

## Evidence for Fusion Between Cardiac and Skeletal Muscle Cells

Hans Reinecke, Elina Minami, Veronica Poppa, Charles E. Murry

**Abstract**—Cardiomyoplasty with skeletal myoblasts may benefit cardiac function after infarction. Recent reports indicate that adult stem cells can fuse with other cell types. Because myoblasts are “fusogenic” cells by nature, we hypothesized they might be particularly likely to fuse with cardiomyocytes. To test this, neonatal rat cardiomyocytes labeled with LacZ and green fluorescent protein (GFP) were cocultured with unlabeled C2C12 myoblasts. After 3 days, we observed a small population of skeletal myotubes that expressed LacZ and GFP, indicating cell fusion. To test whether such fusion occurred *in vivo*, LacZ-expressing C2C12 myoblasts were grafted into normal nude mouse hearts. At 2 weeks after grafting, cells at the graft-host interface expressed both LacZ and cardiac-specific myosin light chain 2v (MLC2v). To test more definitively whether fusion between skeletal and cardiac muscle could occur, we used a Cre/lox reporter system that activated LacZ only upon cell fusion. When neonatal cardiomyocytes from  $\alpha$ -myosin heavy chain promoter ( $\alpha$ -MHC)-Cre mice were cocultured with myoblasts from floxed-lacZ reporter mice, LacZ was activated in a subset of cells, indicating cell fusion occurred *in vitro*. Finally, we grafted the floxed-lacZ myoblasts into normal hearts of  $\alpha$ -MHC-Cre<sup>+</sup> and  $\alpha$ -MHC-Cre<sup>-</sup> mice (n=5 each). Hearts analyzed at 4 days and 1 week after transplantation demonstrated activation of LacZ when the skeletal muscle cells were implanted into hearts of  $\alpha$ -MHC-Cre<sup>+</sup> mice, but not after implantation into  $\alpha$ -MHC-Cre<sup>-</sup> mice. These data indicate that skeletal muscle cell grafting gives rise to a subpopulation of skeletal-cardiac hybrid cells with a currently unknown phenotype. The full text of this article is available online at <http://circres.ahajournals.org>. (*Circ Res.* 2004;94:e56-e60.)

**Key Words:** cardiomyoplasty ■ skeletal myoblast ■ cell transplantation ■ cell fusion ■ Cre/lox

Cellular cardiomyoplasty has emerged as a promising therapy for myocardial infarct repair. Skeletal myoblasts and bone marrow-derived stem cells have been tried in various animal models and are now undergoing clinical trials.<sup>1-4</sup> A recent study has shown that circulating cells derived from transplanted bone marrow can fuse with cardiomyocytes, Purkinje cells and hepatocytes.<sup>5</sup> Because skeletal myoblasts are “fusogenic” cells by nature, we tested if myoblasts can fuse with cardiomyocytes. Our study shows that skeletal myoblast grafting into the heart results in fusion with host cardiomyocytes, giving rise to a subpopulation of hybrid cells.

### Materials and Methods

#### Cell Culture

Mouse neonatal cardiomyocytes and primary neonatal skeletal myoblasts were isolated as described previously.<sup>6,7</sup> Spontaneous cell fusion (coculture) experiments were set up in gelatin-coated 6-well plates at a total density of  $2 \times 10^4$  cells/cm<sup>2</sup> (1:1 ratio) in DMEM/M199 (4:1) supplemented with 10% horse serum, 5% FBS, and 6  $\mu$ g/mL insulin. These conditions were optimized for cardiomyocytes while allowing terminal differentiation of skeletal myoblasts into multinucleated myotubes.

Forced fusion was induced by polyethylene glycol (Hybrimax-PEG, 50% solution; Sigma). Neonatal cardiomyocytes were infected with the Cre recombinase adenovirus (AdCre; Microbix Biosystems) and C2C12 skeletal myoblast were infected with Adfloxed-lacZ adenovirus (Microbix Biosystems) at 300 virus particles per cell. After infection, cells were extensively washed, trypsinized, and cocultured overnight at ratios of 10:1 (cardiomyocytes:C2C12 myoblasts) and 1:10, respectively. Cell fusion was induced by addition of PEG (50% solution for 90 seconds). PEG was rinsed off and regular medium was added. After 3 days, cultures were fixed with 2% paraformaldehyde and subjected to X-GAL assays.

