Cardiac Cellular Transplantation: Transarterial Myoblast Transplantation in Dogs

Mark A. Oyama, DVM[*]
Valentina Merola, DVM[†]
Thomas E. Eurell, DVM, PhD[†]

[*]*Department of Veterinary Clinical Medicine*
[†]*Department of Veterinary Biosciences*
College of Veterinary Medicine
University of Illinois
Urbana, IL

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ABSTRACT

Cardiac cellular transplantation seeks to replace cardiac myocytes irretrievably damaged by injury with adult progenitor cells capable of maturing into cardiac-like tissue. We sought to harvest and culture adult skeletal muscle myoblasts (satellite cells), to evaluate fluorescent labeling of myoblasts, and to develop a technique for cardiac cellular transplantation using a transcoronary arterial transplantation (TCAT) method in healthy dogs. Four adult purpose-bred dogs underwent skeletal muscle biopsy and culture of myoblasts in vitro. Isolated myoblasts were labeled with 1 of 3 different fluorescent dyes and injected into the left coronary artery using selective coronary artery catheterization. The safety of transplanting myoblasts via TCAT was demonstrated by measurement of cardiac hemodynamics, plasma cardiac troponin-I level, and ambulatory electrocardiographic monitoring. The successful entrapment of myoblasts within the coronary capillaries was demonstrated by simultaneous sampling of coronary sinus blood. Survival and migration of transplanted cells into the ventricular myocardium was demonstrated by histological identification of labeled cells 30 minutes and 7 days after transplantation. Two fluorescent cell labels resulted in the positive identification of transplanted cells in situ, while 1 fluorescent cell label was unsuccessful due to autofluorescence of the native tissue. Our results describe a method of isolation, culture, and labeling of canine skeletal myoblasts. Furthermore, our TCAT method resulted in the entrapment and survival of autologous myoblasts and may represent a useful animal model for further study of cellular transplantation by transarterial injection.

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INTRODUCTION

Loss of myocardial cells secondary to cardiac disease is thought to be an irreversible process due to the inability of the differentiated myocyte to return to the cell cycle.[1] While this long-held belief has been recently challenged,[2] the death and reduction of viable cell numbers is a prominent feature of both ischemic and non-ischemic heart disease.[3,4] Loss of cardiac tissue affects both diastolic and systolic function and contributes to the formation of cardiac arrhyth-

mias. Dilated cardiomyopathy (DCM), a disease of progressive systolic failure and ventricular hypertrophy, is a common cardiac affliction in dogs. Once begun, the pathology of DCM is incessant, cumulative, and eventually fatal.[5] Medical therapy has assisted in the management of these cases, but mortality and morbidity remain high.[6,7] Treatments designed to replace lost tissue, such as cellular transplantation, could help slow or reverse muscle loss and improve prognosis. Cellular transplantation using stem cells of autologous origin represents a novel method to replace myocardial cells. Adult stem cells, as found in tissues such as bone marrow and skin, possess an ability to transform into a variety of tissues, including cell types outside their organ of origin.[8,9] Other cell populations, such as skeletal muscle myoblasts (satellite cells), possesses stem cell-like properties in that they can replace cell populations of terminally differentiated lineage. Thus, following lethal injury to the mature and terminally differentiated skeletal muscle cell, myoblasts can be recruited to the site of injury, where they transform into new skeletal muscle, thereby rebuilding their tissue of origin. Whereas skeletal muscle possesses a rich population of myoblasts that reside on the periphery of mature muscle fibers, there is no cardiac myoblast equivalent within the heart, and cardiomyocytes lost to injury cannot be replaced.

The ability of skeletal myoblasts to form functional muscle tissue makes them an intriguing prospect for cardiac transplantation. Based on their robust mitotic capacity and ability to form functional myotubules, researchers have transplanted skeletal myoblasts into animal models of ischemic disease with encouraging results.[10–13] Based on these studies, approximately 100 human patients worldwide have undergone myoblast transplantation,[14] usually in conjunction with a coronary artery bypass procedure. The results of these studies are preliminary but encouraging. Transplanted cells survive, engraft, and begin to take on characteristics of adult muscle cells (that is, they express sarcomeric proteins such as skeletal β-myosin heavy chain).[9] The beneficial effect associated with cellular transplantation is presumably related to improvement in diastolic function (a passive girding effect), reduction of post-infarction remodeling, and an increase in regional ventricular contractility.[14] The encouraging results from these studies suggest a broader potential for use of cellular transplantation in patients with other types of heart disease, such as idiopathic DCM.[15] Indeed, cellular transplantation has been shown to have benefit in both rats with doxorubicin-induced disease and in Syrian hamsters with DCM.[16,17]

In cases of ischemic heart disease (which is usually focused on a specific region of the myocardium), transplantation involves direct injection of cells into the epicardial surface during thoracotomy. This method results in isolated "islands" of transplanted cells that are placed adjacent to and within the infarcted area. Canine DCM differs from human ischemic injury in that DCM is largely nonischemic in etiology, is an affliction of global rather than regional ventricular proportion, and would presumably benefit from the wider dissemination of transplanted cells throughout the entire ventricle. Accordingly, we sought to develop a transcoronary arterial transplantation (TCAT) method in dogs. The underlying principle of TCAT involves the injection of myoblasts into the coronary circulation, entrapment within the coronary capillaries, movement through the capillary wall into the surrounding interstitium, and eventual maturation and incorporation into the native cardiac syncytium. The advantage of TCAT over surgical injection is a more uniform and global dissemination of cells throughout the left ventricle.[16,18-21] Furthermore, the use of adult stem cells from the patient's own (autologous) tissues is particularly appealing in that concerns regarding transplant rejection or use of embryonic tissues are minimized.

In the current study, we sought to evaluate the feasibility of skeletal muscle myoblast transplantation using a TCAT

delivery method in healthy dogs. In particular, we attempted to isolate, culture, and label canine myoblasts, develop a catheter-based and fluoroscopically-guided TCAT protocol, and to determine the acute entrapment and short-term behavior of transplanted myoblasts.

MATERIALS AND METHODS

Experimental Animals

The study protocol was approved by the University of Illinois, Institutional Animal Use and Care Committee. Animal use followed the guidelines set forth by the US Department of Agriculture and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health. Four healthy, male purpose-bred mixed breed dogs were used, with an average (SD) weight of 18.6 (2.3) kg. Normal cardiac function was documented in dogs via physical examination, 10-lead (PageWriter Xli, Hewlett Packard, Palo Alto, CA, USA) and standard M-mode, 2D, and color flow echocardiographic (ECG) examinations (VIVID7, GE Medical Systems, Waukesha, WI, USA).

