Hill, MA; Fig. 1). Platinum electrodes were secured to stainless steel wire with silver epoxy glue (Chomerics, Woburn, MA). Within each chamber, the 3-D PGA scaffold was suspended between the electrodes. This configuration allowed 3-D myoblast growth within a physiological and spatially uniform current flux. To provide biochemical cues that are produced by myocardial cells, culture medium that was conditioned for 24 h by mature cardiomyocytes, i.e., conditioned medium (CM), was added to culture chambers in some experiments.

Four culture chambers were attached in series to control electronics that were designed to generate the pulse rates and myocardial current fluxes of multiple species (mouse, rabbit, and human) by adjustment of the stimulation pulse width, pulse rate, and output voltage (Fig. 2). The series configuration ensured uniform current fluxes through the four chambers. The control circuitry consisted of two timers in series with a field effect transistor to provide a constant current to the platinum electrodes for the duration of the pulse width. During

intervening periods between pulses, no voltage was applied to the electrodes. Electrical stimuli with pulse widths of 0.5–250 ms, thresholds of 4 μ A–3 mA, and pulse rates of 0.5–10 Hz could be produced with this device to accommodate other animal models as necessary.

To reproduce the physiological environment of an infarct, we attempted to mimic the current density and voltage gradient that would be found in an infarct area in the center of the ventricular wall in rabbits. A uniform wavefront approaching the infarct creates an interstitial potential of ~ 100 mV with an upstroke of 1 ms (25). For normal rabbit myocardium, the conduction velocity is $\sim 76 \pm 1$ cm/s along the fibers and 26 ± 1 cm/s across the fibers (26). Therefore, if the wavefront is 100 mV traveling at 76 cm/s with an upstroke of 1 ms, then the voltage gradient is $\sim 1,315$ mV/cm. This is the maximum voltage gradient; however, the voltage gradient will vary greatly in an infarct area from the maximum at the peri-infarct area to nearly no voltage gradient at the center of the infarct.

The conductivity of the culture medium was determined by measuring total resistance as a function of medium depth and taking the slope of the resulting line. The measured value of medium conductivity was 0.28 S/m. A current of 1.56 mA was delivered spatially uniformly through the medium, resulting in a current density of 1.56 mA/cm² and a potential gradient of 564 mV/cm in the culture chamber. Hence, the potential gradient across the chamber falls within the range of what would be expected within a rabbit myocardial infarct. The skeletal myoblasts did not contract, and they did not reach $>70\%$ confluence during the course of the experiment; consequently, we did not observe formation of skeletal myotubes.

Tissue engineering substrate. Biodegradable PGA mesh scaffolding (Albany International, Mansfield, MA, as well as a gift from Smith & Nephew, Heslington, UK) was used as the substrate for cell seeding. All meshes were processed (10) into nonwoven sheets of 13- μ m-diameter PGA fibers. A 1-mm-thick mesh with a bulk density of 45 mg/ml and a void volume of 97% was used. The PGA mesh was cut into $3.0 \times 1.0 \times 0.1$ -cm segments, and its surface was treated to increase hydrophilicity, as previously described (11). Prolene suture was used to sew the mesh to a biocompatible, nonconducting nylon support (VWR). The substrate was then immersed in 70% ethanol for sterilization and dried overnight.

Cell isolation and culture. Adult rabbit skeletal myoblasts were isolated from hindlimb soleus muscle biopsies as previously reported (33) and expanded in myoblast culture medium consisting of Dulbecco's modified Eagle's medium (JRH Biosciences), 20% horse serum (GIBCO), and 0.05% gentamicin (GIBCO). For prevention of premature differentiation, myoblasts were maintained at $<70\%$ confluence. For seeding of PGA substrates, 5×10^5 skeletal myoblasts were harvested using trypsin-EDTA and were seeded onto the PGA mesh at a density of 2×10^6 cells/ml. After an incubation period of 30 min, the chamber was filled with 5 ml of culture medium, closed in a sterile fashion, and cultured at 37°C and 10% CO₂.

