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Skeletal Muscle Stem Cells Do Not Transdifferentiate Into Cardiomyocytes After Cardiac Grafting

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H. Reinecke, V. Poppa and C. E. Murry. Skeletal Muscle Stem Cells Do Not Transdifferentiate Into Cardiomyocytes After Cardiac Grafting. *Journal of Molecular and Cellular Cardiology* (2002) **34**, 241–249. Skeletal muscle cell-derived grafts in the heart may benefit myocardial performance after infarction. Several studies have suggested that skeletal muscle stem cells (satellite cells) from adult muscle undergo transdifferentiation into cardiomyocytes after grafting into the heart, but expression of cardiac markers in graft cells has not been rigorously confirmed. To determine the fate of satellite cell-derived grafts in the heart, adult rat satellite cells were tagged *in vitro* with bromodeoxyuridine (BrdU) and grafted into normal hearts of syngeneic rats. At 4 and 12 weeks the graft cells formed multinucleated, cross-striated myofibers that expressed fast skeletal myosin heavy chain (MHC), thus indicating a mature skeletal muscle phenotype. Double staining for the BrdU tag and cardiac-specific markers was employed to identify transdifferentiation. Aside from four questionable cells, none of the 11 grafts examined expressed α-MHC, cardiac troponin I, or atrial natriuretic peptide. At 4 weeks, grafts expressed β-MHC, a hallmark of slow twitch myofibers. By 12 weeks, however, the myofibers had atrophied and downregulated β-MHC. Grafts never expressed the intercalated disk proteins N-cadherin or connexin43, hence electromechanical coupling did not occur. In conclusion, satellite cells differentiate into mature skeletal muscle and do not express cardiac-specific genes after grafting into the heart. Thus, transdifferentiation into cardiomyocytes did not occur.

Key Words: Rat satellite cells; Cell transplantation; Transdifferentiation; Myosin heavy chain; Cadherin; Connexin43.

Introduction

Since the heart lacks functional repair mechanisms, we and others are exploring skeletal muscle cells for cardiac repair.[1–6] Our group showed that primary neonatal skeletal myoblasts formed grafts of differentiated skeletal muscle in normal and injured hearts.[1] The grafts contracted when exogenously stimulated, and showed physiological properties of skeletal rather than cardiac muscle. In contrast, several other investigators have reported that satellite cells (skeletal muscle stem cells) from adult skeletal muscle transdifferentiated into cardiac muscle after grafting into normal or injured hearts.[5–10] Furthermore, there is growing evidence for plasticity of adult stem cells, with reports of skeletal muscle-derived cells capable of repopulating bone marrow[11] and marrow-derived cells repopulating skeletal muscle[12] or myocardium.[13,14] While provocative, the studies purporting skeletal-to-cardiac muscle conversion have not rigorously shown expression of cardiac markers in cells proven to originate from skeletal muscle, a prerequisite to prove transdifferentiation. The current study was undertaken to test whether adult skeletal muscle satellite cells exhibited the anatomical and molecular phenotype of cardiomyocytes after grafting into the heart.

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Methods

Satellite cell isolation

Satellite cells were isolated from the hind limbs of adult male Fischer rats weighing 350–400 g.[15] Tibialis anterior (TA), extensor digitorum longus (EDL), soleus, gastrocnemius, quadriceps and hamstrings were excised and placed into a tissue culture dish containing 10 ml DMEM plus antibiotics [penicillin G (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), and gentamicin (50 μg/ml) (GIBCO, Grand Island, NY, USA)]. Tendons, all bone, and fat were carefully discarded, and the muscle tissue was throroughly minced and then digested at 37°C with 0.1% pronase (Calbiochem, La Jolla, CA, USA) for 1 h. The tissue was triturated vigorously and passed through a 10-μm filter (Millipore, Bedford, MA, USA) and cells were collected by centrifugation. The cells were then plated on gelatin-coated plates in DMEM containing 20% fetal bovine serum (HyClone, Logan, UT, USA), 15% horse serum (ICN Flow, Costa Mesa, CA, USA) and 6 ng/ml recombinant human bFGF (a gift from Scios Inc., Mountain View, CA, USA), plus antibiotics. Basic FGF was added every 24 h for 3 days without media change. Thereafter, bFGF was added every 12 h and the media was changed every 24 h. Cells were not passaged prior to transplantation and were used for grafting after 5 days of culture. The primary satellite cell isolates contained $\sim$70% desmin-positive myoblasts; the other cells were presumably fibroblasts. Myogenic differentiation was induced by switching the cells to DMEM supplemented with 7.5% horse serum, 6 μg/ml insulin (Sigma, St. Louis, MO, USA) and no bFGF. To identify the grafts the cells were tagged with BrdU (10 μM) overnight prior to grafting.