Skeletal Muscle Biopsy and Myoblast Isolation and Culture

Dogs were sedated with buprenorphine (0.008 mg/kg IV) and acepromazine (0.03 mg/kg IV) and general anesthesia induced with pentobarbital (8–15 mg/kg IV to effect). Patients were intubated and maintained on inhalant isoflurane. An intraoperative ECG was monitored (PageWriter Xli). A 1- to 2-cm^3 skeletal muscle biopsy was obtained from the quadriceps muscle of the pelvic limb of each dog and transferred to a tube containing Hanks Balanced Salt Solution (HBSS). A 10-mL blood sample was collected, allowed to clot and the resulting serum frozen. The isolated muscle biopsy was transported to a laminar flow tissue culture hood (within 30 minutes of harvest) and minced into fine pieces using a scissors and sterile technique. The minced tissues were incubated in HBSS containing 0.02% collagenase for 1 hour followed by HBSS containing 2% Dispase for an additional 1 hour. All incubations occurred at 37˚C and 5% CO_2. The tissue suspension was then filtered through 100-µm and 70-µm filters, respectively, to remove large muscle fibers. The resultant cell suspension was transferred to 100-mm tissue culture dishes containing myoblast culture media that consisted of Dulbecco's minimal essential media (DMEM) enriched with 10% horse serum, 10% fetal bovine serum (FBS), 2mM L-glutamine, and 1X antibiotic-antimycotic mixture (penicillin-G/ streptomycin/amphotericin-B). The cells were then incubated at 37˚C and 5% CO_2 for 1 hour. Fibroblasts rapidly adhered to the dish surface (as judged by phase contrast microscopy) and the supernatant, which contained the myoblasts, was transferred to a new dish. This incubation/supernatant transfer procedure served as a myoblast enrichment technique and was repeated at 3 hours and then once every 24 hours for 7 days. Myoblast cultures demonstrating characteristic morphologic features (see Results) and without fibroblast contamination were allowed to grow and subsequently processed for cell labeling and TCAT (Figure 1).

Myoblast Labeling

Isolated myoblasts were labeled to allow their identification in target tissue following transplantation. Three different compounds were used to label the cultured myoblasts, green fluorescent protein (GFP), 4',6-diamidino-2-phenylindole (DAPI), and carbocyanine (DiI).

GFP labeling was accomplished by transfection of cells with an adenovirus vector containing GFP rDNA. The infected cell manufactures GFP, which localizes in the cytoplasm. GFP labeling was accomplished following the supplier's recommended protocol. Briefly, myoblast cultures that were approximately 80% confluent were exposed to 100 multiplicities of infectivity (determined in preliminary experiments following suppliers recommended protocol to be the optimal virus dosage) and incubated in

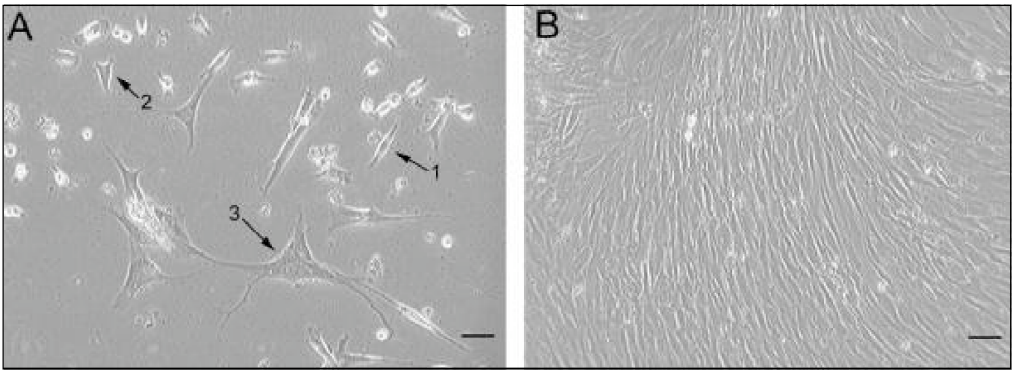

Figure 1. Phase contrast microscopic images of enriched (Frame A) and fibroblast-contaminated (Frame B) canine myoblast cultures taken after the fifth supernatant passage. Note the lower density and characteristic fusiform (arrow 1) and triangular to stellate (arrows 2 and 3) morphologies of the purified myoblasts in Frame A. Bar in lower right-hand corner of image frame equals 20 microns.

myoblast culture media for 72 hours. Cultures were then rinsed 3 times (5 minutes each rinse) in fresh myoblast culture media. After the final rinse, the cells were harvested and standardized cell suspensions for TCAT were made as described below.

DAPI is a blue fluorescing DNA-intercalating dye that binds to A-T rich regions of double stranded DNA. It localizes to the cell nucleus and despite binding to DNA, does not interfere with cell replication or transcription.[22] Fresh stock solutions of DAPI (5mg/mL) were prepared in tissue culture certified water prior to cell labeling. The myoblast culture media overlying an approximately 80% confluent population of myoblast cells growing in a 75 cm^2 tissue culture flask was removed and replaced with 8.95 mL of fresh media + 20 mL HBSS + 1.05 mL DAPI stock solution. The cells were incubated for 2 hours at 37˚C in 5% CO_2 and then rinsed 3 times (1 minute each rinse) with 10 mL aliquots of HBSS + 10% FBS. After the final rinse, the cells were harvested and standardized cell suspensions for TCAT were made as described below.

DiI is a lipophilic membrane dye that fluoresces orange-red and is supplied as a liquid. The myoblast culture media overlying an approximately 80% confluent population of myoblast cells growing in a 75-cm^2 tissue culture flask was removed and replaced with 10 mL of fresh media containing 25 µL of DiI. The cells were incubated for 20 minutes at 37˚C in 5% CO_2 and then rinsed 3 times (1 minute each rinse) with 10-mL aliquots of HBSS + 10% FBS. After the final rinse the cells were harvested and standardized cell suspensions for TCAT were made as outlined below. Two dogs received myoblasts labeled with GFP, 1 dog received myoblasts labeled with DAPI, and 1 dog received two populations of myoblasts, one labeled with DAPI and one labeled with DiI (Table 1).

