

Electromechanical Coupling between Skeletal and Cardiac Muscle: Implications for Infarct Repair[Ⓞ]

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Abstract. Skeletal myoblasts form grafts of mature muscle in injured hearts, and these grafts contract when exogenously stimulated. It is not known, however, whether cardiac muscle can form electromechanical junctions with skeletal muscle and induce its synchronous contraction. Here, we report that undifferentiated rat skeletal myoblasts expressed N-cadherin and connexin43, major adhesion and gap junction proteins of the intercalated disk, yet both proteins were markedly downregulated after differentiation into myotubes. Similarly, differentiated skeletal muscle grafts in injured hearts had no detectable N-cadherin or connexin43; hence, electromechanical coupling did not occur after *in vivo* grafting. In contrast, when neonatal or adult cardiomyocytes were cocultured with skeletal muscle, ~10% of the skeletal myotubes contracted in synchrony with adjacent cardiomyocytes. Isoproterenol increased myotube contraction rates by 25% in coculture without affecting myotubes in monoculture, indicating the cardiomyocytes were the pacemakers. The

gap junction inhibitor heptanol aborted myotube contractions but left spontaneous contractions of individual cardiomyocytes intact, suggesting myotubes were activated via gap junctions. Confocal microscopy revealed the expression of cadherin and connexin43 at junctions between myotubes and neonatal or adult cardiomyocytes *in vitro*. After microinjection, myotubes transferred dye to neonatal cardiomyocytes via gap junctions. Calcium imaging revealed synchronous calcium transients in cardiomyocytes and myotubes. Thus, cardiomyocytes can form electromechanical junctions with some skeletal myotubes in coculture and induce their synchronous contraction via gap junctions. Although the mechanism remains to be determined, if similar junctions could be induced *in vivo*, they might be sufficient to make skeletal muscle grafts beat synchronously with host myocardium.

Key words: skeletal myocytes • cardiomyocytes • electromechanical coupling • N-cadherin • connexin43

Introduction

Mature cardiomyocytes are terminally differentiated and do not reenter the cell cycle to a significant extent after injury (Anversa et al., 1980; Rumyantsev, 1991; Soonpaa and Field, 1998). Hence, the loss of myocardium after myocardial infarction is irreversible. Since the human heart lacks functional repair mechanisms, we are exploring skeletal muscle as a possible replacement tissue. Several aspects favor skeletal muscle rather than cardiac muscle as a source of graft cells for cardiac repair. First, skeletal muscle is much more resistant to ischemia than cardiac muscle (Eckert and Schnackerz, 1991; Wolff and Stiller, 1993). Second, skeletal myoblasts might establish satellite cells, thereby providing resident muscle stem cells for future self

repair (Murry et al., 1996a,b). Third, autologous skeletal muscle satellite cells could be isolated from muscle biopsies and reimplanted into the infarct, thereby circumventing graft rejection. We and others have shown that skeletal myoblasts will form viable grafts of differentiated skeletal muscle in normal and injured hearts (Koh et al., 1993; Chiu et al., 1995; Yoon et al., 1995; Murry et al., 1996a,b; Robinson et al., 1996; Taylor et al., 1998).

The main theoretical drawback to skeletal muscle for cardiac repair is its differing electrical properties. Heart muscle cells are electromechanically coupled by specialized cell-cell junctions, the intercalated disks, which contain adherens and gap junctions for mechanical and electrical coupling, respectively. The major adherens junction protein in the mature mammalian heart is N-cadherin (Volk and Geiger, 1984), whereas connexin43 is the major gap junction protein (Beyer et al., 1987). Electromechanical coupling between cardiomyocytes is a basic require-

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ment for coordinated mechanical activity in myocardium. In contrast to heart muscle cells, skeletal muscle fibers are electrically isolated from one another, a prerequisite for fine motor control. Interestingly, skeletal muscle cells express N-cadherin and connexin43 as replicating myoblasts and then downregulate this expression after terminal differentiation and myotube formation (MacCalman et al., 1992). Functional gap junctions have been detected during the early stages of skeletal muscle development, and gap junctional intercellular communication was shown to play an important role in myoblast fusion (Mege et al., 1994; Proulx et al., 1997). An equally important role in myoblast fusion was described for N-cadherin (Mege et al., 1992; Cifuentes-Diaz et al., 1993; Holt et al., 1994; George-Weinstein et al., 1997; Redfield et al., 1997).

The goals of this study were (a) to investigate the formation of electromechanical junctions between cardiac and skeletal muscle, and (b) to test whether cardiac muscle can provide excitation to skeletal muscle and induce its synchronous contraction.

Materials and Methods

Cell Isolations and Rat Cardiac Injury Model

These studies were approved by the University of Washington Animal Care Committee and were conducted in accordance with federal guidelines. Neonatal skeletal myoblasts and neonatal or adult cardiomyocytes were isolated by enzymatic dispersion from 1–2-d-old or adult Fischer 344 rats (Simonsen Labs) and cultured as previously described (Murry et al., 1996a,b; Reinecke et al., 1997, 1999). Fischer 344 is an inbred strain and was used to avoid graft rejection. Under these conditions, the skeletal muscle cultures were ~50% myogenic. Approximately 90% of neonatal and 70% of adult cardiomyocyte preparations were muscle cells. Skeletal myoblasts were grown in F10 medium (GIBCO BRL) in the presence of 15% horse serum (ICN Flow) and 6 ng/ml bFGF (Scios Inc.), plus penicillin G (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml; GIBCO BRL). Myogenic differentiation was induced by switching the cells to a medium supplemented with 1.5% horse serum, 6 µg/ml insulin (Sigma), 0.9 mM CaCl₂, and no bFGF.

Neonatal cardiomyocytes were grown in DME/M199 (4:1) supplemented with 10% horse serum, 5% FBS (HyClone), penicillin G (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). Both cell types were cultured in gelatin-coated dishes (Murry et al., 1996a,b). Adult cardiomyocytes were resuspended in serum-free M199 (Sigma) supplemented with L-carnitine (2 mM), creatine (5 mM), taurine (5 mM), albumin (0.2%), penicillin G (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml), and then were plated in laminin-coated (1 µg/cm²; Sigma) 60-mm cell culture dishes (Falcon) at a density of 5×10^3 /cm² and incubated for 4 h (5% CO₂, 37°C). Then, the medium was aspirated and replaced by fresh medium supplemented with 15% FBS. The medium was changed every 3 d thereafter.