In vitro characterization of satellite cells

Samples of each satellite cell isolation were processed for immunocytochemistry and reverse transcriptase polymerase chain reaction (RT-PCR). For immunocytochemistry, undifferentiated cell cultures were fixed on day 3 with ice-cold methanol for 10 min. To analyze differentiation cells were grown for 5 days to reach $\sim$80% confluency, switched to differentiation medium for 2 days and then fixed with methanol. By differentiation day 2 spontaneously contracting myotubes had formed. For RT-PCR total RNA was isolated from undifferentiated satellite cells at day 5,[16] and 200 ng total RNA were reverse transcribed in a 25 μl reaction using SuperScript (Gibco) and random hexamer oligonucleotides (Promega, Madison, WI, USA). Two μl of the RT reactions were amplified with specific primer sets by PCR (see Table 1). Total RNA isolated from the mouse skeletal myoblast cell line MM14[17] (generously provided by Dr Stephen D. Hauschka, University of Washington) and from neonatal rat cardiomyocytes served as references.

Animal model

Inbred male Fischer rats (Simonsen Labs, Gilroy, CA, USA) weighing 350–400 g were anesthetized with intraperitoneal ketamine-xylazine (68 and 4.4 mg/kg, respectively), intubated, and mechanically ventilated with room air. The heart was exposed aseptically via left thoracotomy as described and $3.5–7 \times 10^6$ satellite cells were injected into the uninjured left ventricular wall as described previously.[1] Rats were sacrificed after 4 and 12 weeks ($n=4$ and $n=7$, respectively), the hearts perfusion fixed with methyl Carnoy's solution (60% methanol, 30% chloroform, 10% glacial acetic acid), transversely sectioned, and embedded in paraffin.

Antibodies and immunocytochemistry

Immunostaining was performed using immunoperoxidase methods as described.[1,18,19] For BrdU staining slides were pretreated with pepsin (0.1 mg/ml) for antigen retrieval and the DNA denatured in 0.1 N HCl to expose the BrdU epitope for 30 min at 37°C. After a brief wash in dH_2O slides were treated with 1.5 N HCl for 15 min at 37°C. After a brief rinse in dH_2O, slides were incubated in 0.1 M boric acid for 10 min at RT. Then antibody staining was performed as described.[1,18,19] The following antibodies were used for immunostaining: anti-human desmin[20] (D33, 1:5; DAKO, CA, USA), anti-MyoD[21] (5.8A, 1:100; generous gift from Drs Peter Houghton and Peter Dias, St. Jude Children's Hospital, Memphis, TN, USA), anti-pan cadherin[22] (rabbit antiserum, 1:2000; Sigma), anti-connexin43[23] (mouse monoclonal antibody MAB3068, 1:200; Chemicon, Temecula, CA, USA), anti-cardiac α-myosin heavy chain[24] (BA-G5, mouse monoclonal antibody, hybridoma supernatant, 1:50; ATCC, Rockville, MA, USA), anti-sarcomeric MHCs[25] (MF-20, mouse monoclonal antibody, hybridoma supernatant, 1:10; ATCC), slow β-MHC[26] (A 4.951,

Table 1 Primer sets used for PCR

Template	Primer (5′-3′): Forward, Reverse	PCR product size	Annealing temperature
Cardiac troponin I	AAAGTGGATGAAGAGAGATA ATTCTTGCGCCAGTCTCCCA	266 bp	54°C
c-met	TCACATTGACCTCAGCGCTCT CTAAAGCCATCCACTTCACCGGTA	605 bp	65°C
Connexin43	GACTTCAGCCTCCAAGGAGTTCCACC AGTTGGAGATGGTGCTTCCGGCC	1064 bp	60°C
Desmin	TGATGAGGCAGATGAGGGAG TGAGAGCTGAGAAGGTCTGG	246 bp	60°C
Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)	CCTCTGGAAAGCTGTGGCGT TTGGAGGCCATGTAGGCCAT	430 bp	65°C
MRF4	TCGTCGGAAAGCAGCTACCCT CTGGGGAGTTTGCGTTCCTCT	426 bp	64°C
Myf5 (based on mouse sequence[15])	GAGCCAAGAGTAGCAGCCTTCG GTTCTTTCGGGACCAGACAGGG	440 bp	65°C
MyoD	CACACTTCCCCACTACGGTGC CACTGTAGTAGGCGGCGTCGTAG	464 bp	62°C
Myogenin	CCATCCAGTACATTGAGCGCCTA GGGGCTCTCTGGACTCCATCTT	551 bp	65°C
N-cadherin	ATGATCCAAATGCCCTGAAT GACTGAGGTGGGTGCTGAAT	565 bp	55°C

mouse monoclonal antibody, hybridoma supernatant, 1:50; ATCC), anti-fast skeletal MHC[27] (types IIA and IIB, MY-32, mouse ascites, 1:2000, Sigma), anti-bromodeoxyuridine[28] (BU1/75, rat monoclonal antibody, 1:5; Harlan Sera-Lab, England).