Harvesting of Labeled Myoblasts and Preparation for TCAT

The overlying media from tissue culture flasks containing labeled myoblasts was aspirated and discarded. Myoblasts were then exposed to 4 mL ice-cold dilute Trypsin/EDTA [0.1X in HBSS diluted from 10X trypsin/ethylenediaminetetraacetic acid (EDTA)] for approximately 30 seconds. This solution was aspirated and discarded and 4 mL of fresh, ice-cold 0.5X Trypsin/EDTA was added to each flask. The cells were then incubated at 37˚C for 8 to12 minutes and gently mixed to break up cell aggregates. When cells were observed to release from the bottom of plate (using phase contrast microscopy), 5 mL of trypsin inhibitor (1:5 dilution of FBS in DMEM) was added to the culture dish and the media was gently passed back and forth through a

Table 1. Fluorescent Label, Number of Cells Transplanted, and Time to Sacrifice for 4 Dogs Undergoing Cardiac Cellular Transplantation

	Cell Type	Cell Label	No. of Cells	Time to Sacrifice
Comparison of cell number used for transplantation				
Dog 1	HMC*	GFP	1×10^6	5 days
Dog 2	HMC	GFP	5×10^6	7 days
Acute entrapment of DAPI-labeled cells				
Dog 3	AMB	DAPI	1×10^6	30 minutes
Entrapment of DAPI and DiI- labeled cells†				
Dog 4	AMB	DAPI	1.5×10^6	7 days
Dog 4	AMB	DiI	1.5×10^6	

*HMC indicates heterogenous mixed culture (fibroblasts and myoblasts); AMB, autologous myoblasts; GFP, green fluorescent protein; DAPI, 4',6-diamidino-2-phenylindole; DiI, carbocyanine.
†Dog 4 received 2 different types of cells, DAPI and DiI.

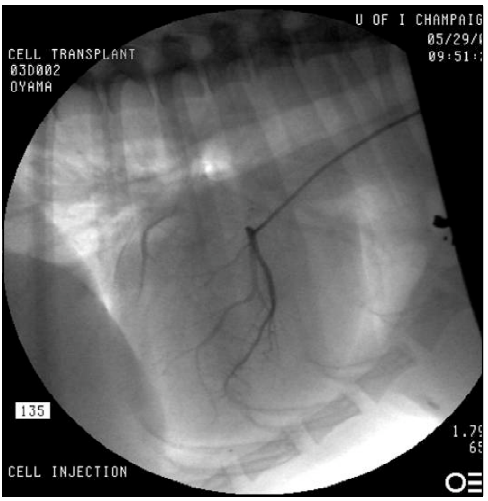

Figure 2. Angiogram demonstrating placement of the catheter tip in the proximal portion of the left anterior descending coronary artery in a dog undergoing cardiac cellular transplantation. Following placement and flushing of the catheter, intra-arterial injection of skeletal muscle myoblasts was performed.

5-mL pipette to facilitate removal of all cells from the plate surface and to break up cell clumps. The cell suspension was then transferred to a 50 mL centrifuge tube and spun at 180X G for 7 minutes. The cells were resuspended in HBSS containing 5% autologous dog serum and passed through a 40-µm filter in preparation for TCAT. The cells were counted using a hemocytometer and viability was estimated using trypan blue exclusion.[23]

Transcoronary Arterial Transplantation

To assess the feasibility and efficacy of TCAT, 4 separate experiments were performed. The cell type, number, and labeling are presented in Table 1. Approximately 4 weeks after muscle biopsy, dogs were anesthetized as previously described. The right carotid artery was surgically isolated and a 6-Fr introducer catheter (Check-Flo Performer, Cook, Bloomington, IN, USA) placed. A 5- or 6-Fr multipurpose end-hole catheter (Torcon NB Advantage, Cook, Bloomington, IN, USA) was positioned within the proximal left anterior descending coronary artery under fluoroscopic guidance. Small boluses of radiographic contrast media were used to confirm the position of the catheter (Figure 2). A 4-port cardiac manifold was constructed by attaching four 3-way stopcocks in series, which allowed simultaneous connection of the catheter to a physiologic pressure recorder (Biopac MP150, Biopac Systems, Goleta, CA, USA), the myoblast transplant solution, and syringes containing heparinized saline flush, and contrast media. During catheter placement and cell injection, coronary arterial pressure waveforms were monitored to avoid accidental occlusion of the coronary artery. Once the catheter was positioned, the labeled myoblasts were injected into the coronary artery over a 2-minute duration.

Comparison of Different Cell Numbers Used for TCAT

We compared the number of cells that could be safely transplanted via TCAT by injecting 1×10^6 and 5×10^6 heterologous GFP-labeled cells (consisting of a mixture of

canine skeletal muscle fibroblasts and myoblasts) into 2 different dogs. The acute effect of TCAT was determined via ECG monitoring and measurement of cardiac output before and after the procedure via thermodilution (E for M V2213A, Honeywell, Morristown, NJ, USA). Ambulatory Holter monitoring (Tracker, Del Mar Reynolds Medical, Irvine, CA, USA) was performed during the first 24 hours post-procedure, and plasma cardiac troponin-I concentrations were measured up to 120 hours post-procedure using a human immunoasssay system validated for use in dogs. (Access AccuTnI, Beckman Coulter, Fullerton, CA, USA). Five days after transplantation, dogs were euthanized and the hearts removed for histological examination.

Acute Myocardial Entrapment of Myoblasts following TCAT

Successful TCAT requires that myoblasts are entrapped within the coronary capillaries and do not simply wash through into the venous circulation. To demonstrate efficacy of entrapment, we injected 1 x 10^6 autologous DAPI-labeled myoblasts into 1 dog. During cell injection, 18 mL of coronary sinus blood were drawn from a preplaced 7-Fr multipurpose catheter in the coronary sinus and collected in heparinized blood tubes. The coronary sinus blood was processed using a self-generating Percoll gradient centrifugation method to isolate labeled myoblasts that may have entered the coronary sinus circulation. We followed the manufacturer's recommendations for self-generating Percoll gradients. Briefly, 49.5 mL Percoll were mixed with 5.5 mL of 1.5 M NaCl to make a stock isotonic Percoll solution (SIPS). Ten milliliters of SIPS were mixed with 10 mL 0.15 M NaCl to make a working Percoll suspension. This suspension was transferred to centrifuge tubes and placed in an angle-head rotor and centrifuged at 30,000X G for 15 minutes at a temperature maintained between 10˚C and 15˚C. This procedure produces a self-forming gradient used for cell isolation. Five milliliters of coronary blood was then carefully layered upon the self-formed Percoll gradient and centrifuged at 400X G for 30 minutes at a temperature maintained between 10˚C and 15˚C. All runs were calibrated using density marker beads. Cells isolated using this procedure were evaluated for positive DAPI-staining using an Olympus CK-40 inverted fluorescent microscope with a DAPI filter set (XF03 Vivid Standard Set, Omega Optical, Brattleboro, VT, USA). The dog was euthanized 30 minutes after cell injection and the left ventricle and interventricular septum dissected and processed for histological examination.