Hearts of adult male Fischer 344 rats were cryoinjured by placing a 6-mm aluminum rod, precooled with liquid nitrogen, in contact with the surface of the anterior left ventricular wall as previously detailed (Murry et al., 1996a,b; Reinecke et al., 1999). Normal control and acutely cryoinjured hearts were injected with 3×10^6 skeletal myoblasts as described previously (Murry et al., 1996a,b). Rats were killed at timed intervals (1 and 3 d; 1, 2, and 4 wk; 3 mo; $n = 4$ /group). All hearts were perfusion fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, and 10% glacial acetic acid), transversely sectioned, and embedded in paraffin.

Immunocytochemistry and Western Blot Analysis

Immunostaining was performed using immunoperoxidase and immunofluorescent methods as described (Murry et al., 1996a,b; Reinecke et al., 1999). Expression of cadherin and connexin43 in undifferentiated myoblasts and differentiated myotubes was determined by Western blot analysis. Skeletal myotubes were maintained in differentiation medium for 10 d before lysis. On day 5 cytosine arabinofuranoside (5 µM; Sigma) was

added to kill proliferating cells (e.g., fibroblasts). Cells were lysed in standard sample buffer containing protease inhibitors (1 mM Pefabloc[®] SC, 10 µg/ml leupeptin, and 10 µg/ml aprotinin; Boehringer Mannheim). 20 µg of total soluble protein were separated via SDS-PAGE (Bio-Rad) using a 7.5% resolving gel for cadherin and a 12% resolving gel for connexin43 detection. Proteins were electroblotted to Hybond-ECL nitrocellulose membrane (Amersham Corp.) and immunoreactions were carried out as described using the ECL detection kit (Reinecke et al., 1996, 1997). N-Cadherin was detected using an anti-pan-cadherin mouse monoclonal antibody (Geiger et al., 1990; Sigma), diluted 1:2,000 for light microscopy, 1:200 for immunofluorescence, and 1:1,000 for Western blotting. Connexin43 was detected using a mouse monoclonal antibody (Kanter et al., 1993; Chemicon), diluted 1:200 for light and fluorescence microscopy and 1:1,000 for Western blotting.

Coculture of Neonatal or Adult Cardiomyocytes with Skeletal Myotubes

Neonatal cardiomyocytes and skeletal myoblasts were mixed in ratios of 1:3 and 1:1 in DME/M199 with 10% horse serum and 5% FBS and plated at a total cell density between 6×10^3 and 1×10^4 cells/cm². It appeared that the best results were obtained when 1×10^4 total cells/cm² were plated at a ratio of 1:1, allowing formation of smaller, unbranched myotubes and enough surrounding cardiomyocytes to provide excitation (note that the fusion of myoblasts into multinucleated myotubes leads to a substantially lower number of myotubes compared with the original number of myoblasts). 60-mm gelatin-coated (Difco) or laminin-coated (for adult cardiomyocytes) tissue culture plates (Falcon) were used for video analysis and microinjection studies, Lab-Tek II glass chamber slides (Nunc) for confocal microscopy, and Lab-Tek II chambered coverglass (Nunc) for calcium imaging. Neonatal cardiomyocytes and skeletal myoblasts were mixed and cocultured in 60-mm plates for 2 d, by which time the myoblasts had fused to form multinucleated myotubes. Cocultures were then evaluated under an inverted microscope equipped with a heated chamber (37°C) and a video camera. Isoproterenol (25 nM; Sigma) was used as a β-adrenergic agonist and 1-heptanol (0.5 mM; Sigma) as a gap junction inhibitor (Christ et al., 1999). Plates were incubated with the respective drug for 20 min before the analysis. For statistical quantification, three different plates were evaluated and the contraction frequencies of eight different fields on each plate were counted. The field areas were encircled with a marker pen, and specific morphological characteristics were noted in order to find the same fields before and after treatments. Monocultures of cardiomyocytes and skeletal myotubes, respectively, served as controls.

In one set of experiments, cocultures of adult cardiomyocytes and skeletal myoblasts were set up on the day of cardiomyocyte isolation. Adult cardiomyocytes were plated at 5×10^3 /cm² and 1.5×10^4 /cm² skeletal myoblasts were added (ratio 1:3). Cocultures were maintained in M199 supplemented with L-carnitine (2 mM), creatine (5 mM), taurine (5 mM), albumin (0.2%), 15% FBS, 1.5% horse serum, and 6 µg/ml insulin. Under these conditions myoblast fusion and differentiation into multinucleated myotubes took place between 24–48 h. In serum-containing medium, adult cardiomyocytes undergo a morphological change described as dedifferentiation/redifferentiation, hallmarked by the loss of the rod shape and myofibrillar disintegration and subsequent spreading, and reorganization of the contractile apparatus (Eppenberger et al., 1987, 1988, 1995). On day 3, cytosine arabinofuranoside (10 µM) was added to prevent fibroblast overgrowth. Most of the cardiomyocytes were redifferentiated by day 8 and contractile activity was observed frequently. In a second set of experiments skeletal myoblasts (1.5×10^4 /cm²) were added to the cardiomyocyte cultures on day 9 (i.e., after completion of dedifferentiation/redifferentiation) and cells were cocultured as before. Cytosine arabinofuranoside (5 µM) was added back after myoblast differentiation.

Confocal Microscopy

For confocal microscopy neonatal or adult cardiomyocytes were cocultured with skeletal muscle cells for 2–3 d (neonatal cardiomyocytes) or 4–9 d (adult cardiomyocytes). Cocultures were fixed for 2 min in 3% paraformaldehyde in PBS, 0.2% Triton X-100, 5 mM EGTA (pH 7.2) followed by fixation with 3% paraformaldehyde in PBS for 20 min. Specimens were incubated with pan-cadherin or connexin43 antibodies for 60 min. Primary antibodies were detected with a rabbit anti-mouse FITC-conjugated secondary antibody (DAKO). Counterstaining for f-actin was performed using rhodamine-phalloidin (Molecular Probes). Vectashield (Vector) was used as mounting medium. Optical sections of 0.1-µm thick-

ness were obtained using a BioRad MRC 600 confocal microscope equipped with a krypton-argon laser.