Adult rabbit cardiomyocytes were harvested by a modification of the Langendorff method, an enzymatic digestion. Briefly, the heart was removed and placed on the Langendorff apparatus, attached by the aorta, and washed. Enzyme buffer solution (500 ml of Joklik solution and 5 ml of HEPES) was added, and 38 mg of collagenase and 30 mg of hyaluronidase were added to the enzyme buffer and perfused through the heart for 11 min. After 32 mg of pronase were added to 20 ml of enzyme buffer solution, the solution was perfused for 35 min. At this time, the heart was removed from the apparatus, placed in enzyme buffer, moved to the tissue culture hood, minced, processed, and plated (3). Immediately after enzymatic extraction, rabbit cardiomyocytes were plated at 1×10^6 rods/60-mm dish (Falcon) coated with laminin (10 μ g/ml; GIBCO). The cells from the isolated heart preparation were maintained in cardiomyocyte culture medium consisting of medium 199 (GIBCO), 20% horse serum, 15 mM HEPES, and 100 U/ml of penicillin-streptomycin. To ensure that the primary adult cardiomyocytes retained their cardiac phenotype, the culture was observed daily, and a rod-shaped morphology with

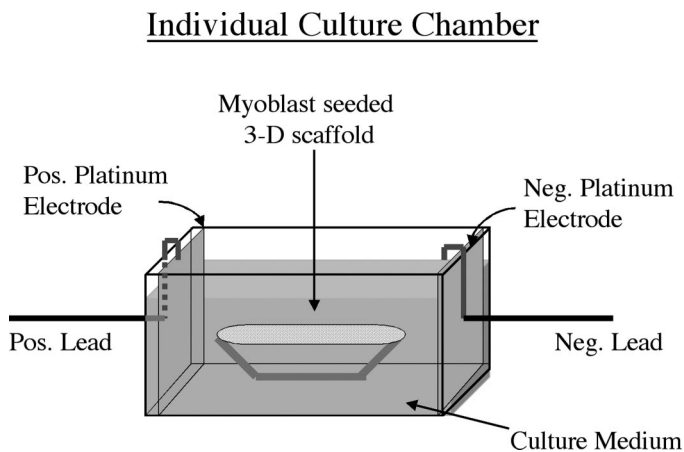


Fig. 1. Individual culture chamber. Each chamber is fitted with 2 platinum electrodes that provide a spatially uniform electric field across the length of the chamber. The chamber is filled with culture medium, which acts as the conductor for current. The highly porous polyglycolic acid (PGA) scaffold is suspended in the culture medium and is seeded with myoblasts. 3-D, 3-dimensional.

Culture Setup

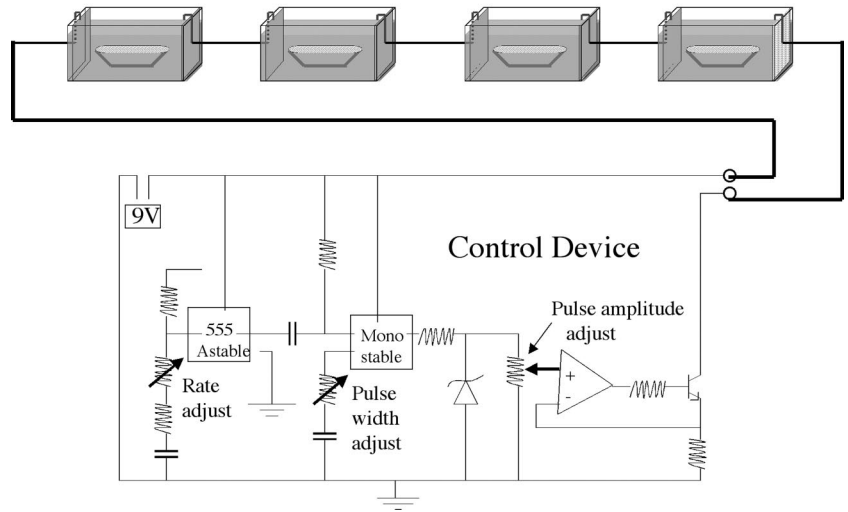


Fig. 2. Culture setup to provide electrical current to multiple chambers. Four chambers are assembled in series and connected to control electronics that deliver pulses of tunable frequency and intensity.