Acute Myocardial Entrapment of DAPI and DiI-Labeled Myoblasts Following TCAT

To compare the survival and behavior of transplanted autologous myoblasts labeled with different fluorescent markers, 1 dog underwent transplantation with 3 x 10^6 myoblasts (1.5 x 10^6 DAPI-labeled cells and 1.5 x 10^6 DiI-labeled cells). The dog was euthanized 7 days after transplantation and left ventricular and interventricular septal tissue processed for histological examination.

Tissue Processing and Histological Examination

One cubic centimeter samples of the left ventricular free wall and interventricular septum were embedded in optimal cutting temperature freezing medium and quick-frozen in a hexane/liquid nitrogen bath. Frozen specimens were held at -80˚C until processed for histology. Eight-micron tissue sections were stained using Gill's #3 hematoxylin for 30 seconds, rinsed in tap water for 5 minutes, and counterstained using eosin/phloxine for 30 seconds. Stained sections were then dehydrated in a series of alcohols, dipped in xylene, and mounted using Eukitt mounting medium. Tissue images were captured using a Leitz Orthoplan microscope equipped with a SpotRT digital camera (Diagnostic Instruments, Sterling Heights, MI, USA) and an epifluorescence attachment.

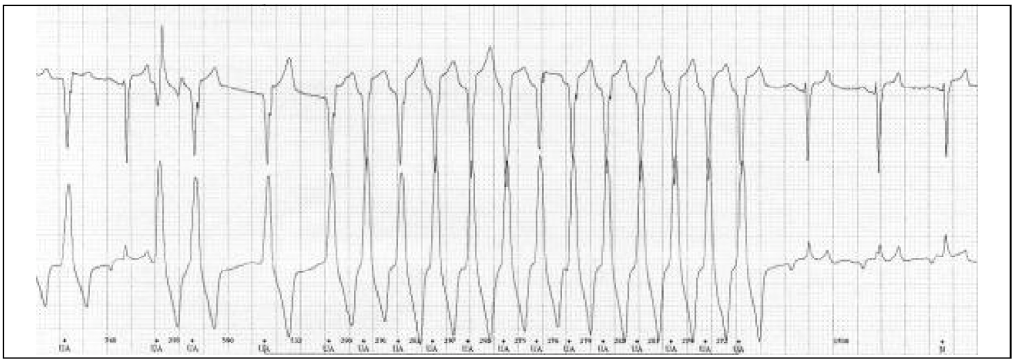

Figure 3. Twenty-four hour ambulatory electrocardiographic (ECG) (Holter) recording from dog 2 3 hours after the coronary injection of 5 x 10^6 cells, demonstrating nonsustained ventricular tachycardia. Myocardial infarction was suspected. Paper speed = 25 mm/sec.

Fluorescent images were detected using GFP, DAPI, and DiI optimized filters.

RESULTS

Myoblast Isolation and Culture

Fibroblasts that contaminated the muscle preparation rapidly adhered to the surface of the culture dish (judged by phase contrast microscopy) within 45 minutes of culture at 37˚C in 5% CO_2. By the fourth or fifth passage of the supernatants during the myoblast enrichment technique, cell cultures that continued to be contaminated with fibroblasts showed characteristic dense patterns of fusiform cells (Figure 1B) and were discarded. Cell cultures demonstrating characteristic morphologic features of myoblasts (Figure 1A) and without fibroblast contamination were allowed to grow and were subsequently processed for cell labeling and TCAT. There were 2 characteristic morphologies of the canine myoblast identified during cell culture when the cells were attached to the surface of plastic culture ware: fusiform (type 1) and triangular to stellate (type 2). Type 1 cells ranged from 10 to 40 microns in length, whereas the type 2 cells ranged from 10 to 100 microns.

Comparison of Different Cell Numbers Used for TCAT

One million cells were successfully injected into the main left coronary artery of dog 1. No arrhythmias were noted during or immediately after the injection. Cardiac output, measured in triplicate before and 5 minutes after the injection was not significantly different [pre-injection = 2.90 ± 0.25 L/min, heart rate = 122 beats per minute (bpm) versus postinjection = 3.16 ± 0.18 L/min, heart rate = 125 bpm; $P = 0.34$). Recovery from anesthesia was uneventful. Ambulatory ECG monitoring during the first 24 hours post-transplant revealed one single ventricular premature beat. The predominant rhythm was sinus arrhythmia, with an average heart rate of 88 bpm (range, 54–205 bpm). Cardiac troponin-I concentration was 0.01 ng/mL during recovery from anesthesia, 0.03 ng/mL at 24 hours, 0.11 ng/mL at 48 hours, and 0.02 ng/mL at 120 hours (normal reference range for our laboratory is < 0.11 ng/mL).

Dog 2 received 5 x 10^6 cells with no complications or arrhythmias during or directly after the procedure. No cardiac output data were available from this dog. Recovery from anesthesia was uneventful. Ambulatory ECG monitoring over the first 24 hours post-transplant revealed 87,900 ventricular premature beats (representing 52% of the total number of heart beats), and included 2,000 couplets, 1,002 triplets, and numerous runs of sustained ventricular tachycardia with heart rates as high as 326 bpm (Figure 3). The average heart rate was 123 bpm (range, 57–326 bpm). Cardiac troponin-I concentration was 0.13 ng/mL during recovery from anesthesia, 23.5 ng/mL at 24 hours, 7.53 ng/mL at 48 hours, and 4.06 ng/mL at 72 hours. Five days after injection, histological

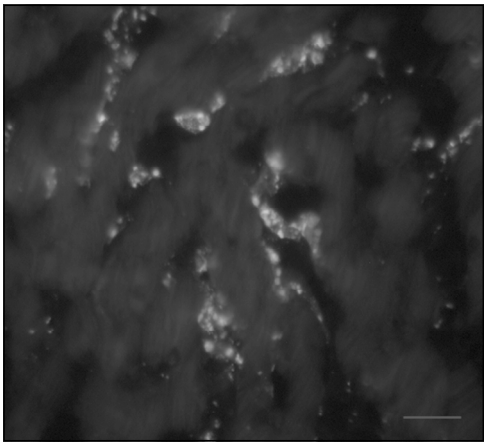

Figure 4. Fluorescent microscopic image of control canine myocardium (right ventricle) from dog 2 showing prominent autofluorescence characteristic of lipofuscin. Bar in lower right-hand corner equals 20 microns.

examination of left ventricular and septal tissues from dogs 1 and 2 was performed. The presence of GFP-labeled cells could not be definitively demonstrated due to background fluorescence from lipofuscin within native myocardiocytes (Figure 4).