Microinjection Studies

Gap junctions were evaluated functionally by microinjecting cells with the gap junction permeable dye, Lucifer yellow (Molecular Probes). Pilot studies showed that injecting cardiac myocytes resulted in excessive dilution of the dye after transfer into the much larger myotubes, so we chose to inject myotubes instead. It was necessary to inject quiescent instead of actively contracting myotubes, since injecting beating myotubes often caused hypercontracture and cell death. Therefore, beating cocultures were rendered mechanically quiescent by pretreatment with the calcium chelator BAPTA-AM (40 μ M, 30 min; Sigma), and subsequent addition of the cross-bridge cycle inhibitor 2,3-butanedione monoxime (BDM¹, 5 mM; Sigma) to the medium. Microinjections were performed as described (Reinecke et al., 1999). The dye solution was composed of 10% Lucifer yellow (gap junction permeable) and 10% tetramethylrhodamine-dextran (gap junction impermeable; Molecular Probes) in sterile ddH₂O. Skeletal myotubes in close approximation to cardiomyocytes were pressure-injected (time setting 0.4 s, pressure setting 180 kPa). To visualize cell nuclei cocultures were vitally counterstained with Hoechst 33342 (4 μ g/ml). Dye transfer was evaluated under a Olympus BH-2 fluorescence microscope.

Calcium Imaging of Cocultures

For calcium imaging (Zirpel and Rubel, 1996; Zirpel et al., 1998) neonatal cardiomyocytes and skeletal myoblasts were cocultured for 5 d. Beating cocultures were loaded with the membrane permeant acetoxymethyl ester derivative of the fluorescent calcium indicator, Calcium Green. Cells were loaded with 5 μ M Calcium Green-AM (Molecular Probes), 0.02% Pluronic (Molecular Probes) and 0.15% dimethylsulfoxide (Sigma) in DMEM/M199 with 10% horse serum and 5% FBS for 45 min at 37°C/5% CO₂. The cultures were gently given two changes of fresh medium at the end of dye loading to remove extracellular dye. Cells were imaged on a Diaphot TMD (Nikon) inverted microscope equipped with a 40 \times , NA 1.3 Fluor objective, Nikon B-2E fluorescent filter set and a 75 W Xenon lamp. The dye was excited at 450–490 nm, whereas an emission band from 520 to 560 nm was directed to a Hamamatsu II Intensifier coupled to a Hamamatsu CCD-77 camera. The intensified images were recorded directly onto an optical memory disk recorder (OMDR; Panasonic TQ-3038F) at 30 frames/s. Black level and gain were set at each imaging session and blank images were collected for flat field correction before analysis. The analogue OMDR images were converted to TIFF files with Image/1 (version 4.17; Universal Imaging Corp.) and then transferred to a Macintosh PPC9600/200MP computer for analysis and movie conversion. To address the question of whether myotube contractions were induced by stretch, cocultures were mechanically arrested with BDM. BDM was present during loading with Calcium Green and throughout the evaluation. Pretreatment with BAPTA-AM was omitted in the latter experiments since the calcium chelator interfered with Calcium Green activation.

Supplemental Materials: Evidence for Electromechanical Coupling of Cardiac and Skeletal Muscle

The supplemental data for this manuscript are presented in video format that further depicts Fig. 3 (Pharmacological experiments) and Fig. 6 (Calcium imaging). For these experiments cardiomyocytes from 1–2-d-old neonatal or from adult rats were plated with primary rat skeletal myoblasts. The myoblasts differentiated in the cardiomyocyte medium and fused to form multinucleated skeletal myotubes. Control monocultures of each cell type also were studied. After 2 d (cocultures with neonatal cardiomyocytes) or 7 d (cocultures with adult cardiomyocytes) in culture the dishes were transferred to a videomicroscope equipped with a thermostatically controlled chamber. Over 3 h of videotape was prepared from multiple isolates of cells and analyzed in detail. The microscopic fields presented are representative examples of multiple experiments. The video is available at <http://www.jcb.org/cgi/content/full/149/3/731/DC1>.

¹Abbreviations used in this paper: BDM, 2,3-butanedione monoxime; MT, myotubes; OMDR, optical memory disk recorder.

Results

Differentiation and Electromechanical Integration of Skeletal Muscle Grafts

The histology and differentiation pattern of skeletal myoblast grafts were described in a previous study from our group (Murry et al., 1996a,b). In brief, at day 1 after grafting the myoblasts were undifferentiated. By day 3, the grafted cells fused into multinucleated myotubes and expressed structural proteins such as myosin heavy chain. At 1 wk the myotubes had developed recognizable cross striations, while at 2 wk many had peripheral nuclei and clearly identifiable sarcomeres, signs of maturation. Progressive hypertrophy was observed over the entire time course, and by 3 mo the grafts appeared very similar to normal adult skeletal muscle. Interestingly, the skeletal muscle grafts began to express the slow fiber marker, β -myosin heavy chain, between 2 and 4 wk after grafting. This suggests the grafts were converting to a more fatigue-resistant phenotype in the cardiac environment (Murry et al., 1996a,b).

To investigate electromechanical integration of grafted skeletal muscle with host myocardium, immunostaining was performed for components of the intercalated disk. Fig. 1 shows a representative graft at 2 wk, when the grafted myoblasts had already fused and differentiated (Fig. 1, A and B). An antibody to the COOH terminus of N-cadherin (anti pan-cadherin), the principal component of the cardiac adherens junction, did not stain graft cells (Fig. 1, C and D). Similarly, antibodies to connexin43, the principal gap junction protein of ventricular myocytes, did not stain grafted skeletal muscle cells (Fig. 1, E and F). Both antibodies, however, gave intense staining at the intercalated disks of host cardiomyocytes where N-cadherin and connexin43 typically colocalize. The skeletal muscle grafts did not contain detectable amounts of N-cadherin or connexin43 at any time from 3 d up to 3 mo after grafting into injured myocardium. Hence, the grafts appeared not to form any persistent electromechanical junctions in vivo. In the injured hearts the skeletal muscle grafts were often separated from the host myocardium by intervening scar tissue. To test whether scarring alone might have prevented junction formation, skeletal myoblasts were grafted into normal, uninjured hearts. Although scarring was minimal and the two muscle cell types were closely apposed, N-cadherin- and connexin43-positive junctions still were not detected in the skeletal muscle cells. Thus, it is likely that scarring and the lack of junctional protein expression in skeletal muscle grafts are two separate problems.