#### Animal Procedures

The study protocol was approved by the Institutional Animal Care and Use Committee. Alpha-MHC-Cre/FVB mice (kindly provided by Dr M.D. Schneider, Baylor College of Medicine, Houston, Tex) express Cre recombinase only in cardiomyocytes.<sup>8</sup> To achieve graft-host tolerance,  $\alpha$ -MHC-Cre/FVB mice were crossed with C57Bl/6 wild-type mice, and F1 animals were used as graft recipients. Accordingly, Cre-reporter R26R/C57Bl/6 were crossed with wild-type FVB mice, and F1 animals were used to isolate neonatal skeletal myoblasts as described.<sup>7</sup> The R26R mouse carries a lacZ allele that is separated from its constitutively active ROSA26 promoter by a loxP-flanked neomycin cassette (floxed-lacZ), thus lacZ is expressed only upon Cre-mediated excision of the neomycin cassette.<sup>9</sup> Nude mice were used for grafting of C2C12 myoblasts tagged with a lacZ retrovirus (n=4).  $\alpha$ -MHC-Cre<sup>+</sup>, or  $\alpha$ -MHC-Cre<sup>-</sup>

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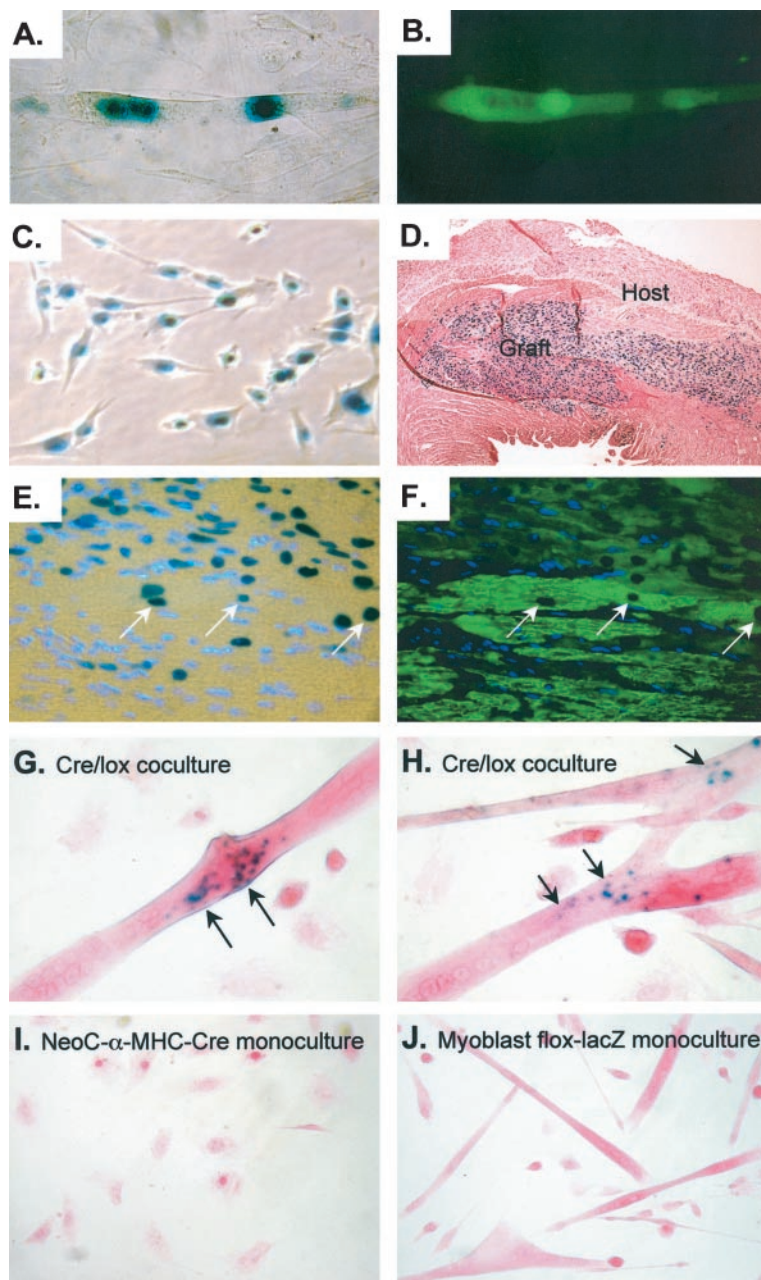
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**Figure 1.** A and B, Coculture of neonatal rat cardiomyocytes and mouse C2C12 skeletal myoblasts. Double-labeled cardiomyocytes (adenoviral nuclear-targeted LacZ<sup>+</sup>, GFP<sup>+</sup>) were cocultured with nonlabeled C2C12 myoblasts. After 3 days in coculture, multinucleated myotubes with LacZ-positive (blue) nuclei and green fluorescent cytoplasm were observed, suggesting cell fusion. Note the presence of a LacZ (blue) gradient in the nuclei, suggesting second-order uptake. C through F, Grafting of nuclear LacZ-tagged C2C12 myoblasts (C; X-GAL assay in vitro) into normal nude mouse hearts (D; X-GAL assay with contrast red). At 2 weeks, we observed cells that were positive for LacZ and the cardiac marker myosin light chain 2v (MLC2v, green fluorescence) (E and F; arrows; note the relatively high background in F results from merging brightfield and fluorescence images). These cells were located at the graft-host interface. G through J, Cre/lox recombination in vitro. Neonatal cardiomyocytes from  $\alpha$ -MHC-Cre mice were cocultured for 3 days with neonatal skeletal myoblasts from floxed-lacZ mice. Activation of LacZ expression (G and H, punctate blue staining, arrows) was observed in cocultures but not in monocultures of the respective cell type (I and J). X-GAL assay with contrast red. Note that in this model Cre-mediated LacZ expression is not nuclear-targeted and punctate LacZ staining is not associated with cell nuclei.