Acute Myocardial Entrapment of DAPI-Labeled Myoblasts Following TCAT

One million DAPI-labeled myoblasts were successfully injected into the left coronary circulation of dog 3. No arrhythmias were noted during or immediately after the injection. The venous blood that was simultaneously drawn from the coronary sinus to test for a flow-through effect was negative for the presence of DAPI-labeled cells. Histological examination of myocardial tissue revealed clusters of DAPI-positive cells scattered within the myocardium and distributed throughout the left ventricular wall and interventricular septum (Figure 5).

Myocardial Entrapment of DAPI- and DiI-Labeled Myoblasts Following TCAT

Dog 4 received 3 x 10^6 autologous myoblasts, half of which were labeled with DAPI and half with DiI. Cells were injected into the left anterior descending coronary artery and no arrhythmias were noted during or after injection. Recovery from anesthesia

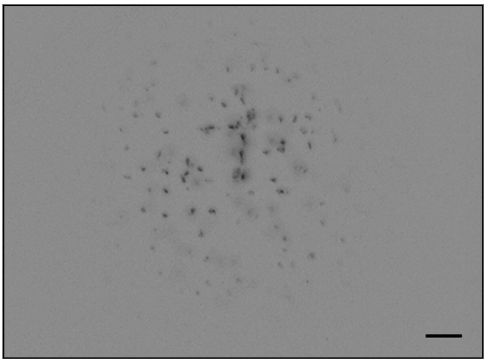

Figure 5. Fluorescent microscopic image of canine myocardium (left ventricle) showing a cluster of 4′,6-diamidino-2-phenylindole (DAPI)-labeled cells. Tissue is from dog 3, which received 1 x 10^6 autologous myoblasts 30 minutes prior to sacrifice. Bar in lower right-hand corner equals 30 microns.

and the 7-day post-transplant period was uneventful. Evaluation of cardiac tissues using fluorescent microscopy revealed that the populations of DAPI- and DiI-labeled cells could be detected within left ventricular and interventricular septal tissues (Figure 6). The advantage of this approach is that the fluorescent images and the brightfield images could be superimposed to facilitate individual cell identification. No evidence of myocardial infarction, emboli, or perivascular inflammation was found using standard histologic methods.

DISCUSSION

To our knowledge, this is the first report investigating cardiac cellular transplantation using a catheter-based TCAT technique in dogs. Our results describe the successful harvesting and isolation of canine skeletal muscle myoblasts, determine the cellular labeling potential of 3 different fluorescent stains, and describe a TCAT method that results in the successful distribution, entrapment, and survival of transplanted cells in healthy dogs. Our work was motivated by previous literature that indicated the overall benefit of cellular transplantation in both animals and humans, but also a high incidence of complications using the epicardial injection method. Borenstein et al. reported results of direct injection of myoblasts into

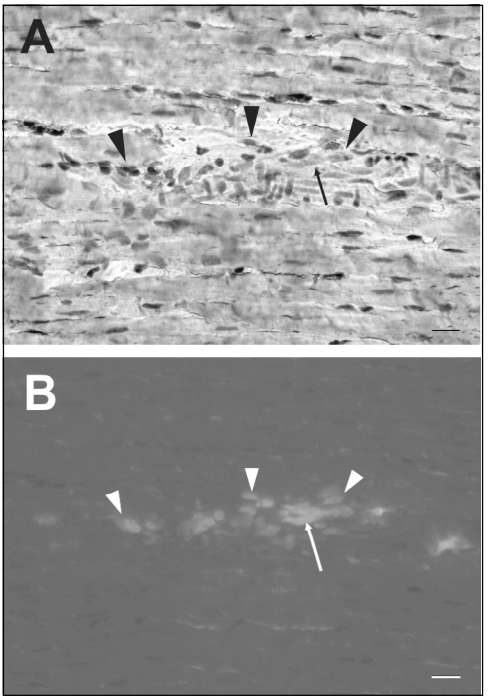

Figure 6. Brightfield (A) and fluorescent (B) microscopic image of canine myocardium from dog 4 that received 3 x106 autologous myoblasts. A) Prominent features of this image include a blood vessel cut in longitudinal section and a lack of inflammatory neutrophils. Note that it is difficult to positively identify transplanted cells (arrowhead and arrow) using conventional staining. B) Fluorescent microscopic image of the tissue shown in (A) indicating an area of co-localization for 4′,6-diamidino-2-phenylindole (DAPI)-(arrow heads) and carbocyanine (DiI)-(arrow)-labeled myoblasts in close proximity to the blood vessel seen in (A). Bar in lower right-hand corner equals 10 microns.

the epicardial surface of 5 dogs with naturally occurring DCM.[24] Three of the 5 dogs died during the postoperative period, 2 due to intractable arrhythmias and 1 due to acute pulmonary thromboembolism. The 2 surviving dogs both demonstrated clinical and echocardiographic improvement 4 months post-procedure. This study underlined both the potential benefit as well as the difficulty in performing direct epicardial injection in diseased dogs. By its nature, surgical injection creates some degree of local tissue damage and inflammatory response, both of which can adversely affect cardiac function and contribute to arrhythmia formation, and a high incidence of perioperative complications, including arrhythmic death, has limited the utility of surgical cellular transplantation in both animal and human subjects.[25,26] Furthermore, surgical injection tends to form localized islet-like clusters of cells that may not be ideal for diseases of global ventricular involvement, such as DCM.[11,12,27] In an effort to address these concerns, novel transplant methods such as TCAT were developed.