Electromechanical Coupling In Vitro

Western blot analysis of skeletal muscle cells in vitro showed that undifferentiated primary rat myoblasts expressed N-cadherin and connexin43 at high levels (Fig. 2). In contrast, cultures containing differentiated myotubes showed markedly decreased (but still detectable) levels of both N-cadherin and connexin43 at day 10 of differentiation (Fig. 2). It should be mentioned that our primary myoblast cultures contained contaminating non-myocytes, which may partially contribute to the signal of both proteins. For the differentiated myotubes, proliferating non-

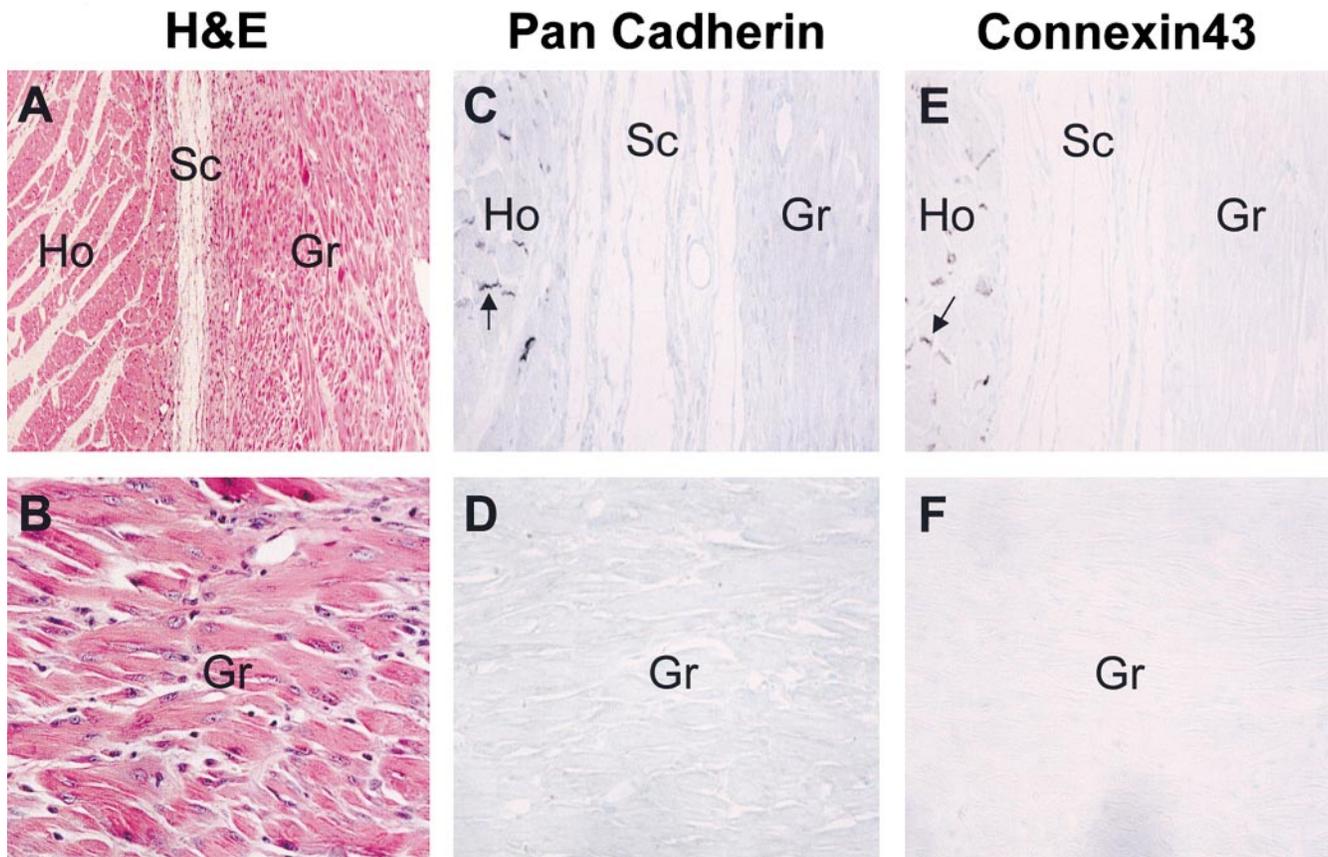


Figure 1. Skeletal muscle cell grafting into the injured heart at 2 wk. (A and B) Hematoxylin and eosin staining. Skeletal muscle cells formed a viable graft (Gr) that was often separated from the host myocardium (Ho) by scar tissue (Sc). No immunostaining for cadherin (C and D) or connexin43 (E and F) was found in the skeletal muscle graft. In contrast, the host myocardium showed a typical staining pattern for both molecules as they colocalize in the intercalated disc (arrows). B, D, and F show high power micrographs of the graft area.

myocytes were killed with cytosine arabinofuranoside and are unlikely to confound the experiment. To verify that cadherin and connexin43 were expressed by myoblasts, the Western blot analysis was also repeated with the mouse myoblast line C2C12. The C2C12 cells also expressed cadherin and connexin43 and similarly downregulated these proteins after differentiation (data not shown).

Given the lower N-cadherin and connexin43 levels with ongoing differentiation, it seemed unlikely that skeletal myotubes would form electromechanical junctions with cardiomyocytes. To our surprise, however, when skeletal myoblasts and neonatal or adult cardiomyocytes were cocultured, some of the resulting skeletal myotubes contracted in synchrony with the cardiomyocyte network. Clearly identifiable synchronous contractions were observed in ~10% of myotubes, although there was some variation among cultures, with differing cell densities, etc.

Myotube formation occurred over the first 2 d of coculture, and synchronous contractions were noted shortly thereafter when neonatal cardiomyocytes were used. When adult cardiomyocytes were studied in monoculture, it took ~7 d for these cells to spread and form a synchronously beating network. In coculture with skeletal muscle cells, myotube contractions began as soon as con-

traction of the cardiomyocyte network started. Synchronously contracting networks were observed for as long as 15 d of coculture, the longest time tested.

Monocultures of primary skeletal myotubes can contract or twitch erratically when plated at high density. In striking contrast to skeletal myotube monocultures, however, the contractions of myotubes in coculture with cardiomyocytes were very regular (heartbeat-like) and the frequency of contractions appeared highly synchronized. For example, myotubes and neonatal or adult cardiomyocytes would skip the same beat and then resume contracting again in synchrony.