(control) mice were used as recipients of floxed-lacZ myoblasts (n=8 each). In a separate set of experiments, C2C12 myoblasts were infected with an adenovirus carrying a floxed-lacZ gene (Adfloxed-lacZ; 300 particles/cell) and grafted into  $\alpha$ -MHC-Cre<sup>+</sup> and  $\alpha$ -MHC-Cre<sup>-</sup> (control) mice (n=6 and n=4, respectively). Normal hearts were injected with a standard dose of  $1 \times 10^6$  myoblasts as previously described.<sup>10</sup> Mice were euthanized at 4 days and 1 week ( $\alpha$ -MHC-Cre mice) or 2 weeks (nude mice). Hearts from  $\alpha$ -MHC-Cre mice injected with Adfloxed-lacZ-infected C2C12 myoblasts were harvested at 4 days. At this early time point, no immune response to the adenovirus-infected allograft was noted. At the defined time points, hearts were excised and routinely processed for cryosections.

### Adenovirus Infections

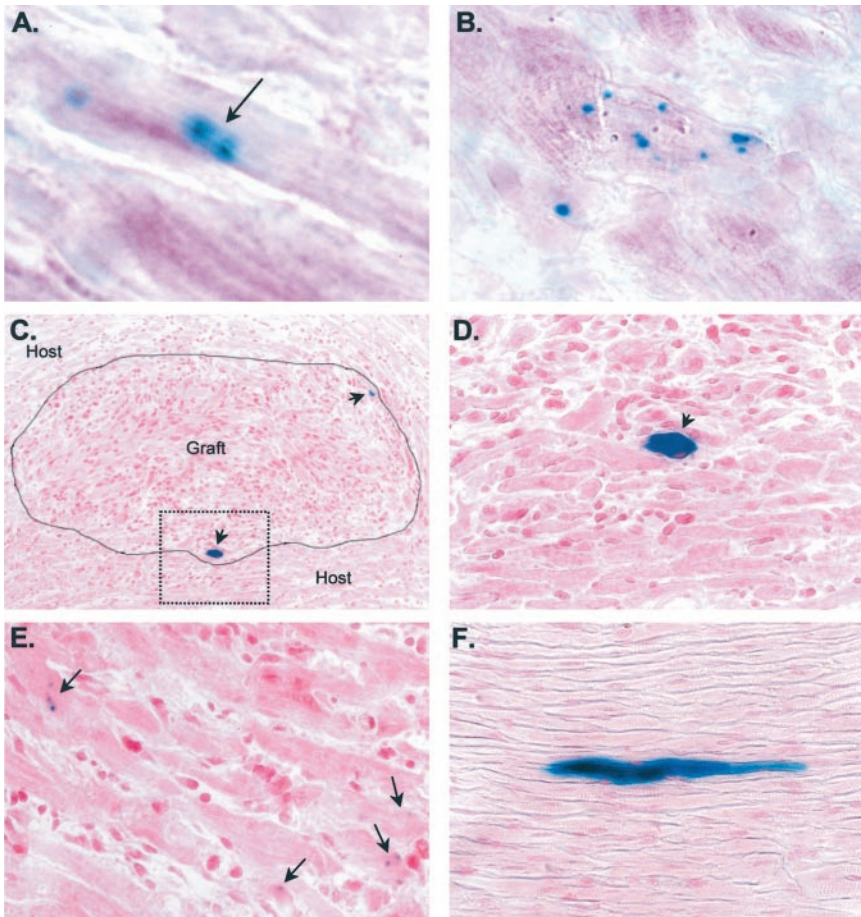
Neonatal rat cardiomyocytes were infected at a total of 300 virus particles per cell with adenoviruses encoding nuclear targeted LacZ<sup>11</sup> and cytoplasmic GFP,<sup>12</sup> respectively. Then, cultures were washed extensively and fresh medium was added. Cocultures were set up the following day. The integrity of the reporter cells (floxed-lacZ