The feasibility of TCAT was first postulated by Robinson et al.[19] and performed in rats by Suzuki et al.[18] in 2000. Suzuki et al. successfully injected 1 x 10^6 skeletal muscle myoblasts into rat hearts via TCAT and identified transplanted cells in all layers and regions of the ventricular myocardium 28 days later. In many of these areas, myoblasts had differentiated into multinucleated myotubules and had aligned along the axis of native cardiac fibers, suggesting an active role in myocardial contraction. Since this initial report, arterial delivery of stem cells has been successfully performed in both human and experimental animals.[16,28–30] Of particular interest is the study by Zhong et al. in which dogs received 2 identical sets of myoblasts, one by direct epicardial injection and one by transarterial injection into the distal left anterior descending coronary artery using a tuberculin syringe.[20] Results demonstrated that cells delivered by epicardial injection were devoid of developing myotubules and grew in a haphazard orientation to the axis of the host myocardium. In contrast, myoblasts delivered via coronary artery injection demonstrated striated muscle fibers and intercalated discs, and were distributed parallel with the host tissue. These results suggest that arterially delivered cells both survive and mature in canine cardiac tissues.

In our study, successful delivery and survival of myoblasts was evidenced by the loci of labeled cells found in dogs 3 and 4, sacrificed at 30 minutes and 7 days post-transplant, respectively. Labeled cells were

distributed in the regions perfused by the left coronary artery (that is, the interventricular septum and left ventricular wall) while none were detected in samples of right ventricular tissue. That transplanted cells began to assimilate into the surrounding tissue was indicated by the alignment of labeled cells in dog 4 along the cardiac fiber axis. Survival and viability of these cells was suggested by the absence of a local inflammatory response that typically accompanies cellular necrosis. The histological findings of our study compare favorably to TCAT studies performed in other animal models. Suzuki et al. reported the identification of DAPI and *lacZ*-labeled myoblasts within the ventricular muscle 4 weeks after transplantation.[16,18] Robinson et al. demonstrated entrapment of myoblasts within the coronary capillaries minutes after injection, and migration of cells into the interstitium within the first 7 days.[19] Zhong et al. reported survival of transplanted myoblasts up to 8 weeks post-injection.[20] In all of these previous studies, transplanted cells had elongated and aligned themselves with the axis of the native cardiac fibers, which is similar to our findings in dog 4.

The percentage of cells that survive the transplantation procedure and successfully mature is unknown. Accurate assessment of cell survival post-transplant is very difficult[19,30] and previous studies have employed elaborate and tedious counting procedures.[18] In our study we did not attempt to assess the survival rate of myoblasts. In humans receiving direct surgical injections of myoblasts, the survival rate of cells is thought to be very low, perhaps as low as <1%.[9] When using TCAT, the efficacy of cell entrapment must be accounted for when calculating cell survival. Our results in dog 3, which indicated that labeled cells were not recovered from coronary sinus blood, suggest that TCAT resulted in efficient entrapment of myoblasts in the coronary circulation. This finding agrees with previous work indicating that as many as 90% of cells become lodged in the lumina of small capillaries following coronary injection.[18] Assuming effective entrapment, estimates of cell survival and differentiation following TCAT range from 1.4% to 50%.[16,18,19] The presumably low survival rate of cells delivered by either technique seems incongruous with a substantial contribution to ventricular contractile function, suggesting that the beneficial results of cell transplantation may be due to more indirect effects, such as simulation of angiogenesis, passive girding that limits progressive cardiac hypertrophy, elaboration of paracrine factors that improve function of surrounding myocytes, and enhanced recruitment of native stem cells.[31]

Clearly, the eventual number of cells that survive transplant also depends on the number of cells that are originally injected. The human left ventricle is estimated to contain approximately 5 billion cells, and the ideal number of cells needed to replenish the injured ventricle is not known. Our results indicate that TCAT of up to 3×10^6 myoblasts was well tolerated, whereas injection of 5×10^6 cells resulted in severe arrhythmia and probable cardiac infarction (dog 3). Post-transplant, dog 3 demonstrated cardiac troponin concentrations that were well above the value considered diagnostic of myocardial infarction in humans (> 0.50 ng/mL). The risk of embolic complications is likely related to the number of cells injected. Both Robinson et al.[19] and Suzuki et al.[18] reported the successful injection of 1×10^6 myoblasts into the coronary circulation of mice whereas the injection of 1×10^7 and 1×10^8 cells induced acute myocardial infarction and loss of organ viability.[18] Below the embolic threshold, improvement in heart function is likely proportional to the number of cells that are injected,[32] so that the ideal TCAT procedure requires a balance between injecting a maximal cell number and the risk for thromboembolic events. In any given patient, the tolerance of cell injection may be related to the severity of underlying disease and presence of pre-existing ischemia, as well as technical considerations (for example, effective filtering of large cell clumps

prior to injection, rate of injection, etc.). In a clinical setting, it would be very difficult to predict the optimal cell load that an individual patient could tolerate. To compensate for both the apparently low rate of cell survival and the relatively small number of cells being injected (compared to the total cell number in the ventricle), a series of multiple injections of modest numbers of cells may be beneficial. Whether the sets of injections could be done over a single anesthetic episode or would require serial catheterizations is unknown, but by virtue of its minimally invasive nature, TCAT would be well-suited for repeated injections.

In addition to the cell number, risk of thromboembolism is likely affected by cell size. In humans, the relatively large size of myoblast compared to other candidate stem cell populations (such as bone marrow progenitor cells) may be of particular concern during TCAT.[33] In our study, histomorphometry of the myoblast cultures indicated that the majority of canine myoblasts (especially type 1 cells) were approximately the size of the typical adult bone marrow progenitor cell (20 to 40 microns). In addition, we employed several technical methods to help reduce the risk of acute embolism. To remove large cell clumps, cell suspensions were filtered through a 40-μm filter just prior to TCAT. A 4-port cardiac manifold was used so that coronary artery pressure could be monitored and accidental occlusion of the vessel avoided during catheter placement and cell injection. In our study, the overall safety of the TCAT procedure was demonstrated when injecting up to 3 x 10^6 myoblasts; however, due to the small number of dogs studied, further investigation should be performed to better define the maximal cell load achievable by the TCAT method.

