

# How can cellular grafts be kept alive and synchronized with the rest of the heart?

**Charles E. Murry, MD, PhD; Hans Reinecke, PhD**

*Department of Pathology - University of Washington - Seattle, Wash - USA*

*Myocardial infarction and subsequent heart failure can be viewed as diseases of cellular deficiency. Cellular cardiomyoplasty is evolving as a promising therapy. Most studies have used cardiac or skeletal myocytes and each cell type has advantages as well as disadvantages, with impact on survival and integration. Cardiomyocytes are poor survivors in the injured heart, however, they are capable of forming electromechanical junctions with the host. In contrast, the more ischemia-resistant skeletal myoblasts survive much better in the injured myocardium, but their differentiated phenotype precludes formation of electromechanical junctions. Heat shocking graft cells prior to implantation significantly improves survival of both cell types. Genetic modification of the graft cells may further foster survival, and also may allow skeletal myocytes to better integrate with the host myocardium.*

**Keywords:** myocardial infarction; cell transplantation; cardiomyocyte; skeletal myoblast; cell death; cell survival; electromechanical coupling

**Address for correspondence:**

Dr Hans Reinecke, University of Washington, Department of Pathology, Box 357470, Room D-514 HSB, Seattle, WA 98195-7470, USA (e-mail: hreineck@u.washington.edu)

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**A**lthough infarct size limitation remains a highly desirable goal, it has been extremely difficult to achieve clinically. A fundamental problem is the fact that ischemic myocardium dies quite rapidly. Infarcts achieve ≈80% of their potential size within 3 hours of coronary occlusion.<sup>1</sup> Thus, myocardial infarction and subsequent heart failure are likely to remain major health problems. Cellular cardiomyoplasty has been explored as a strategy for repairing the infarcted heart. Quite naturally, most studies have used myocytes, reasoning that they would be best suited for the task of replacing the lost cardiomyocytes and restoring systolic wall motion. Here, we will focus on the use of cardiac and skeletal myocytes to achieve this ambitious goal.

## HOW CAN THE GRAFTED MYOCYTES BE KEPT ALIVE?

### Cardiomyocytes

Ideally, dead myocardium should be replaced by living cardiac tissue and, therefore, cardiac myocytes should be a first choice for cardiomyoplasty. Initial studies generated significant excitement after demonstrating that cardiomyocytes from fetal mice formed viable grafts after injection into normal myocardium.<sup>2</sup> In order to restore systolic function,

it seems likely that grafts need to replace a substantial fraction of the lost myocardium. Reasoning that more grafted cardiomyocytes would give rise to larger grafts, we performed a dose-escalation study in injured rat hearts using neonatal cardiomyocytes.<sup>3</sup> Disappointingly, all grafts were small (<2.5% of the left ventricular mass), and there was no increase in graft size with increasing cell dose. This indicated that cell death was likely limiting the amount of new myocardium formed. Poor survival of cardiomyocytes in injured hearts was seen by other investigators as well.<sup>4,5</sup> In fact, depending on the transplantation protocol, injury (cryoinjury, infarct) and animal model (mouse, rat, rabbit, dog, pig), cell survival after transplantation may range anywhere from 0% to ≈20%.

The next question becomes, “why do the cells die?” Information on mechanisms of graft cell death is currently limited, but it appears likely that ischemia plays a major role. Cell survival is better in normal hearts than acutely injured hearts, and vascularized granulation tissue supports cell survival better than acutely necrotic myocardium, although not as well as normal myocardium.<sup>3</sup> The dilemma here is that cardiomyocytes are extremely sensitive to ischemic injury, exactly the reason they die in the ischemic heart in the first place. Similarly, an old