myoblasts) was confirmed by infection with a Cre recombinase adenovirus (AdCre) and subsequent 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-GAL) staining. When C2C12 myoblasts infected with the Adfloxed-lacZ adenovirus were used for grafting experiments, cells were infected the day before grafting (300 virus particles per cell). On the day of grafting, cultures were washed extensively before cell transplantation. An aliquot of the cells was used to confirm the infection efficiency and integrity of the reporter by infection with the Cre recombinase adenovirus.

## Results

### Cell Fusion Detected by Virus-Mediated Cell Tagging

Neonatal rat cardiomyocytes tagged with GFP and nuclear LacZ were cocultured with unlabeled C2C12 myoblasts. After 3 days, we observed multinucleated myotubes that carried both the GFP and the nuclear LacZ tag (Figures 1A



**Figure 2.** A and B, Cre/lox recombination in vivo. Skeletal myoblasts from floxed-lacZ mice were grafted into  $\alpha$ -MHC-Cre<sup>+</sup> hearts.  $\alpha$ -MHC-Cre<sup>-</sup> hearts served as controls. Activation of LacZ expression (blue punctate staining) was observed in  $\alpha$ -MHC-Cre<sup>+</sup> but not in  $\alpha$ -MHC-Cre<sup>-</sup> hearts (not shown). X-GAL assay with contrast red. C through F, Mouse C2C12 myoblasts were infected with Adfloxed-lacZ and grafted into normal hearts of  $\alpha$ -MHC-Cre<sup>+</sup> or into  $\alpha$ -MHC-Cre<sup>-</sup> mice (control, not shown). Approximate outline of the graft area showing 2 cells (arrows) with abundant cytoplasmic LacZ expression. The boxed area is shown in D at higher magnification. Some of the hybrid cells exhibited abundant cytoplasmic LacZ expression (D and F). More typically, however, we observed a punctate LacZ staining pattern (E). F, Fusion-derived cell with abundant LacZ expression that was observed outside of the main graft area. Images were derived from 2 hearts of  $\alpha$ -MHC-Cre<sup>+</sup> mice. No activation of LacZ expression was observed after grafting of  $\alpha$ -MHC-Cre<sup>-</sup> mice. X-GAL assay with contrast red.

and 1B), suggesting fusion of double-labeled cardiomyocytes with unlabeled C2C12 myoblasts. Because replication-deficient adenoviruses were used for labeling the cardiomyocytes and cells were washed extensively before coculture, it is unlikely that free virus was carried over into cocultures. To test whether fusion between skeletal and cardiac muscle cells could occur in vivo, we generated a C2C12 myoblast clone retrovirally tagged with nuclear-targeted LacZ, and grafted these cells into normal hearts of nude mice (Figures 1C and 1D). At 2 weeks after grafting, we performed X-GAL assays in conjunction with immunostaining for the cardiac-specific myosin light chain 2v (MLC2v). At the border zone between host and graft, we detected a small number of cells that expressed the nuclear LacZ tag and were positive for MLC2v (Figures 1E and 1F), suggesting fusion between myoblasts and cardiomyocytes had occurred in vivo.