slow but spontaneous beating was confirmed for up to 1 wk after harvest. CM was prepared as follows: culture medium was aspirated from the dishes every 24 h and passed through a 0.22- μ m filter to remove cellular debris. The resulting medium contained soluble factors secreted by cardiomyocytes over a 24-h period. For the cardiomyocyte CM experimental groups, culture chambers were filled with 4 ml of CM and 1 ml of myoblast culture medium.

Immunostaining. 3-D specimens were harvested at 1 or 14 days, fixed in 4% formaldehyde for 1 h, and embedded in paraffin. MyoD primary antibody (Oncogene, Boston, MA) was diluted to 2 μ g/ml, and proliferating cell nuclear antigen (PCNA; Dako) primary antibody was diluted 1:400; each antigen was individually applied for 1 h. Antibody-antigen complexes were amplified with the Vectastain Elite ABC Kit (Vector Laboratories) and visualized using diaminobenzidine (Vector Laboratories). TdT-mediated dUTP nick-end labeling was performed using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (catalog no. S7100, Chemicon) and visualized using diaminobenzidine, according to the manufacturer's instructions (19). F-actin fibers were stained with rhodamine phalloidin (catalog no. R415, Molecular Probes), 20 μ l of stock per 200 ml of 1% BSA, for 25 min at 37°C. The slides were rinsed with PBS-Tween 20 five times for 5 min each time to remove any nonspecific staining and then mounted with Cytoseal.

DNA quantification. To quantify final myoblast number on 3-D scaffolds, cell pellets from 10- to 20-mg wet wt harvested specimens were suspended and incubated in 1 ml of papain solution (0.7 μ g papain/ml, 5 mM EDTA, and 5 mM Cysteine-HCl) at 60°C overnight. Hoechst 33258 dye (Polysciences; 2.0 ml of 0.1 μ g/ml) was added to 0.1 ml of the dissolved sample, and the result was read in a spectrofluorometer at an excitation wavelength of 365 nm and an emission wavelength of 458 nm. Calibration curves were determined from type I calf thymus DNA (Sigma) at a concentration of 10 μ g/ml (15).

Western analysis. 3-D specimens were suspended in lysis buffer (20 mM Tris, 1% Triton X-100, 10% glycerol, 137 mM NaCl, and 2 mM EDTA) in the presence of protease inhibitors (0.25 mM PMSF, 5 μ g/ml leupeptin, 10 nM microcystin, and 150 μ M Na_3VO_4) and homogenized at 4°C. Protein concentrations in homogenates were determined by Bradford protein assay. Twenty-four micrograms of total protein were solubilized in sodium dodecyl sulfate 5 \times Laemmli buffer, boiled for 5 min, and subjected to 10% polyacrylamide gel electrophoresis using a mini-gel apparatus (Bio-Rad). Proteins were transferred to nitrocellulose membranes, blocked with wash buffer [10

mM Tris, 100 mM NaCl, and 0.1% (vol/vol) Tween 20] and nonfat dry milk (50 g/l) at 4°C overnight. Membranes were probed with primary antibodies [myogenin (1:100), MyoD (1:100), GATA-4 (1:500), and Nkx2.5 (1:100)] for 1 h at room temperature. The membranes were washed (5 times for 5 min each time) with wash buffer and further incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-goat IgG (Santa Cruz Biotechnology) secondary antibodies at 1:500 dilution for 1 h at room temperature. Membranes were washed as described above, and blots were visualized by chemiluminescence (National Diagnostics) for autoradiography.