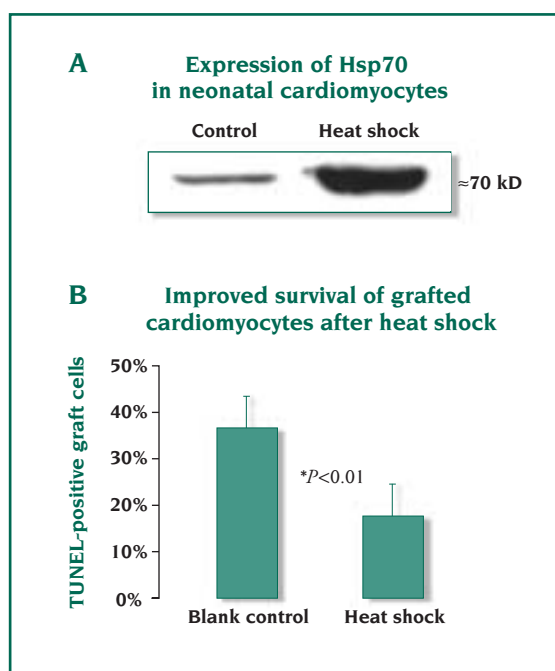
infarct scar represents a relatively ischemic tissue and, therefore, one might expect poor survival of this highly metabolic cell type after transplantation. We sought to protect the neonatal cardiomyocytes to better withstand environmental stresses after grafting into cryoinjured rat hearts. Only modest benefits in survival were noted when the cytoprotective kinase, Akt (protein kinase B), was overexpressed in the graft cells. However, significantly better results were obtained when cardiomyocytes were heat shocked the day before grafting (*Figure 1*), ie, a 54% reduction in cell death was observed.<sup>3</sup> Heat shock is thus a simple and effective approach to enhancing graft cell survival.

### Skeletal myocytes

Because of some of the limitations of cardiomyocytes, our group and others have studied skeletal muscle as a repair cell for the infarcted heart. Before describing these studies, it is worthwhile to review a few points of basic skeletal muscle biology. Mature skeletal muscle fibers originate from undifferentiated, mononucleated progenitor cells, which are termed myoblasts. When local growth factors become depleted, myoblasts withdraw irreversibly from the cell cycle, activate expression of muscle-specific genes (eg, actins, myosins, creatine kinase) and fuse to form multinucleated cells called myotubes. Myotubes undergo progressive maturation and hypertrophy to form differentiated myofibers characteristic of adult skeletal muscle. Not all myoblasts fuse into myotubes, however. Rather, some become quiescent stem cells, or satellite cells, residing in close apposition to the muscle fiber. Satellite cells can reenter the cell cycle in response to muscle injury and are responsible for the ability of skeletal muscle to regenerate. In contrast

to cardiac myocytes, skeletal muscle cells are among the most ischemia-tolerant in the body and, consequently, are capable of forming large grafts in the injured heart. When injected into acutely cryoinjured myocardium, skeletal myoblasts proliferate for up to 3 days and then differentiate to form multinucleated myotubes, eventually forming hypertrophic myofibers.<sup>6</sup>

the early stage after cell transplantation, when grafted cells are subjected to various pathological processes caused by environmental stress, such as ischemic and mechanical injury known to result in both the necrosis and apoptosis of grafted myoblasts.<sup>7,9</sup> Given that angiogenesis takes a minimum of several days to vascularize an infarct, and that cell death is most exten-



**Figure 1.** Heat shock protects grafted cardiomyocytes. Neonatal cardiomyocytes were heat shocked at 43°C for 30 min and 1 day later  $5 \times 10^6$  cells were grafted immediately after cryoinjury. (A) Western blotting showed marked upregulation of Hsp70. (B) Heat shocked cardiomyocytes showed a significant reduction in TUNEL staining 20 h after grafting into acutely necrotic myocardium.

**Abbreviations:** Hsp, heat shock protein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP “nick-end labeling” (a measure of DNA damage).