Measurement of myofiber diameter

The diameter of satellite cell-derived myofibers was measured in longitudinal sections containing nuclei, using a calibrated microscope scale. Measurements were performed on sections stained for fast skeletal myosin heavy chain from three animals in each group (30 myofibers measured per animal; total $n=90$/group). Diameters were expressed as mean $\pm$ S.D. Fiber diameters were compared using Student's *t*-test, and a $P<0.05$ was considered statistically significant.

Results

Characterization of satellite cells *in vitro*

Figure 1 (A–F) describes the phenotype of satellite cells *in vitro*. At day 1–2 after isolation the cells were round, refractile and slowly starting to adhere to the gelatin substratum [Fig. 1(A)]. Spindle-shaped cells increased after 2 days. By 6 days most cells were spindle-shaped and firmly attached [Fig. 1(B)]. The isolates used for grafting contained $\sim$70% desmin- and MyoD-positive cells [Fig. 1(C, D)]. A differentiation assay followed by immunostaining for fast skeletal MHC revealed the presence of skeletal myotubes [Fig. 1(E,F)]. RT-PCR revealed expression of hepatocyte growth factor receptor c-met, a marker for satellite cells,[29,30] and the skeletal muscle-specific transcription factors MyoD, Myf5, myogenin, and MRF4 in day 5 satellite cell cultures [Fig. 2(A)]. The undifferentiated satellite cells expressed connexin43 (gap junction) and N-cadherin (adherens junction). Desmin (intermediate filament) was present in both skeletal and cardiac muscle cells whereas cardiac troponin I was present only in cardiomyocytes [Fig. 2(B)].

Histology and differentiation patterns of graft cells

At 4 weeks after grafting the satellite cells had fused to form well developed myotubes with recognizable cross striations [Fig. 3(A,B inset)], and were identified by the BrdU tag [Fig. 3(C,D)]. The overall appearance of the grafts was similar to neonatal myoblast-derived cardiac grafts.[1] Single-label immunostaining for cardiac α-MHC gave intense staining of the host myocardium but was absent from the graft [Fig. 3(E,F)]. Conversely, fast skeletal MHC was expressed by all muscle cells within grafts, but was absent from the host myocardium [Fig. 3(G, H)]. ANP was detected in cardiomyocytes bordering the graft [Fig. 3(N)] and in fibroblast-like cells in the graft (data not shown). A recent study reported