In addition to demonstrating feasibility of the TCAT method, we attempted to evaluate potential complications of nuclear (DAPI), cytoplasmic (GFP), and membrane (DiI) staining of the transplanted cells. Identification of transplanted cells in situ was demonstrated using DAPI and DiI stains, but could not be confirmed using GFP. Reliable cell labeling is crucial when attempting to assess cell survival and differentiation. Previous reports have applied a variety of staining techniques, including GFP,[34–37] DAPI,[16,20] 5-bromo-2'-deoxyuridine (BrdU),[17,38,39] and a *lacZ* reporter gene that encodes for B-galactosidase activity.[11,18,19,30] Technical complications affecting cell labeling include inadequate stain uptake, loss of fluorescent signal, and autofluorescence of native tissue.[40,41] Due a high degree of autofluorescence in canine myocardial tissue (Figure 4), we could not document the presence of transplanted cells in either dog that received GFP-labeled myoblasts. Difficulty with GFP autofluorescence is of particular concern when using tissues of muscle origin.[42] Several other factors may have also contributed to our trouble in identifying GFP-labeled myoblasts. Both dogs receiving GFP-labeled cells (dogs 1 and 2) received a population of heterologous cells, which may have been destroyed by triggering an immune-mediated reaction. This is possible yet unlikely in the absence of an identifiable histological inflammatory response in either dog. Alternatively, GFP may have interfered with the cytoplasmic functions needed for cell survival and engraftment into the native myocardium.[35,36] In contrast to GFP, use of DAPI and DiI stains was successful in identifying transplanted cells and did not appear to affect the survival of myoblasts, at least to the extent that positively labeled cells were identified within the native tissues. The behavior of the DAPI and DiI cell populations was ostensibly similar in that the differentially labeled cells co-localized within the myocardial tissue.

We chose to perform TCAT using skeletal muscle myoblasts. Myoblasts are an attractive transplant population due to their ease of cell culture, ability to produce functional muscle fibers, high mitotic potential, and relative resistance to ischemia.[43] Of the various candidate adult stem cells, namely skeletal muscle myoblasts, smooth muscle

myoblasts, bone marrow mesenchymal cells, and vascular endothelial cells, the superiority of any one type not been established. Potential differences in survival, plasticity, response to underlying disease state (that is, ischemic versus non-ischemic disease) and ability to express cardiac specific structures (such as gap junctions) require further investigation.[44] A common feature of all adult stem cell populations is the difficulty in obtaining pure cultures. With regard to myoblast isolation, the cultured population is imperfect and often contaminated with fibroblasts.[45,46] The relative purity of our cultures was achieved by the enrichment technique and was demonstrated by the in vitro differentiation of isolated cells into primitive multinucleate myotubules. Previous investigations have utilized a variety of techniques to enrich and purify their cultures, including fluorescence activated cell sorting (FACS), Percoll density centrifugation, and in vitro differentiation and staining for anti-sarcomeric proteins.[8,9,16,47,48] The purification of cultures is important because grossly contaminated transplant cultures can impede evaluation of cell survival, differentiation, and biological relevance, as well as confound the comparisons between different studies.[14]

Additional study of cellular cardiac transplantation is required to determine the possible impact of this new technique. Specifically, the biologic behavior of the transplanted canine myoblasts needs to be studied. Previous reports have demonstrated the successful in vivo differentiation of stem cells through positive staining for components such as skeletal muscle myosin,[9,13,36] actin,[17] myogenin,[12] cardiac troponin-I[27], as well as expression of connexin-43,[10,18,19] a component of cardiac gap junctions. Further work is needed to better understand the course of differentiation and ways to optimize this process. For instance, to promote the expression of pro-survival factors and increase survivability of transplanted cells, it may be possible to precondition stem cells prior to transplant.[49] Our study involved only healthy dogs, and the safety and efficacy of TCAT in dogs with heart disease needs to be evaluated.[50,51] Finally, carefully designed studies are required to discover the mechanisms whereby transplanted cells mediate their beneficial effect.

In conclusion, our results demonstrate the feasibility of TCAT using autologous skeletal muscle myoblasts in healthy dogs. Myoblasts can be isolated from muscle biopsies, expanded in cell culture, and successfully labeled with DAPI or DiI. Transplantation is possible using a catheter-based approach, and when injected into the left coronary arterial circulation, myoblasts are entrapped, engraft, and survive in the host myocardial tissue.

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REFERENCES

1. Rumyantsev PP. Interrelations of the proliferation and differentiation processes during cardiac myogenesis and regeneration. *Int Rev Cytol*. 1977;51:186–273.
2. Nadal-Ginard B, Kajstura L, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. *Circ Res.* 2003:92:139–150.
3. Kostin S, Pool L, Elsasser A, et al. Myocytes die by multiple mechanisms in failing human hearts. *Circ Res.* 2003:92:715–724.
4. Shimomura H, Terasaki F, Hayashi T, et al. Autophagic degeneration as a possible mechanism of myocardial cell death in dilated cardiomyopathy. *Jpn Circ J*. 2001:65:965–968.
5. Tidholm A, Haggstrom J, Borgarelli M, Tarducci A. Canine idiopathic dilated cardiomyopathy. Part I: Aetiology, clinical characteristics, epidemiology and pathology. *Vet J*. 2001:162:92–107.
6. The COVE Study Group. Controlled clinical evaluation of enalapril in dogs with heart failure: results of the Cooperative Veterinary Enalapril Study Group. *J Vet Intern Med.* 1995:9:243–252.
7. Fuentes VL, Corcoran B, French A, et al. A double-blind, randomized, placebo-controlled study of pimobendan in dogs with dilated cardiomyopathy. *J Vet Intern Med*. 2002:16:255–261.

8. Passier R, Mummery C. Origin and use of embryonic and adult stem cells in differentiation and tissue repair. *Cardiovasc Res.* 2003:58:324–335.

9. Pagani FD, DerSimonian H, Zawadzka A, et al. Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. *J Am Coll Cardiol*. 2003:41:879-888.

10. Wang JS, Shum-Tim D, Galipeau J, et al. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential clinical advantages. *J Thorac Cardiovasc Surg.* 2000:120:999–1005.

11. Chiu RC, Zibaitis A, Kao RL. Cellular cardiomyoplasty: myocardial regeneration with satellite cell implantation. *Ann Thorac Surg.* 1995:60:12-18.

12. Atkins BZ, Hueman MT, Meuchel J, et al. Cellular cardiomyoplasty improves diastolic properties of injured heart. *J Surg Res.* 1999:85:234–242.

13. Rajnoch C, Chachques JC, Berrebi A, et al. Cellular therapy reverses myocardial dysfunction. *J Thorac Cardiovasc Surg*. 2001:121:871–878.

14. Chachques JC, Acar C, Herreros J, et al. Cellular cardiomyoplasty: clinical application. *Ann Thorac Surg*. 2004:77:1121–1130.

15. Ohno N, Fedak PW, Weisel RD, et al. Cell transplantation in non-ischemic dilated cardiomyopathy. A novel biological approach for ventricular restoration. *Jpn J Thorac Cardiovasc Surg*. 2002:50:457–460.