Despite their hardiness, a significant fraction of the skeletal myocytes will undergo cell death due to limited resources (eg, nutrients, oxygen) in the graft bed. Promising strategies to improve survival of grafted myoblasts include again heat shock prior to grafting<sup>7</sup> and enhancement of vascularization in the graft bed, eg, by the use of vascular endothelial growth factor (VEGF).<sup>8</sup> The latter is based on the hypothesis that the cellular cardiomyoplasty effect could be reinforced by improved graft survival resulting from an improved blood supply to the graft through both vasodilation and enhanced angiogenesis induced by VEGF. This would be particularly beneficial in

the first day after grafting, it seems that prevascularizing a graft cell bed would offer significantly more benefit than an angiogenic therapy given at the time of myoblast implantation.

### HOW CAN THE GRAFT BE SYNCHRONIZED WITH THE REST OF THE MYOCARDIUM?

A successful myocyte graft in the heart should not only fill the void left by dead myocardium, it should also be in sync with the rest of the heart. Again, the choice of the myocytes to be grafted, ie, cardiac or skeletal, has significant impact. To



understand how synchronization may be accomplished, it is first necessary to briefly review normal electromechanical coupling between cardiac myocytes. The heart acts as a functional syncytium, meaning that all the myocytes in the heart act together as an electromechanical unit. This is in contrast to skeletal muscle, where cells are truly syncytial, ie, have fused to form multinucleated fibers, but the individual fibers are insulated from one another. Electromechanical coupling in the heart is achieved by specialized cell–cell junctions, the intercalated disks, which contain adherens junctions and gap junctions for mechanical and electrical coupling, respectively. Adherens junctions are the first to form during development and are composed of N-cadherin molecules anchored in the sarcolemma,<sup>10</sup> providing binding to N-cadherin molecules in neighboring cells. Electrical coupling is achieved by gap junctions. Connexin43 is the major gap junction protein in the mammalian left ventricle.<sup>11</sup> In contrast to heart muscle cells, mature 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 require these proteins for fusion to form myotubes. As the cells differentiate further, however, N-cadherin and connexin43 expression are markedly downregulated.<sup>12-14</sup>

### Coupling of cardiomyocyte grafts

It is without doubt that cardiomyocytes fulfill all the requirements to couple electromechanically with the host myocardium. Indeed, several studies have shown development of intercalated disks, complete with gap junctions, between grafted fetal or neonatal cardiomyocytes and

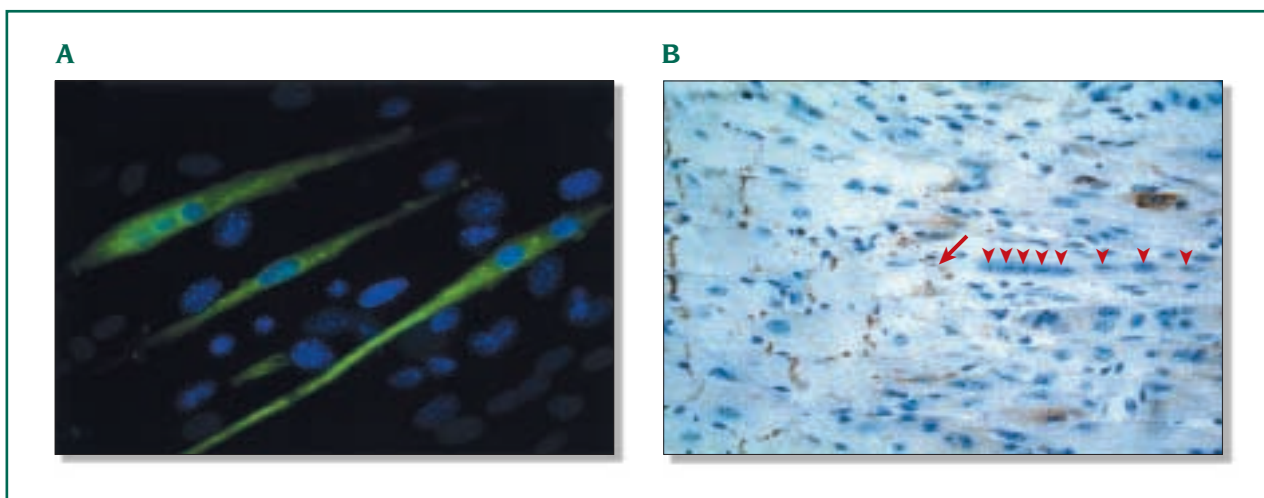
host cardiomyocytes.<sup>2,15</sup> Importantly, Rubart et al<sup>16</sup> recently demonstrated that grafted cardiomyocytes exhibit synchronous calcium transients with host cardiomyocytes in normal mouse hearts. The principal impediment to coupling of cardiomyocyte grafts with the host myocardium is formation of scar tissue. When implanted into the injured heart, grafted cardiomyocytes couple well with one another. At early time points, it is possible to identify adhesive and gap junctions between graft cardiomyocytes and host myocytes. As infarct healing proceeds, however, the grafted cardiomyocytes typically become infiltrated by scar tissue. The scar forms a physical barrier between the graft and the host cardiomyocytes, and in our studies, we were unable to demonstrate coupling of graft and host at later time points.<sup>15</sup> Thus, any cell-based therapy that aims to induce such electromechanical coupling will need to address the issue of insulation by scar tissue.