RESULTS

Myoblast culture experiments. We examined the separate and combined effects of cardiomyocyte CM and cardiac-like electrical current fluxes (EF) on myoblast growth and differentiation. Control experiments were performed under conditions of no EF and no CM. Experiments to assess effects of EF were maintained for 1 or 14 days to assess short- and long-term effects of EF on myoblasts. All experiments in CM were maintained for 14 days. After culture, myoblasts were assayed for cell number, cellular proliferation and apoptosis, and markers associated with skeletal or cardiac muscle differentiation.

When samples were maintained for 14 days, histological analyses revealed that myoblasts adhered to PGA fibers throughout the scaffold in all experiments. EF and CM cultures appeared visually to have greater cell numbers overall than did control specimens, and CM combined with EF appeared to be additive for increased myoblast number and density (Fig. 3D). F-actin staining revealed a possibly more organized cytoskeletal structure in specimens grown in CM with EF (Fig. 3, A and D).

Current flux stimulates myoblast proliferation. Number of myoblasts on PGA scaffolds was determined from DNA assay. Interestingly, cell number appeared increased in EF cultures compared with controls after 1 and 14 days of growth (Fig. 4, A and B). This difference was significant at 14 days and corresponded to a twofold increase in cell number ($22.0 \pm 7.7 \times 10^6$ vs. $11.6 \pm 2.9 \times 10^6$ cells/ml, $n = 4$, $P < 0.05$; Fig. 4B).

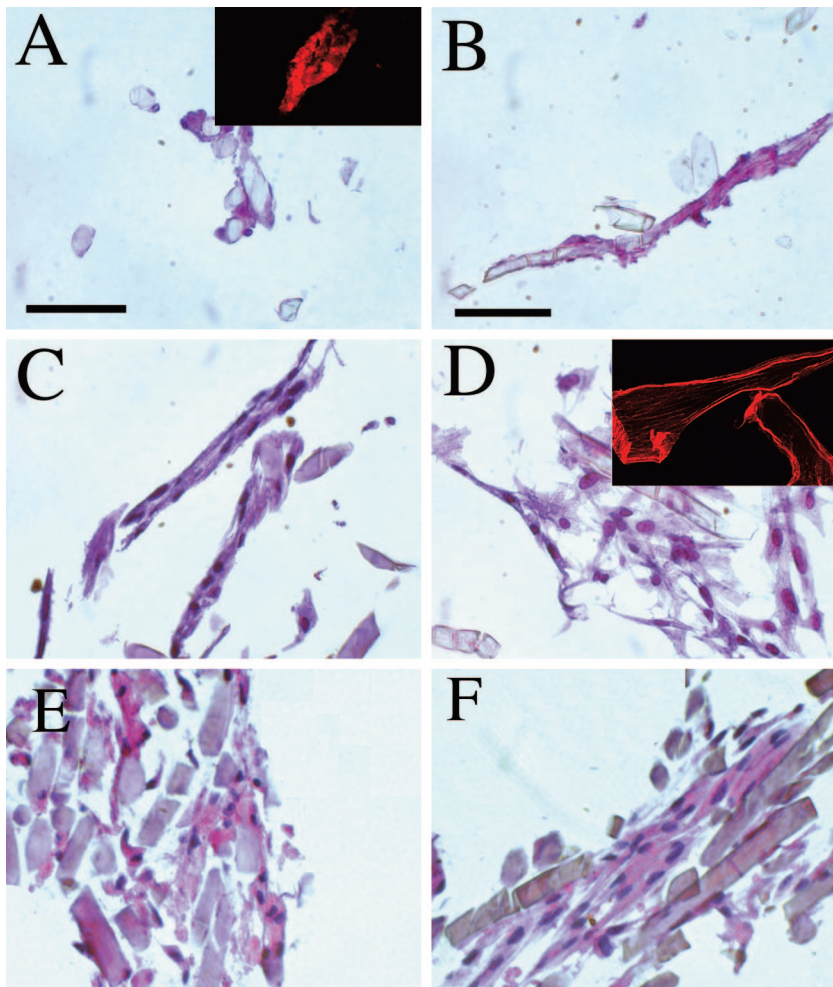


Fig. 3. Myoblasts on PGA scaffold at 14 days of cultivation with hematoxylin and eosin stain. *A*: control conditions, without electrical current flux (EF). *Inset*, rhodamine-phalloidin staining. *B*: EF conditions. *C*: conditioned medium (CM) conditions, without EF. *D*: CM conditions, with EF. *Inset*, rhodamine-phalloidin staining. *E*: verapamil (100 μ M), without EF. *F*: verapamil (100 μ M), with EF. Varying quantities of polymer fragments appear as rectangular objects in images. Scale bars, 50 μ m (applies to *A–F*).