We hypothesized that some of the cardiomyocytes had electrically captured closely adjacent myotubes and were pacing them via gap junctions. Pharmacological experiments to test this hypothesis are summarized in Fig. 3. Isoproterenol, a well-known β -adrenergic agonist, caused a ~25% increase in the contraction frequency of beating myotubes in cocultures. A similar effect of isoproterenol was observed in cardiomyocyte monocultures, whereas no effect was seen in typically noncontracting skeletal myotube monocultures (data not shown). These data implicate cardiomyocytes as the pacemakers. In contrast, the gap junction inhibitor 1-heptanol completely aborted myotube

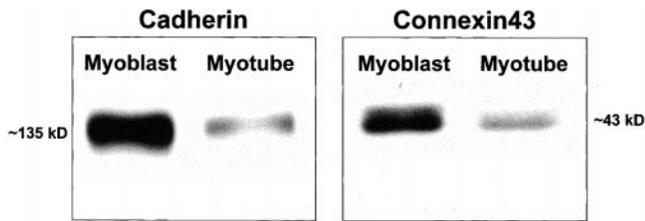


Figure 2. Western blot analysis of N-cadherin and connexin43 in undifferentiated myoblasts and differentiated myotubes. N-cadherin and connexin43 were expressed at high levels in myoblasts; however, the differentiated myotubes showed a marked down-regulation of both molecules at day 10 after induction of differentiation. Cytosine arabinofuranoside (5 μ M) was added to myotube cultures on day 5 to prevent fibroblast overgrowth.

contractions but left spontaneous contractions of individual cardiomyocytes intact, suggesting that excitation of the myotubes required gap junctions. Washing out of the drugs returned the contraction frequencies to baseline levels (Fig. 3). Interestingly, heptanol also inhibited the spontaneous irregular contractions in high density skeletal myotube monocultures. This suggests that low levels of connexin43 in differentiated myotubes may be sufficient for the formation of functional gap junctions between myotubes *in vitro* as well (data not shown).

If electromechanical coupling had indeed occurred between cardiomyocytes and skeletal myotubes, the responsible molecules should be detectable at myotube-cardiomyocyte interfaces. Therefore, immunofluorescent staining for N-cadherin and connexin43 in conjunction with confocal microscopy was performed. Fig. 4 shows abundant expression of N-cadherin at contact sites between a neonatal (A) or an adult (C and E) cardiomyocyte and a skeletal myotube. The adherens junction was often seen as N-cadherin-positive, finger-like cell processes extending from the actin cytoskeleton (Fig. 4 A). Similarly, connexin43 was detected at contact sites between neonatal (Fig. 4 B) or adult (D and F) cardiomyocytes and skeletal myotubes. The formation of gap junctions between cardiomyocytes and skeletal myotubes appeared to lag somewhat behind the formation of adherens junctions and to increase with coculture time. No cadherin or connexin43 proteins were detected in myotubes in areas remote from contacts with cardiomyocytes. When adult cardiomyocytes were cocultured with skeletal muscle cells, day 4 was the earliest time point that N-cadherin- and connexin43-positive junctions were observed. At this time point cardiomyocytes had spread a little at the periphery, but the original architecture of the contractile apparatus was largely intact. Contractions were rarely seen at day 4. By 7 d the cardiomyocytes had established numerous contacts via cell processes and synchronous contractions with skeletal muscle cells were observed.

To test directly whether skeletal myotubes can form functional gap junctions we performed microinjection studies. Skeletal myotubes were microinjected with a combination of Lucifer yellow (\sim 250 D), which readily passes through gap junctions (Becker et al., 1995), and rho-

damine-dextran (\sim 10,000 D), which is not transferable through gap junctions. Fig. 5 (A) shows two injected myotubes in the field (rhodamine-dextran positive). Fig. 5 B shows Lucifer yellow dye transfer from (at least) one of these myotubes to a cardiomyocyte at day 4 of coculture. Note that this cardiomyocyte subsequently carried out a second order dye transfer to another connected cell, likely a coupled cardiomyocyte. The Hoechst 33342 staining for nuclei in C shows the spatial orientation of all cells in the injection field. Note that dye transfer only occurred between the myotubes (MT) and the binucleated recipient cell (Rec), but not to any of the other cells in the field. Dye transfer between myotubes and adjacent cardiomyocytes was less frequent than the observation of synchronous beating. This likely reflects a greater difficulty for the passage of the 250-D Lucifer yellow molecule versus ions involved in action potential propagation. Technical artifacts induced by microinjection also could have inhibited gap junction transfer in some cells.

We used calcium imaging to ensure that the motions observed in the skeletal myotubes were calcium-dependent contractions and not due to passive tethering by beating cardiomyocytes. Fig. 6 shows that cardiomyocyte calcium transients induced highly synchronized calcium transients in an adjacent skeletal myotube. As noted previously with analysis of contractile activity, occasional pauses in calcium cycling occurred synchronously in the two cell types, indicating tight coupling. These data show that the myotube contractions are an active process requiring action potential-induced calcium transients. An alternate analysis of the synchrony of calcium transients is shown in Fig. 6 D. A square was drawn over the region of maximal fluorescence in an adjacent neonatal cardiomyocyte (NC) and myotube (MT). The average light signal along one side of the square is shown for each cell over an 8.5-s interval. The rapid upstroke of the calcium transient is followed by a slow decay of the signal, giving a ladder-like appearance with sharp lower borders and fuzzy upper borders. The rapid upstrokes of the two calcium transients are precisely aligned. Minor variation in the beat-to-beat interval is also identical in the two cells. Taken together, these data show that the calcium transients are highly synchronized in the cardiomyocytes and skeletal myotubes.

To address the possibility that myotube contractions occurred in response to stretch rather than action potential propagation through gap junctions, cocultures were mechanically arrested by the presence of 2,3-butanedione monoxime throughout the experiment. 2,3-Butanedione monoxime acts as an inhibitor of actin-myosin cross-bridge cycling (Hajjar et al., 1994; Seow et al., 1997). We found that synchronous calcium cycles persisted after mechanical arrest, indicating that myotube contractions were not induced by passive stretch.

Taken together, these experiments demonstrate that skeletal myotubes and cardiomyocytes can indeed achieve electromechanical coupling via N-cadherin-mediated adherens junctions and connexin43-mediated gap junctions. Similar observations were made when adult instead of neonatal cardiomyocytes were cocultured with skeletal muscle cells, that is, synchronized contractions and the presence of N-cadherin and connexin43 protein at interfaces with skeletal myotubes.