### Coupling of skeletal myocyte grafts

Although there have been reports suggesting transdifferentiation of skeletal muscle cells into cardiomyocytes,<sup>9,17</sup> previous studies from our laboratory have shown unambiguously that skeletal muscle cells in the heart appear firmly committed to their fate.<sup>6,18</sup> This is characterized by myoblast fusion and myotube formation, maturation, expression of skeletal muscle specific myosin heavy chains, and the failure to express cardiac markers such as  $\alpha$ -myosin heavy chain, cardiac troponin I, and atrial natriuretic factor.<sup>18</sup> Studies with myocardial wound strips showed that the skeletal muscle grafts would contract when exogenously stimulated.<sup>6</sup> The grafts showed the ability to undergo tetanic contraction under high-frequency

stimulation, a property not shared by myocardium because of its refractory period after depolarization. As the electrical field stimulation was increased, the skeletal muscle grafts showed increasing twitch tension, indicating recruitment of additional fibers. Fiber recruitment implied that the skeletal muscle grafts were electrically insulated from one another, unlike cardiomyocytes, which are electrically coupled by gap junctions.

These observations led us to explore the expression of the intercalated disk proteins N-cadherin and connexin43 in skeletal muscle. Immunostaining revealed that skeletal muscle grafts in the heart had undetectable levels of N-cadherin and connexin43, indicating that the grafts were not electromechanically coupled with one another or with host myocardium. These findings make it unlikely that skeletal muscle grafts in the heart are electrically excited by the host myocardium. However, when skeletal and cardiac muscle cells were placed in coculture, the cells formed a synchronously beating network.<sup>14</sup> Skeletal myotube contractions could be accelerated by the  $\beta$ -adrenergic agonist isoproterenol, suggesting the cardiomyocytes were the pacemakers. Skeletal muscle contraction was inhibited by the gap junction blocker heptanol, suggesting electrical excitation was mediated through gap junctions. Moreover, synchronous calcium transients and dye transfer were observed, indicating tight coupling between the skeletal and cardiac myocytes. Finally, confocal microscopy revealed the presence of N-cadherin-mediated adherens junctions and connexin43-mediated gap junctions between skeletal muscle cells and cardiomyocytes. These experiments indicate that cardiomyocytes have the capacity to form electromechanical junc-