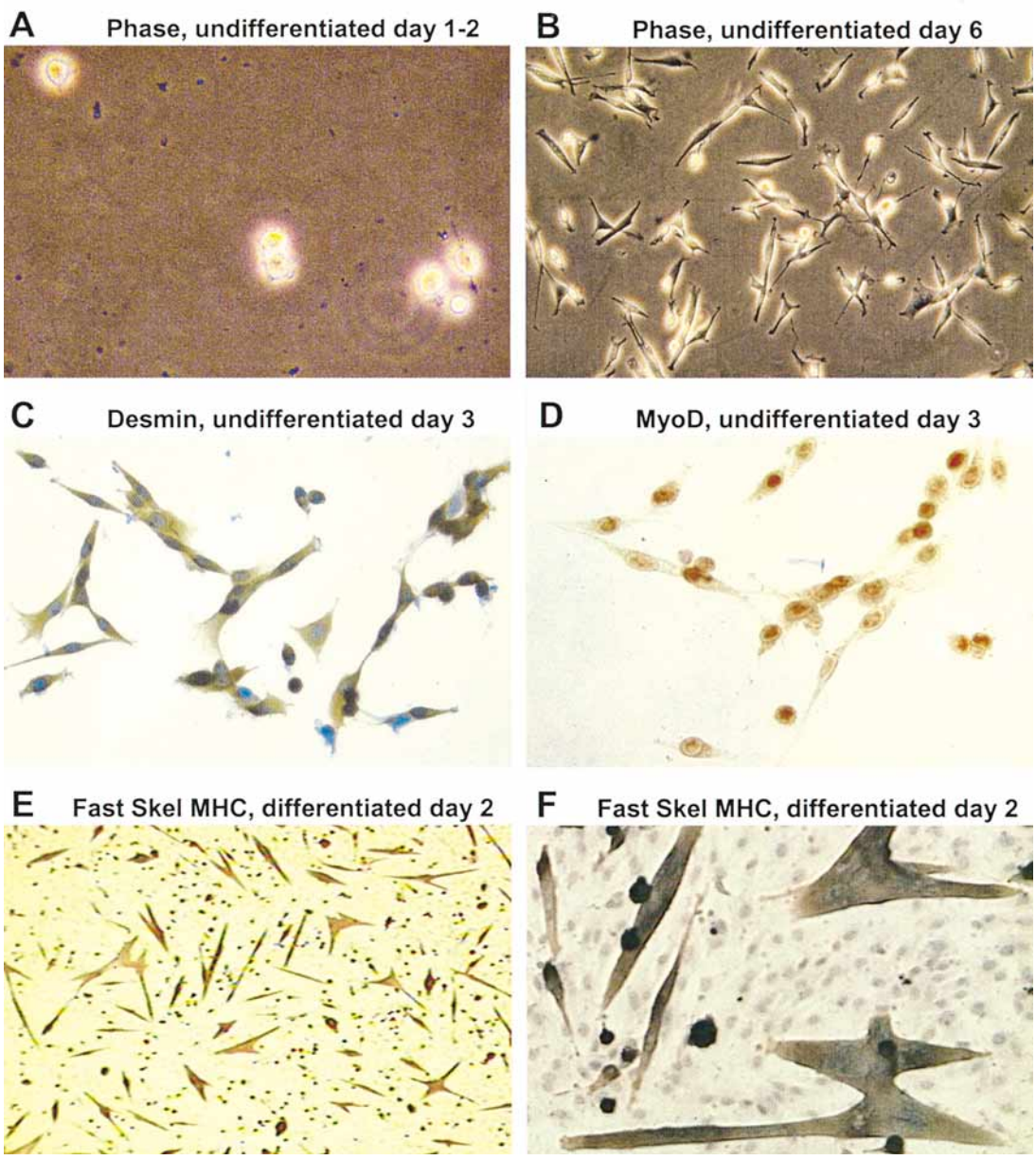

Figure 1 Characterization of satellite cells *in vitro*. (A) At day 1 satellite cells appeared mostly round and slowly started to adhere. (B) By 6 days most cells were spindle-shaped and firmly attached. (C) Approximately 70% of a day 3 cell population stained positive for desmin, the principal intermediate filament of all muscle cells. (D) Similarly, ~70% of the population expressed MyoD, a skeletal muscle-specific transcription factor. (E,F) To check for the ability to differentiate into myotubes, cultures were grown for 5 days and then differentiated for 2 days. Upon differentiation myotubes expressed fast skeletal MHC.

the expression of ANP by cardiac fibroblasts undergoing transition to the myofibroblast phenotype.[31] Figure 4 summarizes the result at 12 weeks. Similar to the 4 week time point, single-label immunostaining revealed the absence of cardiac α-MHC from the graft [Fig. 4(E,F)] and the presence of fast skeletal MHC within the grafts [Fig. 4(G,H)].

We employed double immunostaining for the BrdU tag coupled with fast skeletal α-MHC, cardiac α-MHC, cardiac troponin I, or the fetal/hypertrophic marker ANP to detect transdifferentiation [Fig. 3 (panels K–N)]. In 9 of 11 grafts, no cardiac markers were detected in any BrdU-positive cells. In 2 grafts, a total of 4 cells were identified where BrdU co-localized with α-MHC (2 cells) or cardiac troponin I (2 cells).

Expression of the slow skeletal muscle marker β-MHC was abundant in the grafts at 4 weeks [Fig.