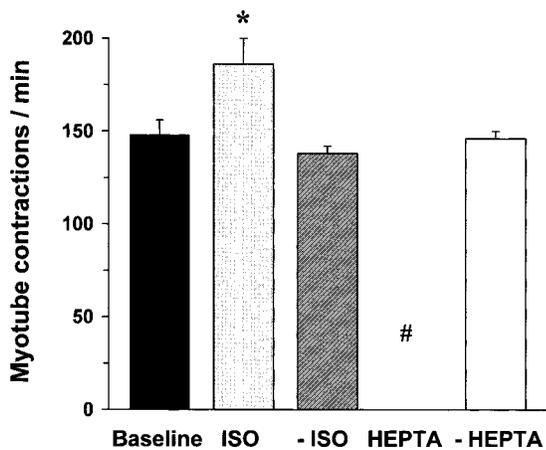


Figure 3. Contraction frequencies of myotubes cocultured with neonatal cardiomyocytes for 6 d. Isoproterenol (ISO; 25 nM) caused a significant increase of the contraction frequency of putative cardiomyocyte-paced myotubes (+25% versus Baseline). In contrast, the gap junction inhibitor heptanol (HEPTA; 0.5 mM) completely inhibited myotube contractions. Myotube contractions returned to baseline levels after wash-out (-ISO and -HEPTA). Values represent means \pm SD from 8 fields of 3 different cocultures (24 fields total) for each treatment; statistical analysis by ANOVA followed by Bonferroni *t* test; * $P < 0.05$ versus Baseline and -ISO, # $P < 0.05$ versus Baseline and -HEPTA. (Mean contraction rates varied from experiment to experiment and were often lower than the rates observed in this experiment; e.g., note in Fig. 6 that the rate is \sim 120/min.) A supplemental video further depicting Fig. 3 is available at <http://www.jcb.org/cgi/content/full/149/3/731/DC1>.

Discussion

The major findings of the current study are: (a) Skeletal myoblasts express significant amounts of N-cadherin and connexin43 but downregulate these proteins as they differentiate into myotubes; (b) mature skeletal muscle grafts in the injured heart do not express significant amounts of N-cadherin and connexin43 and, therefore, would not be expected to form detectable electromechanical junctions with host myocardium; (c) skeletal and cardiac muscle cells form N-cadherin and connexin43-mediated electromechanical junctions in vitro; and (d) cardiomyocytes can pace skeletal muscle cells via these gap junctions, resulting in synchronized heart beat-like contractions.

Comparison with Previous Studies

The finding that skeletal muscle grafts did not express N-cadherin and connexin43 agrees with our previous physiological studies (Murry et al., 1996a,b). In these experiments, we found that the grafts exhibited recruitment of contractile units with increasing voltage, indicating that the muscle fibers were not coupled through gap junctions (Murry et al., 1996a,b). Using a very different protocol, Robinson et al. (1996) injected LacZ-tagged C2C12 myoblasts into the systemic circulation of mice, and found that small numbers of cells engrafted into the myocardium, mostly as isolated cells rather than as a multicellular tissue. Electron microscopy showed apparent junctions, with some characteristics

of adherens junctions and gap junctions, between LacZ-labeled cells and cardiomyocytes. Connexin43 was detected by immunostaining at interfaces between implanted cells (identified by SERCA-1 staining) and cardiomyocytes; but unfortunately, the connexin43-positive cells were not definitively identified as graft by double staining for LacZ. Despite such ambiguities, these data clearly differ from ours. We do not think the difference lies in the cells used for grafting, because our cell culture experiments showed identical downregulation of connexin43 in both C2C12 myoblasts and primary rat myoblasts after differentiation in vitro. Similar data showing downregulation of N-cadherin and connexin43 expression in skeletal muscle cells during development have been reported by other groups as well (Knudsen et al., 1990; MacCalman et al., 1992; Balogh et al., 1993; Dahl et al., 1995). Additionally, we have grafted C2C12 myoblasts into injured rat hearts (albeit with cyclosporine immunosuppression) and could not detect either N-cadherin or connexin43 in differentiated myotubes in vivo (Reinecke, H., and C.E. Murry, unpublished results). One possibility is that isolated skeletal muscle cells without fusion partners, as observed in Robinson's study, might retain high levels of connexin43 expression similar to myoblasts, whereas multinucleated myotubes downregulate this protein. Another possibility is that scar tissue in our cardiac injury model prevented junctions from forming between graft and host tissues. We consider this explanation insufficient, because in this study we showed that skeletal muscle grafts injected into normal, uninjured myocardium also did not express junctional proteins. Thus, whereas scar tissue theoretically could insulate the two cell populations, they do not couple even in its absence.

A recent study from Taylor et al. (1998) reported improved systolic and diastolic function after autologous satellite cell implantation in cryoinjured rabbit hearts. In this study, the grafts formed multicellular tissues that were insulated from the surrounding myocardium by scar tissue. The authors reported that the graft cells appeared mononucleated by electron microscopy and were joined to one another by intercalated disk-like structures. They concluded that the skeletal muscle cells had transdifferentiated into a cardiac muscle phenotype. Other groups have suggested cardiac transdifferentiation of skeletal muscle grafts (Chiu et al., 1995; Yoon et al., 1995) based on morphological criteria. Our studies have found no such evidence for transdifferentiation. Our skeletal muscle grafts formed multinucleated myofibers with no intercalated disks, expressed myosin isoforms specific to skeletal muscle, failed to express cardiac α -myosin, did not express cardiac junctional proteins, and showed contractile properties specific to skeletal muscle (fiber recruitment by voltage, ability to induce tetanus; Murry et al., 1996a,b). Possible explanations for the discrepancies include species differences, the use of adult satellite cells versus our use of neonatal myoblasts, and the lack of definitive markers to delineate grafted skeletal muscle from host myocardial cells.