**Figure 2.** (A) Differentiated C2C12 myotubes overexpress the transgene connexin43 (green fluorescence) *in vitro*. The culture was counterstained with the DNA marker Hoechst 33342 (blue fluorescence). (B) C2C12 myoblasts overexpressing connexin43 were grafted into normal nude mouse hearts and 2 weeks later tested for expression of the transgene *in vivo*. In some cases, there was very close apposition of skeletal graft cells and host cardiomyocytes. Immunostaining for connexin43 revealed presumptive gap junctions between the skeletal myotubes and host myocardium (red arrow). Arrowheads indicate multi-nucleation in the skeletal myotube.

tions with skeletal muscle cells and to use these junctions to induce synchronous beating in the skeletal muscle. Why does this coupling not occur *in vivo* after grafting? Skeletal muscle cells in culture are less differentiated than *in vivo* graft cells, and in culture the cells still have low levels of N-cadherin and connexin43. It appears that this low-level expression is sufficient to permit physiologic coupling. As the graft cells mature *in vivo*, however, N-cadherin and connexin43 appear to be downregulated to undetectable levels, thereby precluding coupling. We hypothesized that forced expression of adherens and gap junctions in differentiated skeletal myocytes should permit electromechanical coupling with cardiac myocytes. In an attempt to achieve electrical coupling, we overexpressed connexin43 in C2C12 myoblast (a mouse myoblast line). Indeed, the differentiated myotubes showed expression of the gap junction protein *in vitro* (Figure 2 A). We then grafted these cells into mouse hearts and 2 weeks later tested for expression of the transgene *in vivo*. Figure 2 B shows a section of a left ventricle

grafted with the C2C12 cells overexpressing connexin43. In some cases, we observed very close apposition of skeletal graft cells and host cardiomyocytes. Immunostaining for connexin43 revealed presumptive gap junctions between the skeletal myotubes and host myocardium (Figure 2 B; arrow). A similar approach by Suzuki's group showed that connexin43 overexpression in rat L6 myoblasts resulted in enhanced intercellular dye transfer, although this group did not study the cells after differentiation into myotubes, when gap junctions are normally downregulated.<sup>19</sup> Although encouraging, we also have observed reductions in the viability of skeletal muscle grafts that express connexin43 (unpublished observations). It is therefore possible that differentiated skeletal muscle has not evolved mechanisms to accommodate gap junctions without cell injury. The greatest concern for attempts to couple skeletal and cardiac muscle is possible arrhythmogenesis. Skeletal muscle cells have much faster action potentials and fiber conduction velocities than cardiac myocytes. Hence, it is possible that suc-

cessful coupling of the two muscle types would result in an arrhythmogenic substrate. Nevertheless, the possible gain from coupling the two muscle types justifies additional, careful animal experimentation. Finally, it is clear that separation of muscle grafts from host myocardium by scar tissue is just as problematic with skeletal muscle as it is with cardiac muscle grafts, and would need to be solved before proper coupling could be achieved.

## SUMMARY

New muscle tissue can be generated by grafting either cardiac or skeletal myocytes into the injured heart. However, the amount of new muscle tissue is limited by ischemic cell death. Heat shocking the cells prior to grafting protects against ischemic cell death and thus benefits graft cell survival. Moreover, heat shock is simple and feasible in the clinical situation. Angiogenic therapy along with or prior to myocyte grafting has not been explored thoroughly, however, long-term survival of myocyte grafts may benefit from enhanced angiogenesis.



Synchronization between graft and host remains a major challenge for cellular cardiomyoplasty. Both cell types, cardiac and skeletal myocytes, are likely to be separated from the host by scar, thus precluding coupling. Principally, synchronization is likely to occur if grafted cardiomyocytes can be brought into close contact with the host myocardium. In contrast, differentiated skeletal muscle cells do not express the proper molecules to couple to myocardium in vivo. Thus, it appears unlikely that coupling will occur using naïve skeletal muscle cells. Expression of transgenes mediating electromechanical coupling in these cells may prove feasible in the laboratory, but this approach is potentially arrhythmogenic and is certainly quite far from a clinical application.

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