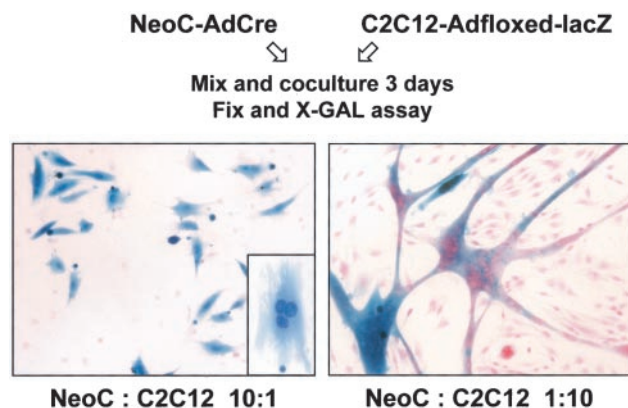
#### Cell Fusion Detected by Cre/lox Recombination

To test more definitively whether fusion had occurred, we isolated neonatal cardiomyocytes from mice where expression of Cre recombinase was driven by the cardiac-specific  $\alpha$ -MHC promoter.<sup>8</sup> These cardiomyocytes were cocultured with myoblasts from floxed-LacZ reporter mice where the LacZ expression is normally blocked by the presence of a Cre-excisable stuffer sequence.<sup>9</sup> After 3 days in coculture, X-GAL assays revealed the presence of LacZ-positive multinucleated myotubes (Figures 1G and 1H), strongly

indicating fusion between the two cell types. Monocultures of the respective cell types did not yield any LacZ-positive cells (Figures 1I and 1J). We then used Cre/lox recombination for cell grafting experiments. Floxed-lacZ skeletal myoblasts were grafted into the uninjured hearts of  $\alpha$ -MHC-Cre<sup>+</sup> or into  $\alpha$ -MHC-Cre<sup>-</sup> mice (control). At 4 days and 1 week after cell implantation, LacZ expression was observed in cells at the graft-host interface (Figures 2A and 2B). In contrast, LacZ expression was not detected in grafted hearts of  $\alpha$ -MHC-Cre<sup>-</sup> mice (data not shown).

As a final test for fusion, C2C12 myoblasts were infected with an adenovirus carrying a floxed-lacZ gene and grafted into normal hearts of  $\alpha$ -MHC-Cre<sup>+</sup> or into  $\alpha$ -MHC-Cre<sup>-</sup> mice (control). Similar to the results with primary floxed-lacZ myoblasts, we observed LacZ expression primarily in cells at or close to the graft-host interface (Figures 2C through 2F). In these experiments, some cells displayed punctate LacZ staining (Figure 2E), whereas others showed abundant LacZ staining (Figures 2C, 2D, and 2F). Again, no LacZ expression was detected in grafted hearts of  $\alpha$ -MHC-Cre<sup>-</sup> mice (data not shown).

A final experiment focused on how the stoichiometry of the fusion partners affected morphology of the hybrid cells. We hypothesized that the morphology of fused cells may be dependent on the number of nuclei and/or cytoplasm contributed by each cell type. Since spontaneous cell fusion was infrequent we explored forced fusion by means of polyethyl-



**Figure 3.** Forced fusion induced by polyethylene glycol (PEG). Neonatal cardiomyocytes were infected with the Cre recombinase adenovirus (AdCre) and C2C12 skeletal myoblast were infected with Adfloxed-lacZ adenovirus. After infection, cells were extensively washed, trypsinized, and cocultured overnight at ratios of 10:1 and 1:10, respectively. Then, cell fusion was induced by addition of PEG. After 3 days, fused cells were detected by X-GAL assay. Ad-Cre-infected neonatal cardiomyocytes were mixed with Adfloxed-lacZ-infected C2C12 skeletal at a ratio of 10 cardiomyocytes to 1 myoblast. PEG-forced fusion resulted in LacZ-positive cells that were mono-, bi-, and trinucleated (left and inset). In contrast, when the ratio was reversed (1 cardiomyocyte:10 C2C12 myoblasts), LacZ-positive fusion cells appeared as typical skeletal myotubes showing multinucleation and branching (right).