To determine whether this increase in myoblast number was due to differences in cell proliferation or decreased cell death or apoptosis, we performed immunochemical staining on fixed culture specimens. PCNA staining revealed significantly more positive nuclei in EF cultures than in controls ($48.7 \pm 6.3\%$ vs. $21.7 \pm 6.5\%$, $n = 4$, $P < 0.01$), indicating that cells in EF cultures were more proliferative (Fig. 4*E*). In contrast, TdT-mediated dUTP nick-end label nuclear staining for cellular necrosis/apoptosis revealed no significant differences between control and EF cultures (Fig. 4*D*). This indicates that EF applied in these experiments were likely not cytotoxic and that differences in programmed cell death were not responsible for the observed increase in cell number in EF cultures. These data

indicate that cardiac-like EF stimulates proliferation in skeletal myoblasts.

Cardiocyte CM stimulates myoblast proliferation. CM was prepared from culture with mature rabbit cardiomyocytes. When myoblasts were grown in CM for 14 days, significant increases in cell number were observed compared with standard culture medium ($28.3 \pm 4.1 \times 10^6$ vs. $11.6 \pm 2.9 \times 10^6$ cells/ml, $n = 4$, $P < 0.01$; cf. Fig. 4, *B* with *C*). In addition, the proliferative effects of EF conditions were maintained in cardiomyocyte CM. EF samples in CM exhibited a twofold increase in cell number compared with CM alone ($55.8 \pm 11.6 \times 10^6$ vs. $28.3 \pm 4.1 \times 10^6$ cells/ml, $n = 4$, $P < 0.01$; Fig. 4*C*). Taken together, these results indicate that cardiomy-

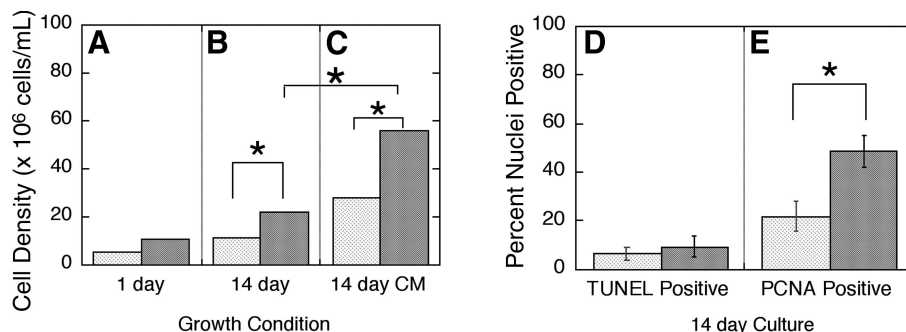


Fig. 4. Myoblast number on PGA scaffolds. Light gray bars, control (without EF); dark gray bars, with EF. *A* and *B*: effects of EF on myoblasts grown for 1 and 14 days, respectively, in 3-D. Cell number is significantly increased by EF after 14 days. *C*: EF also significantly increases myoblast number in the presence of CM. In comparison of EF conditions between *C* and *B*, the presence of CM also significantly increases myoblast number, independent of EF. *D*: numbers of TdT-mediated dUTP nick-end labeling (TUNEL)-positive nuclei are similar between control and EF conditions. *E*: numbers of proliferating cell nuclear antigen (PCNA)-positive nuclei are significantly increased in EF cultures.

ocyte CM, combined with cardiac-like EF, increases the number of myoblasts fourfold in vitro. These two effects appear to be statistically independent and not synergistic ($P = 0.56$ for an interaction effect, as assessed by 2-way ANOVA).