16. Suzuki K, Murtuza B, Suzuki N, et al. Intracoronary infusion of skeletal myoblasts improves cardiac function in doxorubicin-induced heart failure. *Circulation*. 2001:104(12 suppl 1):I213–I217.

17. Yoo KJ, Li RK, Weisel RD, et al. Autologous smooth muscle cell transplantation improved heart function in dilated cardiomyopathy. *Ann Thorac Surg.* 2000:70:859–865.

18. Suzuki K, Brand NJ, Smolenski RT, et al. Development of a novel method for cell transplantation through the coronary artery. *Circulation*. 2000:102(19 suppl. 3):III359–III364.

19. Robinson SW, Cho PW, Levitsky HI, et al. Arterial delivery of genetically labelled skeletal myoblasts to the murine heart: long-term survival and phenotypic modification of implanted myoblasts. *Cell Transplant*. 1996:5:77–91.

20. Zhong H, Zhu H, Zhang Z. Affects of different access routes on autologous satellite cell implantation stimulating myocardial regeneration [in Chinese]. *Chin Med J.* 2002:115:1521–1524.

21. Taylor DA, Silvestry SC, Bishop SP, et al. Delivery of primary autologous skeletal myoblasts into rabbit heart by coronary infusion: a potential approach to myocardial repair. *Proc Assoc Am Physicians*. 1997:109:245–253.

22. Kapuscinski J. DAPI: a DNA-specific fluorescent probe. *Biotech Histochem.* 1995:70:220–233.

23. Montanaro F, Lindenbaum M, Carbonetto S. alpha-Dystroglycan is a laminin receptor involved in extracellular matrix assembly on myotubes and muscle cell viability. *J Cell Biol.* 1999:145:1325–1340.

24. Borenstein N, Chetboul V, Rajnoch C, et al. Successful cellular cardiomyoplasty in canine idiopathic dilated cardiomyopathy [Letter to the Editor]. *Ann Thorac Surg.* 2002;74:294–295.

25. Minami E, Reinecke H, Murry CE. Skeletal muscle meets cardiac muscle. Friends or foes? *J Am Coll Cardiol*. 2003:41:1084–1086.

26. Strauer BE, Kornowski R. Stem cell therapy in perspective. *Circulation.* 2003:107:929–934.

27. Tomita S, Mickle DA, Weisel RD, et al. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg.* 2002:123:1132–1140.

28. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation*. 2002:106:1913–1918.

29. Assmus B, Schachinger V, Teupe C, et al. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation.* 2002:106:3009–3017.

30. Wang JS, Shum-Tim D, Chedrawy E, Chiu RC. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. *J Thorac Cardiovasc Surg*. 2001:122:699–705.

31. Scorsin M, Hagege AA, Dolizy I, et al. Can cellular transplantation improve function in doxorubicin-induced heart failure? *Circulation*. 1998:98(19 suppl):II151–II155.

32. Pouzet B, Vilquin JT, Hagege AA, et al. Factors affecting functional outcome after autologous skeletal myoblast transplantation. *Ann Thorac Surg*. 2001:71:844–850.

33. Perin EC, Geng YJ, Willerson JT. Adult stem cell therapy in perspective. *Circulation.* 2003:107:935–938.

34. Leobon B, Garcin I, Menasche P, et al. Myoblasts transplanted into rat infarcted myocardium are functionally isolated from their host. *Proc Natl Acad Sci USA.* 2003:100:7808–7811.

35. Min JY, Yang Y, Converso KL, et al. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J Appl Physiol.* 2002:92:288–296.

36. Tomita S, Nakatani T, Fukuhara S, et al. Bone marrow stromal cells contract synchronously with cardiomyocytes in a coculture system. *Jpn J Thorac Cardiovasc Surg.* 2002:50:321–324.

37. Orlic D, Hill JM, Arai AE. Stem cells for myocardial regeneration. *Circ Res*. 2002:91:1092–1102.

38. Yoo KJ, Li RK, Weisel RD, et al. Heart cell transplantation improves heart function in dilated cardiomyopathic hamsters. *Circulation*. 2000:102(19 suppl 3):III204–III209.

39. Yokomuro H, Li RK, Mickle DA, et al. Transplantation of cryopreserved cardiomyocytes. *J Thorac Cardiovasc Surg.* 2001:121:98–107.

40. Jackson KA, Majka SM, Wulf GG, Goodell MA. Stem cells: a minireview. *J Cell Biochem Suppl*. 2002:381–386.

41. Watson TF. Fact and artefact in confocal microscopy. *Adv Dent Res*. 1997:11:433–441.

42. Jackson KA, Snyder DS, Goodell MA. Skeletal muscle fiber-specific green autofluorescence: potential for stem cell engraftment artifacts. *Stem Cells.* 2004:22:180–187.

43. Eckert P, Schnackerz K. Ischemic tolerance of human skeletal muscle. *Ann Plast Surg*. 1991:26:77–84.

44. Hassink RJ, Brutel dlR, Mummery CL, Doevendans PA. Transplantation of cells for cardiac repair. *J Am Coll Cardiol*. 2003:41:711–717.

45. Pouzet B, Vilquin JT, Hagege AA, et al. Intramyocardial transplantation of autologous myoblasts: can tissue processing be optimized? *Circulation.* 2000:102(19 suppl 3):III210–III215.

46. Blanco-Bose WE, Yao CC, Kramer RH, Blau HM. Purification of mouse primary myoblasts based on alpha 7 integrin expression. *Exp Cell Res.* 2001:265:212–220.

47. Jankowski RJ, Haluszczak C, Trucco M, Huard J. Flow cytometric characterization of myogenic cell populations obtained via the preplate technique: potential for rapid isolation of muscle-derived stem cells. *Hum Gene Ther.* 2001:12:619–628.

48. Dorfman J, Duong M, Zibaitis A, et al. Myocardial tissue engineering with autologous myoblast implantation. *J Thorac Cardiovasc Surg.* 1998:116:744-751.

49. Puceat M, Travo P, Quinn MT, Fort P. A dual role of the GTPase Rac in cardiac differentiation of stem cells. *Mol Biol Cell*. 2003:14:2781–2792.

50. Mangi AA, Noiseux N, Kong D, et al. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med.* 2003:9:1195–1201.

51. Akita T, Murohara T, Ikeda H, et al. Hypoxic preconditioning augments efficacy of human endothelial progenitor cells for therapeutic neovascularization. *Lab Invest.* 2003:83:65–73.