Electromechanical Coupling between Cardiac and Skeletal Muscle

Before performing these studies it was unknown whether

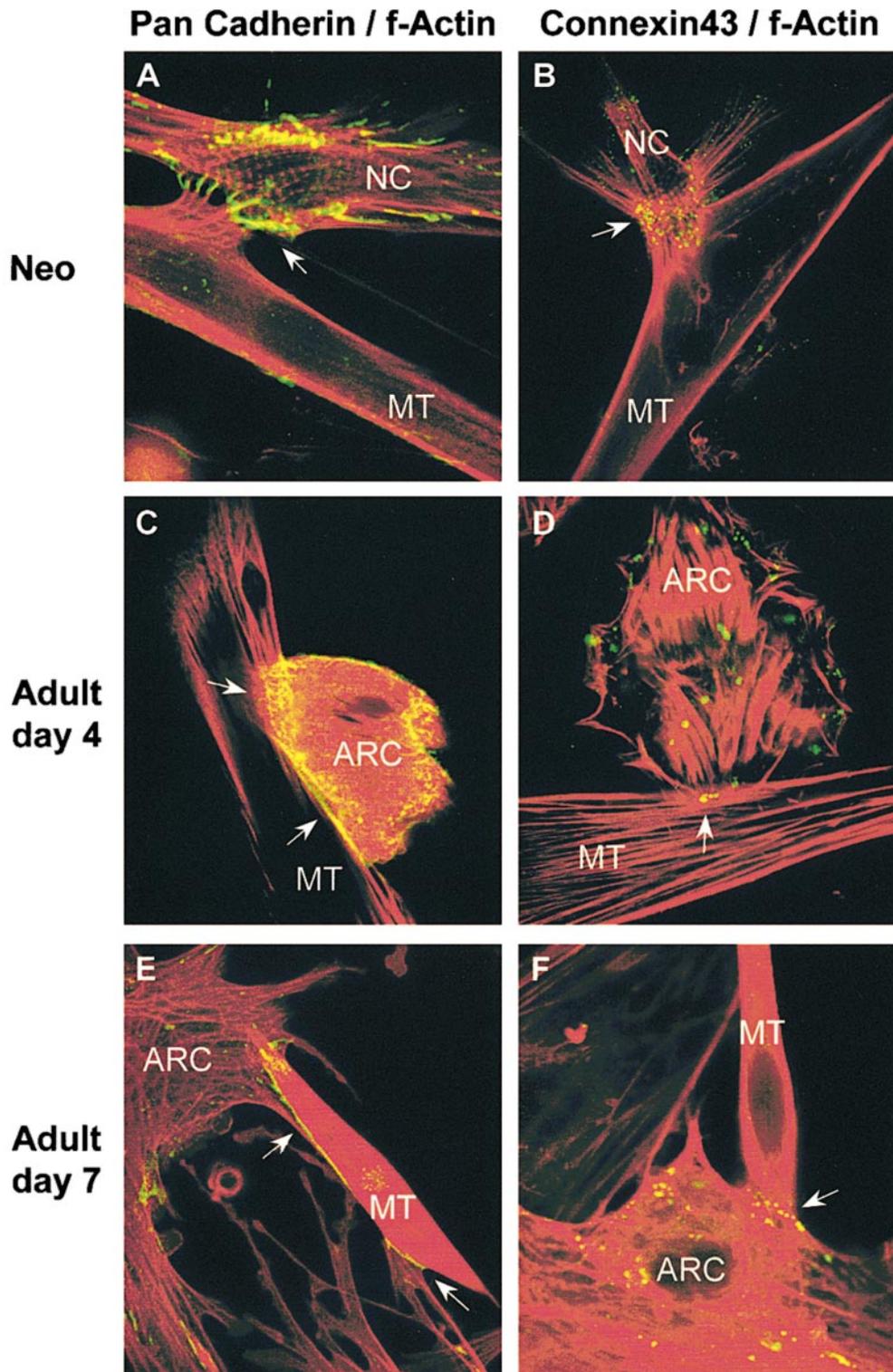


Figure 4. Confocal microscopy of neonatal cardiomyocytes (NC; upper panels, A and B) or adult rat cardiomyocytes (ARC; middle and lower panels, C-F) cocultured with skeletal myotubes (MT). Cocultures were stained for N-cadherin (A, C, and E) and connexin43 (B, D, and F) in green (FITC-conjugated secondary antibody), and counterstained for f-actin with rhodamine-phalloidin (red fluorescence). Expression of N-cadherin and connexin43 was detected at the interfaces between neonatal (A and B) or adult (C-F) cardiomyocytes and skeletal myotubes. Note that myotubes express N-cadherin and connexin43 only at the point of cardiomyocyte contact. Photographs of cocultures containing neonatal cardiomyocytes were taken at day 2 (A) and day 3 (B), and photographs of cocultures containing adult cardiomyocytes at day 4 (C and D) and day 7 (E and F) after setting up the cocultures. Day 4 was the earliest time point that N-cadherin- and connexin43-positive junctions between adult cardiomyocytes and skeletal myotubes were observed. Synchronous contractions between adult cardiomyocytes and skeletal myotubes started around day 7 when the adult cells had spread and formed a network.

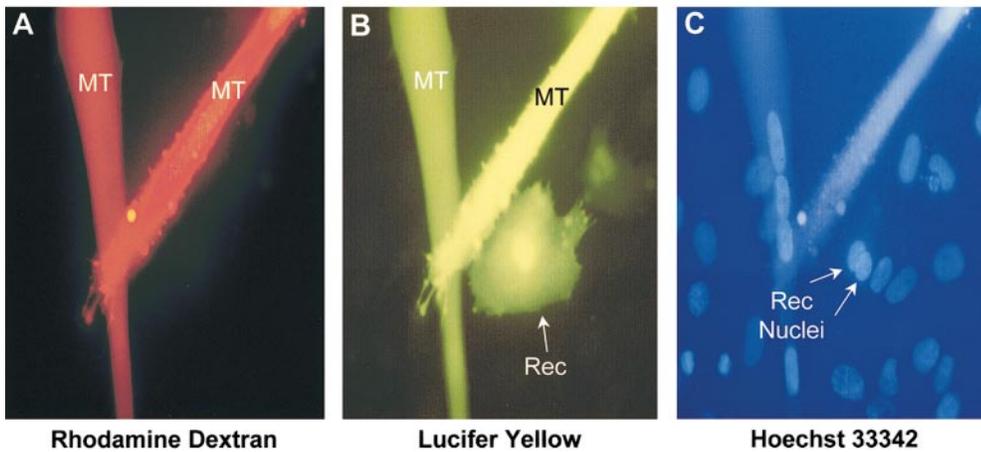
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cardiac muscle could excite skeletal muscle, even if the cells were electrically coupled via gap junctions. It seemed possible that cardiac muscle currents might be insufficient to depolarize skeletal muscle cells to threshold. The current experiments, however, clearly indicate that cardiac muscle cells can trigger depolarization in skeletal muscle. Furthermore, our evidence suggests that the cardiac mus-

cle cells pass depolarizing current directly to the skeletal myotubes via gap junctions.