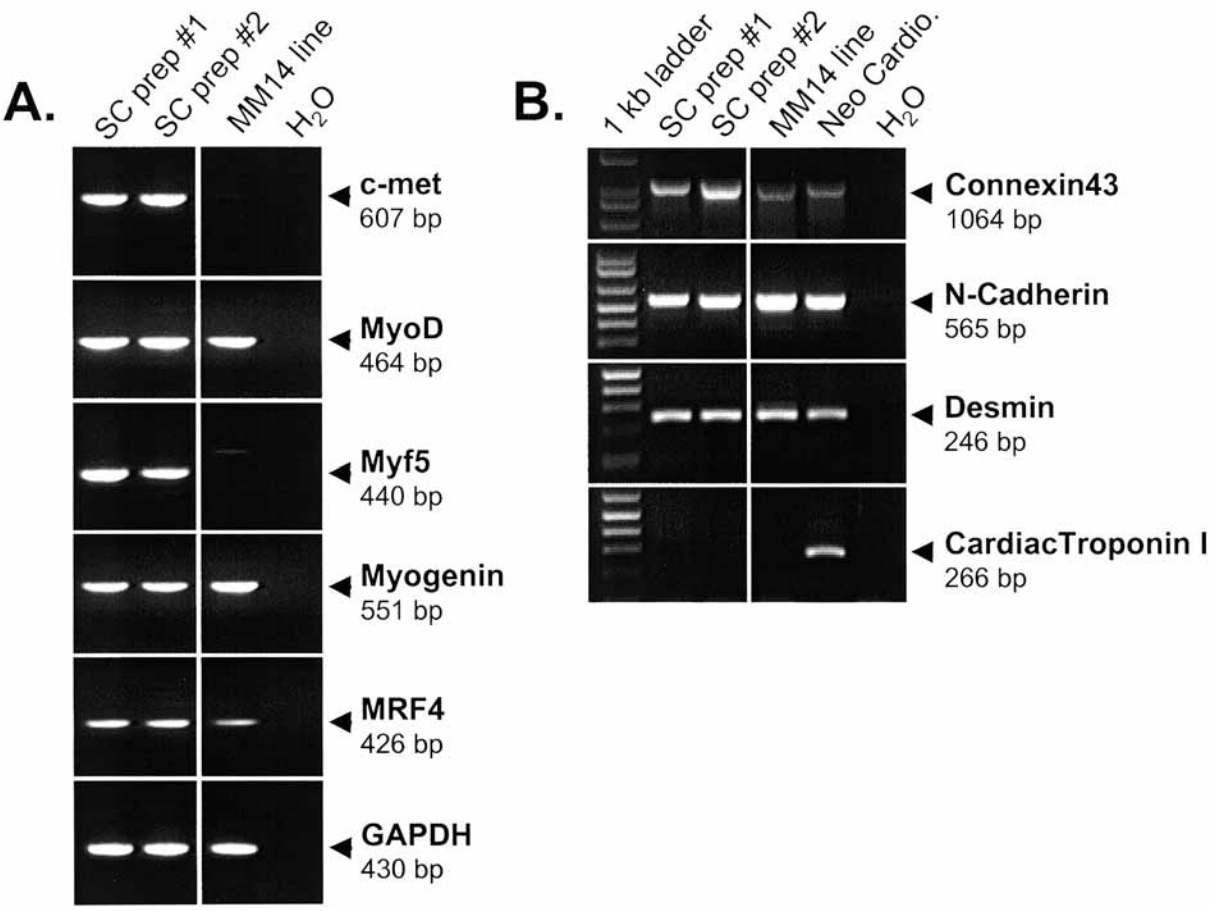

Figure 2 RT-PCR analysis of undifferentiated satellite cells. (A) The cell populations expressed the hepatocyte growth factor receptor c-met, a marker for satellite cells,[29,30] and the skeletal muscle-specific transcription factors MyoD, Myf5, myogenin, and MRF4. (B) N-cadherin and connexin43, major proteins of cardiac adherens junctions and gap junctions, respectively, were also expressed in undifferentiated satellite cells. In contrast, cardiac troponin I was only expressed in cardiac muscle cells but not in skeletal muscle cells, whereas the intermediate filament desmin was expressed in both cardiac and skeletal muscle cells.

3(I,J)], similar to our previous neonatal myoblast-derived grafts.[1] At 12 weeks, however, β-MHC expression was rarely observed [Fig. 4(I,J)] and the grafts had atrophied, with a reduction of diameter from $10.6 \pm 3.0\ \mu$m to $6.0 \pm 1.7\ \mu$m (Fig. 5). Satellite cell-derived grafts did not express detectable amounts of N-cadherin and connexin43 at 4 or 12 weeks (Fig. 6), indicating that electromechanical coupling between host and graft did not occur.

Discussion

Proof of cardiac transdifferentiation has two critical components: (1) unambiguous tracking of the cell lineage, i.e. certainty that the cells in question are derived from graft, not host; and (2) definitive characterization of phenotype, i.e. demonstration that the cells in question are, in fact, cardiomyocytes. In this study satellite cells were tagged with BrdU to track them *in vivo*, and we utilized a panel of antibodies that was carefully developed to stain cardiac muscle but not skeletal muscle (or vice-versa). Using this rigorous approach, the current study clearly shows that unfractionated isolations of satellite cells differentiate into mature skeletal muscle, and not cardiac muscle, after grafting into the heart. The satellite-derived cells expressed skeletal muscle-specific MHCs and failed to express the cardiac-specific markers α-MHC, cardiac troponin I, ANP, or the intercalated disk proteins N-cadherin and connexin43. Furthermore, the satellite cell-derived grafts had numerous, peripherally located nuclei, structural hallmarks of skeletal myocytes.