ene glycol (PEG) as a way to study fusion cell morphology. When Ad-Cre-infected neonatal cardiomyocytes were mixed with Adfloxed-lacZ-infected C2C12 skeletal myoblasts at a ratio of 10:1, PEG-forced fusion resulted in LacZ-positive cells that were mono-, bi-, and trinucleated (Figure 3). In contrast, when the ratio was reversed (1 cardiomyocyte:10 C2C12 myoblasts) LacZ-positive fusion cells appeared as typical skeletal myotubes with multinucleation and branching (Figure 3).

### Discussion

The major finding of the present study is that skeletal myoblasts can fuse with cardiomyocytes *in vitro* and *in vivo*. Cell fusion was detected by using virus-mediated cell labeling (GFP, LacZ) and by Cre/lox recombination. Both coculture models and *in vivo* cardiac grafting experiments yielded a similarly small (less than 0.01% of seeded or grafted cells) but readily detectable population of hybrid cells. Interestingly, *in vitro* the fused cells morphologically resembled skeletal myotubes, whereas *in vivo* they resembled cardiomyocytes. Forced fusion experiments with varying ratios between cardiomyocytes and skeletal myoblasts suggested that the morphology of fused cells was dependent on the stoichiometry of nuclei and/or cytoplasm contributed by each cell type.

It should be mentioned that our Cre/lox experiments were not designed to detect a cardiomyogenic transdifferentiation event (eg, from a multipotent progenitor cell) independent of fusion. The studies involving C2C12 myoblasts would not be expected to contain progenitor cells, because they are an immortalized, clonal cell line. Our primary myoblast cultures

clearly had a heterogeneous mix (although were >85% desmin-positive) and could have contained progenitor cells.

In recent years, it has become apparent that cells of different origin may fuse with one another, thus adopting properties of the fusion partner. Although first described *in vitro*,<sup>13,14</sup> fusion subsequently was shown to explain the *in vivo* transdifferentiation of hematopoietic stem cells into hepatocytes.<sup>15,16</sup> The question arises whether cell fusion can explain previous reports of transdifferentiation of various cell types into cardiomyocytes. Our observations are consistent with the very low levels of transdifferentiation reported by Robinson et al<sup>17</sup> (intraarterial delivery of myoblasts), Jackson et al<sup>18</sup> (hematopoietic stem cell transplantation after lethal irradiation), Malouf et al<sup>19</sup> (intramyocardial injection of liver-derived progenitor cells), and Laflamme et al<sup>20</sup> (“transgender” cardiac transplantation in humans). They are inconsistent, however, with high level transdifferentiation reported in other studies.<sup>21–24</sup> When the hybrid cells expressed cytoplasmic LacZ from the ROSA26 promoter, we typically observed a punctate LacZ-staining pattern *in vitro* (Figures 1G and 1H) and *in vivo* (Figures 2A and 2B). A similar, punctate staining pattern was observed in rare cardiomyocytes ( $\approx 0.02\%$ ) in the peri-infarct region by Jackson et al,<sup>18</sup> after performing bone marrow transplantation with highly enriched ROSA26-derived (LacZ<sup>+</sup>) hematopoietic stem cells. Although the authors proposed that the LacZ<sup>+</sup> cardiomyocytes arose via transdifferentiation, one may hypothesize that these cells in fact resulted from cell fusion. Indeed, while our work was in the final phases of preparation, two other studies showed fusion of adult cells with cardiomyocytes. By using Cre/lox recombination, Alvarez-Dolado et al<sup>5</sup> showed that bone marrow-derived cells could fuse with cardiomyocytes in the uninjured heart, whereas Oh et al<sup>25</sup> showed that intravenously infused progenitor cells from the adult heart could fuse with cardiomyocytes in the setting of myocardial infarction. Our study extends this work by adding skeletal myoblasts as a clinically relevant cell type that can fuse with cardiac myocytes *in vitro* and *in vivo*. Although a rare event, the appearance of cardiac-skeletal hybrid cells with unknown (electrical) properties at the graft-host interface may impact autologous skeletal myoblast grafting for cardiac repair, which is already undergoing clinical trials.<sup>1–3</sup>

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