3-D culture does not accelerate skeletal differentiation. On isolation and expansion in 2-D, myoblasts enter the cell cycle and express MyoD, a transcription factor that is expressed before commitment to a differentiated skeletal lineage. For further differentiation to skeletal muscle, several other transcription factors, including myogenin, are required (34). Myogenin expression in myoblast cultures is dependent on the presence of MyoD and on sufficient cellular density, and it correlates with cell cycle withdrawal and transition to differentiation (40).

Immunoblotting for MyoD showed the presence of this transcription factor in rabbit skeletal myoblasts grown to subconfluence in 2-D flasks (Fig. 5). Expression of this transcription factor did not appear to be altered by 3-D culture or by EF conditions (Fig. 5; densitometric analysis shows <2-fold difference in band intensities between samples). Immunoblotting for the marker myogenin showed that although control rhabdomyosarcoma cells expressed this protein, myoblasts in 3-D culture did not express appreciable levels even after 14 days of culture (Fig. 5). These findings were independent of EF and show that neither 3-D culture conditions nor EF appeared to affect myoblast differentiation along the skeletal lineage under the experimental conditions tested. The lack of myogenin expression corroborates data from PCNA staining (Fig. 4E) showing that 3-D myoblasts had not yet completely withdrawn from the cell cycle and were still proliferating.

Cardiac-lineage differentiation is not stimulated by EF or CM. Early cardiomyogenesis and expression of cardiac α -actin are controlled by multiple transcription factors (31). Among the earliest factors directing cardiac cellular differentiation is Nkx2.5, a MADS box factor that is essential for commitment into the cardiac muscle lineage (14, 17). GATA-4 is another early transcription factor and key activator of the cardiac genetic program (12, 22); it is present in precardiac cells during embryonic development, and its expression characterizes very early stages of heart formation. Nkx2.5 and GATA-4 were chosen as early cardiac markers, because, without the expression of these markers, cardiac cells cannot develop (14, 41). Nkx2.5 and GATA-4 expression in embryonic stem cell con-

version is present at *day 3* and lasts until *day 14*; therefore, the expression of these markers is necessary to demonstrate cardiac commitment (39).

We performed immunoblotting for Nkx2.5 and GATA-4 to determine whether 3-D culture, EF conditions, or CM caused early conversion of myoblasts to cells capable of cardiac gene expression. Murine embryonic cells that were engineered to express high levels of Nkx2.5 and GATA-4 were used as positive controls (Fig. 6) (27). We observed no detectable expression of Nkx2.5 or GATA-4 in 3-D myoblast cultures that were exposed to EF or CM (Fig. 6). Although it is acknowledged that, by immunoblotting, extremely low levels of protein expression may not be detected, with stem cell conversion, GATA-4 can be seen by immunoblotting (15). Therefore, these data argue that cardiac-like electrical stimulation and cardiocyte soluble factors do not induce significant transdifferentiation of myoblasts into cardiac-like cells.

Mechanism for skeletal myoblast proliferation. Electrical stimulation induced proliferation of skeletal myoblasts in vitro. Several other cell types are known to have a proliferative response to electrical stimuli, including skeletal osteoblasts (18, 37, 38). Intracellular calcium levels have been implicated in the mechanism for electric field effects on osteoblasts (37). This potential mechanism has also been proposed for electrical effects on neuronal cells (7, 20). In cultured sensory neurons, patterned electrical stimulation induces changes in gene expression that are mediated by N- and L-type calcium channels. On the basis of these observations, we hypothesized that the mechanism of proliferation by electrical stimulation is due to an increase in intracellular calcium resulting from the EF.