Why are these findings discrepant with those reported by others? Although our previous grafting

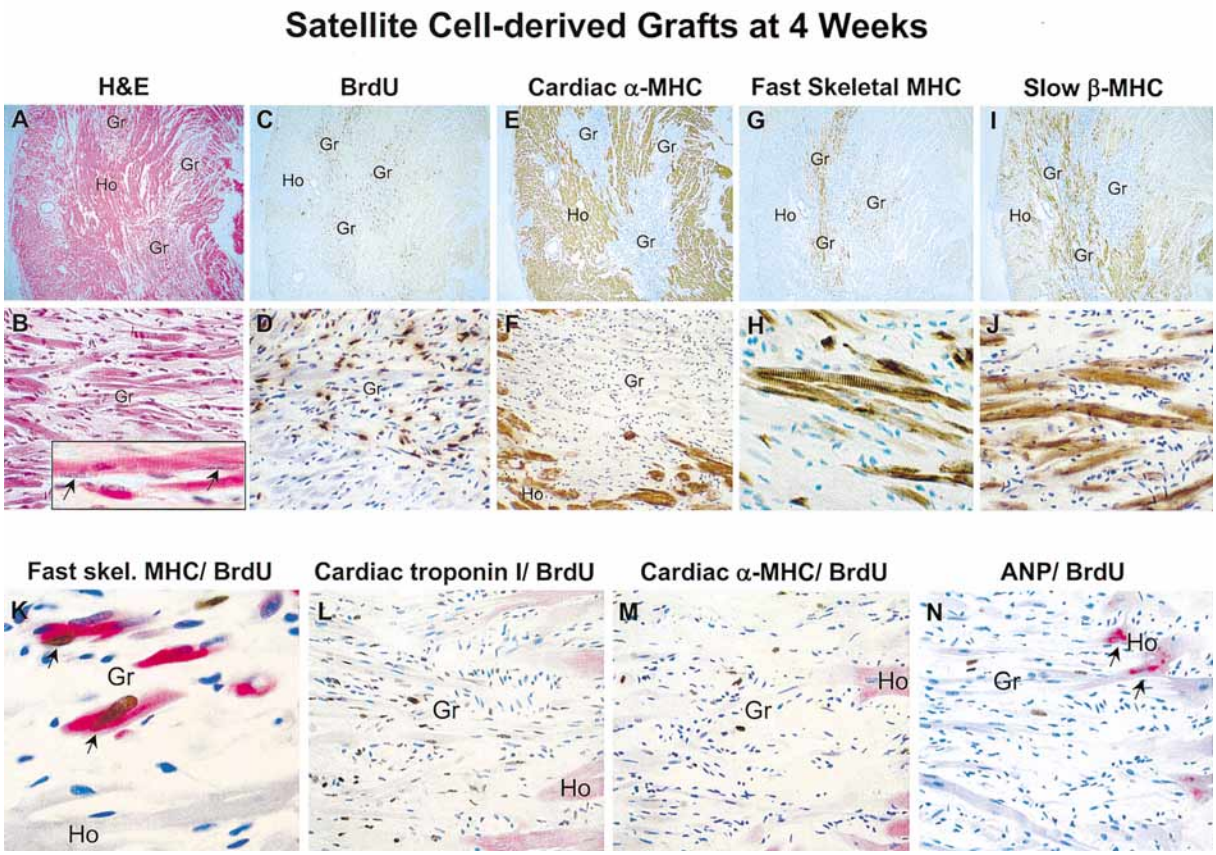

Figure 3 Phenotype of satellite cell-derived grafts at 4 weeks. A–D. Satellite cell grafts were readily detectable in H&E (A,B, inset) and BrdU (C,D) stained sections. The vast majority of grafted cells had fused into multinucleated myotubes with recognizable cross striations and peripheral nuclei (B inset, arrows). Ho, host myocardium; Gr, graft. E,F. In contrast to the surrounding host myocardium, the grafts did not express α-cardiac MHC. G,H. Conversely, the grafts were positive for fast skeletal MHC whereas the surrounding host myocardium was negative. I,J. Expression of slow *β*-MHC was observed in many graft myofibers and also in the host myocardium. (J) High power of *β*-MHC-positive graft myofibers. K. Double staining for fast skeletal MHC (red) and BrdU (brown). Double positive cells were readily detectable in the graft (Gr) but not in the host (Ho). L. Double staining for cardiac troponin I (red) and BrdU (brown). Cardiac troponin I was detected in host cardiomyocytes (Ho) but not in the graft (Gr). M. Double staining for cardiac α-MHC (red) and BrdU (brown). Cardiac α-MHC was detected in the host myocardium but not in graft cells in conjunction with the BrdU tag. N. Double staining for ANP (red) and BrdU (brown). ANP was detected in cardiomyocytes bordering the graft (arrows) and in fibroblast-like cells in the graft area (not shown). ANP was never detected in conjunction with the BrdU tag, however.

work employed neonatal skeletal myoblasts,[1] the current study used adult satellite cells, ruling out developmental stage as a source of variation. Transdifferentiation has been reported in dogs,[7,8] rats[9] and rabbits,[5,10] indicating that species variation is unlikely to account for the difference. Transdifferentiation was also reported in studies where mixed (slow and fast) fibers[7–9] or slow fibers[5,10] were used for satellite cell isolation, indicating that the muscle type of origin cannot explain the discrepancy. Furthermore, transdifferentiation has been reported in both the normal[9] and injured[5,7,8,10] heart, suggesting that the status of the recipient heart is not critical. A previous study from our group has shown that neonatal myoblasts grafted into injured hearts form grafts of mature skeletal muscle at 3 months post-grafting and showed electrical properties typical of skeletal muscle.[1] In our experience the phenotype of the neonatal myoblasts in culture is very similar to cultured satellite cells from adult animals. Therefore, we would not expect satellite cells to behave differently from neonatal myoblasts in an injury model.