The question remains why we see such a discrepancy between our *in vivo* and *in vitro* results. Electromechanical coupling requires the proper junctional molecules to be expressed at the correct anatomical location. It seems that *in vivo* there are problems both with gene expression and

Microinjection of Skeletal Myotubes



of second order dye transfer from the first recipient. (C) Nuclear staining by Hoechst 33342 showing all cells in the injection field. Even though the beating criterion was not available to identify the recipient cell unambiguously (microinjections were carried out in mechanically quiescent cultures), the binucleation (arrows) and second order dye transfer (recipient to adjacent cell) strongly suggest a cardiomyocyte as the recipient cell. It is also notable that first order dye transfer (myotube to recipient) was only observed to this cell and not to any other of the many adjacent cells (see Fig. 5 C, Hoechst nuclei staining), likely fibroblasts.

with anatomy. We believe the critical difference in gene expression lies in the extent to which skeletal muscle cells differentiate. In grafts, the skeletal muscle cells become almost indistinguishable from normal adult skeletal muscle, whereas in culture the myotubes remain relatively immature. We propose that the low levels of connexin43 and

N-cadherin that persist in the cultured myotubes are physiologically significant and permit them to make homotypic molecular junctions with cardiomyocytes. When grafted, however, these junctional molecules are further downregulated as the cells mature, leaving the graft muscle fibers electrically insulated. Our studies showed that adult and

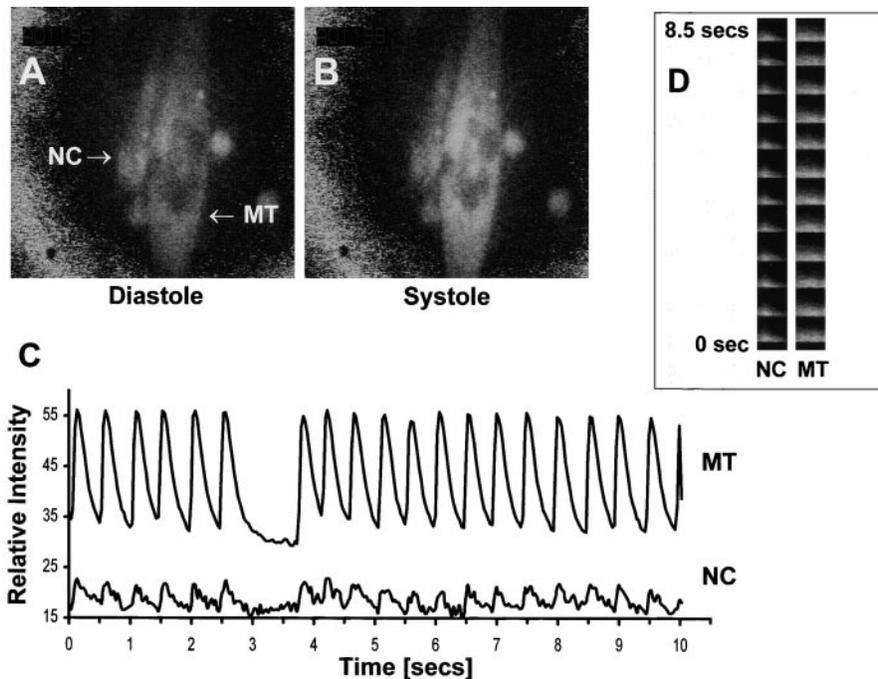


Figure 6. Calcium imaging in cardiomyocyte-skeletal myotube cocultures at day 4. (A and B) Cells were loaded with Calcium Green, and intracellular calcium release-induced fluorescence was evaluated. A shows the cardiomyocyte (NC) and the adjacent skeletal myotube (MT) in diastole (low $[Ca^{2+}]_i$), and B shows it in systole (high $[Ca^{2+}]_i$). (C) The graphs represent the relative fluorescent intensity of a single myotube (upper graph, MT) and an adjacent cardiomyocyte (lower graph, NC) over a time period of 10.2 s. The frequencies of the cardiomyocyte and the myotube were significantly synchronized (correlation coefficient $r = 0.85$). Note that even in a period of skipped beats around the 3-s time point the synchrony persisted. The lower overall intensity of the cardiomyocyte is due to its smaller size versus the skeletal myotube. D shows image analysis of calcium fluxes from an adjacent neonatal cardiomyocyte (NC) and myotube (MT) pair. A 24×24 -pixel region was sampled, and the average light signal

along one of the 24-pixel line segments is shown for each cell. Each contraction was initiated by rapid calcium release that decayed slowly, giving a sharp lower border and a fuzzy upper border to the light signal. The calcium releases are precisely synchronized in the two cell types, indicating tight coupling. A supplemental video further depicting Fig. 6 is available at <http://www.jcb.org/cgi/content/full/149/3/731/DC1>.

neonatal cardiomyocytes formed comparable junctions with skeletal myotubes. Adult cells took longer, however, because they had to dedifferentiate and spread before they formed a network. (In other words, the adult cells did not couple with the skeletal muscle until they could couple with other adult cardiomyocytes.) Previous studies have shown that cardiomyocytes next to infarcted regions remodel their intercalated discs and extend cell processes out into the infarcted region (Vracko et al., 1988; Matsushita et al., 1999a,b). Process extension in vivo appears structurally similar to cardiomyocyte spreading in vitro. Along these lines, our group recently showed that cardiomyocytes in vivo extended similar processes to form junctions with grafted neonatal cardiomyocytes (Reinecke et al., 1999). This shows that adult cardiomyocytes in vivo are capable of remodeling their junctions to couple with other cell types.

In summary, this study has presented structural and functional evidence for the formation of electromechanical junctions between skeletal and cardiac muscle cells in vitro. Furthermore, we have shown that these junctions permit the cardiac muscle cells to trigger synchronous, calcium-dependent contraction of the skeletal muscle cells. We hypothesize that these junctions do not occur after grafting in vivo due to downregulation of N-cadherin and connexin43 in skeletal myofibers. If this hypothesis is correct, it is possible that inducing persistent expression of these two junctional proteins in skeletal muscle cells and by devising strategies for limiting scar tissue barriers, may cause electromechanical coupling between skeletal and cardiac muscle cells in vivo.

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