To test this hypothesis, an L-type calcium channel blocker was selected and added to EF and control experiments (Fig. 3, E and F). Verapamil inhibits passage of calcium through the voltage-gated L-type (long-lasting current) calcium channel, reducing calcium availability (30). We studied the effects of supplementing culture medium in control (non-EF) and EF cultures with 100 μ M verapamil as previously reported (27). In the presence of verapamil, non-EF cultures had fewer cells than those without verapamil, but this difference was not significant ($P = 0.0718$; Fig. 7). However, as hypothesized, the proliferative effect of EF was ablated in myoblast cultures exposed to verapamil ($P = 0.290$; Fig. 7). Hence, blocking the L-type calcium channels and blocking intracellular calcium fluxes eliminated the proliferative effect seen with EF in skeletal myoblasts.

DISCUSSION

Multiple stimuli, including soluble growth factors, the extracellular matrix, and direct cell-to-cell interactions, dictate cell proliferation and differentiation during embryonic development and throughout life. Although each of these signals uniquely regulates gene activity and mitogenic responses, the decision of a cell to proliferate, differentiate, or undergo apoptosis is an integrated response (30). The goal of these experiments was to determine whether electrical current flux or soluble factors derived from cardiomyocytes could serve as a stimulus to skeletal myoblasts to change their proliferation or differentiation.

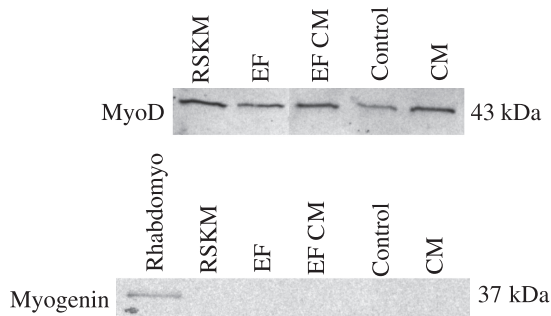


Fig. 5. Skeletal muscle differentiation markers after 14 days of culture. Evaluation of MyoD by immunoblotting shows a single band at 43 kDa, with similar expression in rabbit skeletal myoblasts (RSKM, positive control), EF, and control cultures. Myogenin, a later marker for skeletal muscle, is expressed by rhabdomyosarcoma cells, but not by RSKM or any of the myoblast cultures.

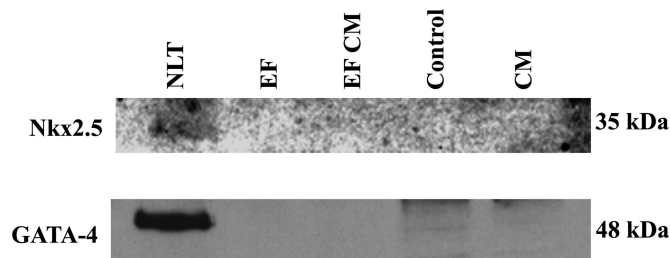


Fig. 6. Cardiac muscle differentiation markers after 14 days of culture. Expression of Nkx2.5, an early transcription factor in cardiac development, is weakly evident in murine embryonic cells that were engineered to express high levels of Nkx2.5 and GATA-4 (NLT, positive control), but is not evident in any of the myoblast cultures. GATA-4 expression is confined to NLT cells as well, with no visible expression in myoblast populations by immunoblotting.

Electrical stimulation, at the levels implemented in this experiment, induced proliferation of skeletal myoblasts in culture. To the best of our knowledge, this is the first report of electrical current flux inducing a mitogenic response in skeletal muscle-derived cells in vitro. Cardiac-like electrical current pulses appear to stimulate myoblast proliferation via an L-type calcium channel-dependent pathway. Calcium is a second messenger that has a role in cell survival, proliferation, motility, apoptosis, and differentiation (23). Voltage-gated calcium channels are activated by depolarization and are primarily involved in mechanical contraction of excitable cells (9, 16, 23). The main role for proliferative calcium signals is the progression from the G_1 to G_1 - G_S transition phase. It has recently been shown that high concentrations of calcium induced the expression of cyclin D in osteoblasts. Another possible mechanism is the activation of early-response genes, such as *c-fos*, which are triggered within 15 min of calcium signaling and lead to cell proliferation. It has been shown that specific inhibition of the L-type calcium channels blocks *fos*-related protein production. Future studies are needed to understand why these specific experimental conditions permitted mitogenic calcium signaling.