We think the discrepancy is probably due to methodological differences. All reports of transdifferentiation either failed to use definitive markers to track cell lineage or did not employ definitive markers of the cardiac phenotype. For example, a graft origin of the cells was inferred by their location within the area of injury,[5,7,8,10] where "con-

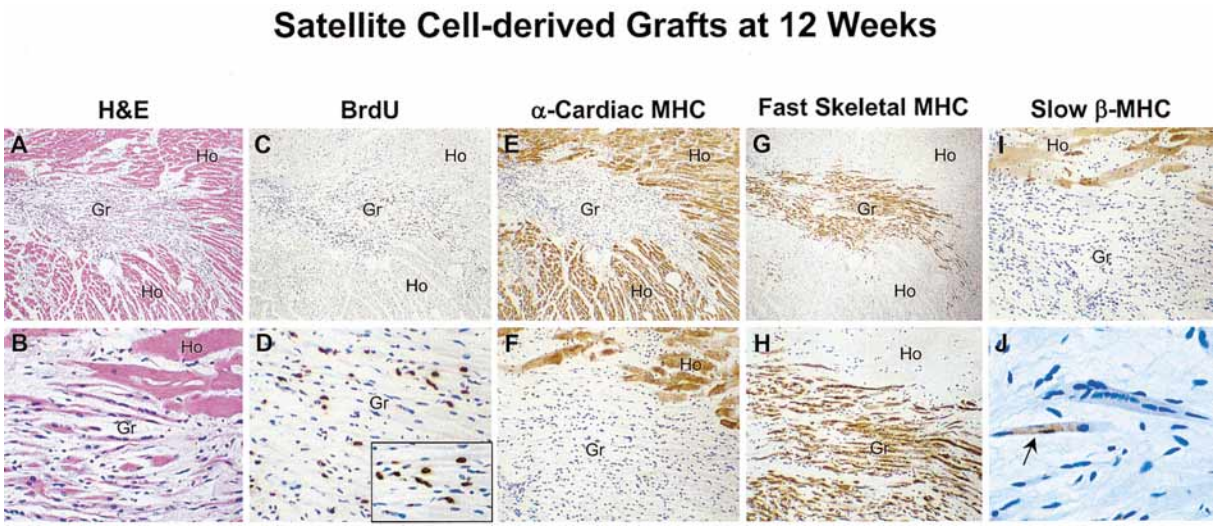

Figure 4 Phenotype of satellite cell-derived grafts at 12 weeks. Satellite cell-derived grafts were identified at 12 weeks by hematoxylin and eosin staining (A,B) and immunostaining for the BrdU tag (C,D). The phenotype of the grafts at 12 weeks was similar to the 4-week time point with respect to the absence of cardiac α-MHC expression (E,F) and the presence of fast skeletal MHC (G,H). Surprisingly, at 12 weeks slow β-myosin heavy chain was rarely observed in the grafts (I,J).

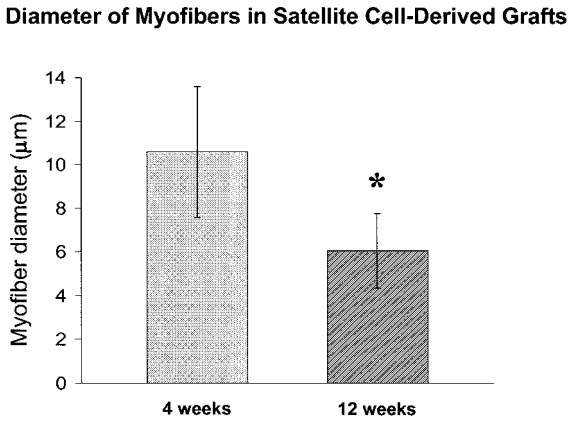

Figure 5 Diameter of graft cell-derived myofibers. The diameter of the satellite cell-derived myofibers was significantly reduced at 12 weeks as compared to the 4-week time point, thus indicating atrophy (data are mean $\pm$ S.D.; *P<0.05).

tamination" by surviving host cardiomyocytes could easily occur. Similarly, a cardiac phenotype was inferred by such imprecise morphological criteria as central nuclei (common in immature or regenerating skeletal muscle) or the presence, in routine light micrographs, of refractile cytoplasmic structures thought to be intercalated disks.[5–7] As stated above, proof of transdifferentiation requires a rigorous combination of lineage determination and phenotypic characterization, preferably using well defined molecular markers.