It is useful to consider the degree to which this in vitro system indeed mimics the electrical environment of the myocardium. Because of the strong dependence of electric field strength on distance from a dipolar source ($1/r^2$), a broad range of current densities might be relevant to the simulation of the electrical environment. We selected a value (564 mV/cm) that is probably well within the bounds of this range. However, the field strength would be significantly higher for cells that are very close to active tissue and significantly lower for those farther from the active tissue. A subsequent study that examines the relative effects of field strength on growth and expression is suggested by this work.

The primary pathways involved in myoblast differentiation in vitro and in vivo have been characterized. In vitro, skeletal precursor cells express MyoD or Myf-5 in the committed state and turn on transcription of myogenin when induced to differentiate (13). In vivo, myoblasts that are activated begin a differentiation program and express a series of myogenic regulatory factors (MRFs), which consist of MyoD, Myf-5, myogenin, and MRF-4 (40). In culture, myoblasts can also begin to execute this differentiation program as a function of their density and the extent of cell-to-cell contact. However,

multiple factors are known to interfere with myoblast skeletal differentiation. For example, myoblasts that express integrin subunit α_{61044t} may remain in the proliferative phase and not differentiate, even in high-density cultures (30). In the studies reported here, myoblasts that were cultured in 3-D for 14 days did not execute the skeletal muscle differentiation program and express myogenin. It is assumed that the large surface area of the PGA mesh increased the time for myoblasts to achieve adequate cell-to-cell contact required for differentiation.

In these studies, we did not observe evidence of cardiac differentiation of skeletal myoblasts with EF or cardiomyocyte CM. This result may be consonant with several in vivo studies of cellular cardiomyoplasty, wherein conversion to a cardiac phenotype has not been clearly demonstrated. However, myoblast proliferation in response to stimuli that are likely present in the cardiac milieu, i.e., EF and soluble growth factors, is a new finding. This proliferation response may underlie some of the successful engraftment results that have been observed with cellular cardiomyoplasty, despite reports of initial cell death after transplantation in vivo (35). These results point to potential new mechanisms governing myoblast proliferation that were not understood previously.

Conclusions. Because EF and cardiac-derived soluble factors stimulate proliferation, the in vivo cardiac environment may be more supportive for myoblast survival than previously thought. Cardiac-like electrical current pulses appear to stimulate myoblast proliferation via an L-type calcium channel-dependent pathway. The in vitro system described here provides a platform for dissecting the effects on progenitor cells of multiple complex stimuli that occur in vivo. Exploitation of this or similar systems for the growth of other types of stem cells, such as bone marrow-derived stem cells, mesenchymal stem cells, and embryonic cells, could be an excellent means to study mechanisms of cell survival and differentiation in the intact heart.

The results of this study provide an intellectual foundation for the growth of precursor cells in a cardiac-like environment in vitro. The insights gained from in vitro study of cells in the cardiac environment have the potential to improve the lives of many end-stage heart failure patients.

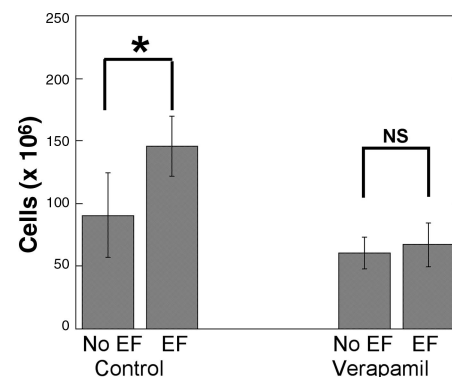


Fig. 7. Effects of verapamil on myoblast replication: number of myoblasts after 14 days of culture under non-EF (control) or EF conditions in the presence and absence of verapamil (100 μ M). *Cell number was increased significantly by EF under control conditions ($P = 0.02$). EF causes no significant change in cell number in the presence of verapamil [not significant (NS), $P = 0.29$].

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