Slow β-MHC expression was observed in the grafts at 4 weeks. We observed similar fast-to-slow fiber type conversion when neonatal myoblasts were used for grafting.[1] Slow twitch fibers have physiological similarities to cardiac muscle suggesting that they may be suited to perform a cardiac type work load.[1] Surprisingly, by 12 weeks the grafts were atrophic and slow β-MHC expression was rarely observed. This contrasts to our previous study in the injured heart, where 12-week-old grafts showed progressive hypertrophy and still expressed β-MHC.[1] We hypothesize that the injured tissue results in different mechanical loading, which in turn promotes hypertrophy and slow fiber differentiation.

In summary, we have shown that satellite cell-derived grafts differentiate into mature skeletal muscle after grafting into the heart. Transdifferentiation into cardiomyocytes, as reported previously, was not observed in our study. Cultured satellite cells thus appear to be committed to the skeletal muscle lineage and our results indicate that the cardiac environment is not capable of reversing this commitment.

Acknowledgments

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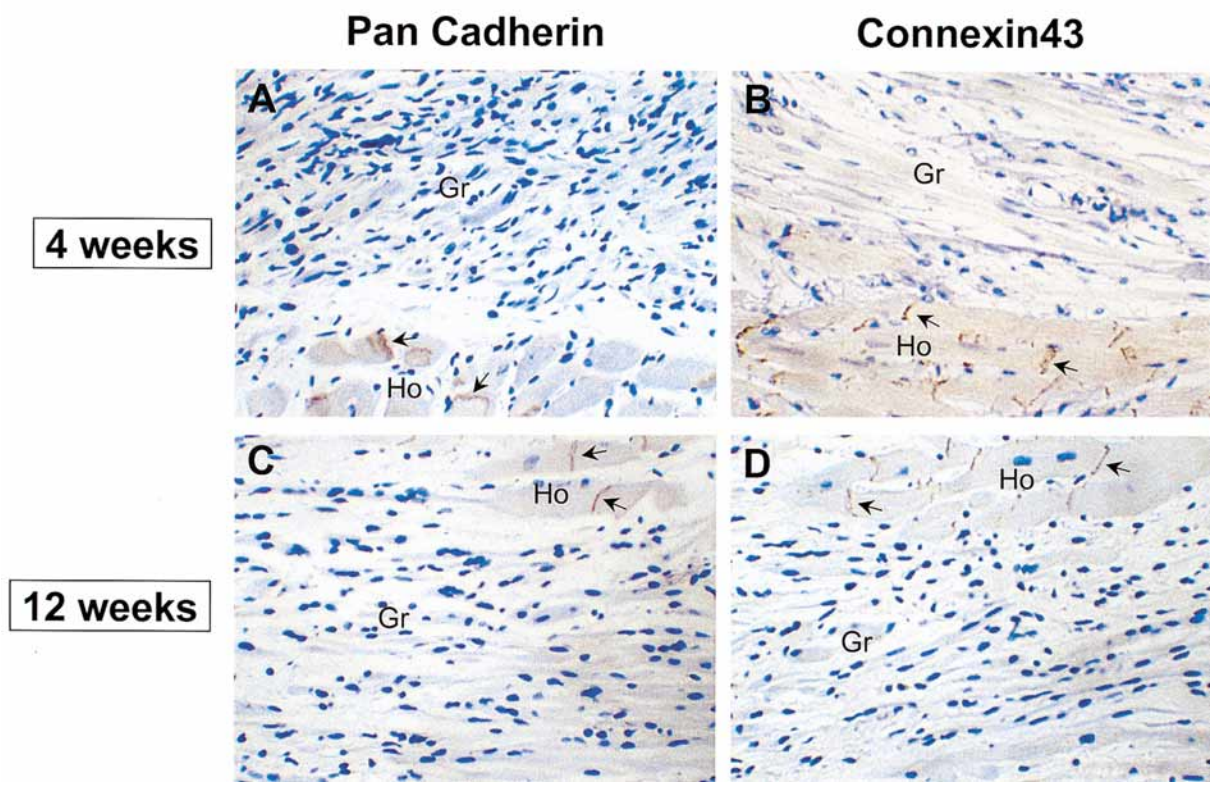

Figure 6 N-cadherin and connexin43 immunostaining. A–D. Major components of the cardiac intercalated disk, that is N-cadherin (A,C) and connexin43 (B,D) were detected in host myocardium (Ho, arrows) but not in the satellite cell grafts (Gr) at 4 and 12 weeks.

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