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BASIC SCIENCE

Intrinsic cardiac origin of human cardiosphere-derived cells

Anthony J. White[1,2], Rachel R. Smith[1], Satoshi Matsushita[1], Tarun Chakravarty[1], Lawrence S.C. Czer[1], Kevin Burton[1,3,4], Ernst R. Schwarz[1], Darryl R. Davis[1], Qi Wang[5], Nancy L. Reinsmoen[5], James S. Forrester[1], Eduardo Marbán[1]*, and Raj Makkar[1]

[1]Cedars-Sinai Heart Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; [2]Department of Medicine, Monash Cardiovascular Research Centre, Monash University, Melbourne, Australia; [3]Minimally Invasive Surgical Technologies Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; [4]Division of Cardiovascular Surgery, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; and [5]Medical Genetics, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA

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Aims Cardiosphere-derived cells (CDCs) are in clinical development as a regenerative cell product which can be expanded *ex vivo* from patient cardiac biopsies. Cardiosphere-derived cells are clonogenic, exhibit multilineage differentiation, and exert functional benefits in preclinical models of heart failure. The origin of CDCs remains unclear: are these cells endogenous to the heart, or do they arise from cells that populate the heart via blood-borne seeding?

Methods and results Right ventricular endomyocardial biopsies were obtained from cardiac transplant recipients ($n = 10$, age 57 ± 15 years), and CDCs expanded from each biopsy. Donor-recipient mismatches were used to probe the origin of CDCs in three complementary ways. First, DNA analysis of short-tandem nucleotide repeats (STRs) was performed on genomic DNA from donor and recipient, then compared with the STR pattern of CDCs. Second, in two cases where the donor was male and the recipient female, CDCs were examined for the presence of X and Y chromosomes by fluorescence *in situ* hybridization. Finally, in two cases, quantitative PCR (qPCR) was performed for individual-specific polymorphisms of a major histocompatability locus to quantify the contribution of recipient cells to CDCs.

In no case was recipient DNA detectable in the CDCs by STR analysis. In the two cases in which a female patient had received a male heart, all CDCs examined had an X and Y chromosome, similarly indicating exclusively donor origin. Likewise, qPCR on CDCs did not detect any recipient DNA.

Conclusion Cardiosphere-derived cells are of endogenous cardiac origin, with no detectable contribution from extra-cardiac seeding.

Keywords Cardiosphere-derived cells • Cell therapy

Introduction

The adult heart was long believed to be incapable of self-regeneration following injury. The documentation that cardiomyocytes are capable of division[1,2] challenged that notion, which was further undermined by the discovery of stem cells located within the heart.[3–7] Furthermore, after cardiac injury, genetic fate-mapping studies have demonstrated that stem cells or precursor cells contribute to the replacement of adult cardiomyocytes.[8]

In a related development, techniques have been developed to coax proliferating cells from cardiac muscle. One such protocol involves production of *cardiospheres* that are self-assembling cell clusters enriched for stem cell characteristics,[9] which are then replated to yield cardiosphere-derived cells (CDCs).[10] Cardiosphere-derived cells have been extensively characterized and developed as a therapeutic product,[10–18] culminating in the first-in-human trial CADUCEUS (CArdiosphere-Derived aUtologous Stem CElls to Reverse ventricULar dySfunction; see clinicaltrials.gov for details). An unresolved issue is whether CDCs are endogenous to the heart, or whether they home to the myocardium from an extra-cardiac origin, perhaps the bone marrow.[3,8]

It does appear that blood-borne seeding contributes, at least in part, to the resident cardiac stem cell population in experimental animals,[19] and it is possible that human cardiac stem cells may be

* Corresponding author. Tel: +1 310 423 7557, Fax: +1 310 423 7637, Email: eduardo.marban@cshs.org

renewed externally in the same way.[20] Similarly, studies of transplanted human hearts have established that after some time, various types of mature cardiac cells are able to be replaced by external (recipient) cell sources[21–23] (endothelial>smooth muscle cells>cardiomyocytes). Hence, it is possible that the cells within the cardiac biopsy that give rise to CDCs may originate outside of the heart. In particular, because ~5–20% of CDCs express the cell surface-receptor c-kit,[10] and because it has been demonstrated that the heart can be populated by bone-marrow derived c-kit$^+$ cells,[24] we hypothesized that a significant proportion of CDCs originate outside the heart. To investigate this hypothesis, we exploited the paradigm of human cardiac transplantation, which allowed identification of CDCs as donor (cardiac) or recipient (extra-cardiac) in origin, or a combination of the two.

Methods

Study participants

The study was approved by the Institutional Review Board of Cedars-Sinai Medical Center, Los Angeles, California and was in compliance with the principles of the declaration of Helsinki. Informed consent was obtained from 10 subjects prior to a planned, clinically-indicated cardiac biopsy procedure, performed for rejection screening after cardiac transplantation. Consent was specifically obtained for taking an extra biopsy sample for research purposes, in addition to the 5–9 samples dedicated to clinically-indicated screening for rejection. Percutaneous biopsy was performed with a 7-French bioptome (Ceres Medical Systems, Texas) via the jugular or femoral vein according to routine clinical methods.[25] Biopsy specimens were obtained from randomly-selected portions of the right ventricular endomyocardium, including the interventricular septum, apex, and occasionally the free wall of the right ventricle. Care was taken to avoid taking samples from the same location in the right ventricle with sequential passes of the bioptome in any given harvesting procedure.

Culture of human cardiosphere-derived cells

Cardiosphere-derived cells, cultured according to published protocols,[9,10] were successfully derived from all 10 biopsied subjects. Briefly, cardiac biopsy specimens (10–40 mg) were minced, and subjected to 5 min digestion with trypsin 0.25%-EDTA (Sigma). These explants were plated onto fibronectin-coated dishes (BD biosciences) in explant medium [IMDM (Invitrogen), 20% foetal bovine serum (FBS) (Invitrogen), 1% penicillin-streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 0.1 mM 2-mercaptoethanol (Sigma)].

Within 7–28 days, a primary outgrowth of adherent cells grew out radially in monolayer, from the biopsy fragments. This outgrowth was harvested (0.25% trypsin-EDTA) and re-plated into poly-D-lysine-coated wells (BD biosciences) in cardiosphere medium [7 mL IMDM (Invitrogen), 13 mL DMEM/F12 (Invitrogen), 0.7 mL FBS (Invitrogen), 0.2 mL penicillin-streptomycin (Invitrogen), 0.2 mL of 200 mM L-glutamine (Invitrogen), 20 U thrombin (Sigma), 0.4 mL of B-27 (Invitrogen), 1.6 μg bFGF, 0.5 μg EGF, and 80 ng cardiotrophin (Peprotech)]. Under these conditions, some of the cells gave rise to free-floating clusters of cells, termed cardiospheres. In a third phase, the adherent cells were discarded, while the cardiospheres were collected and plated onto fibronectin-coated 25 cm^2 flasks (BD Biosciences). The cardiospheres adhered and flattened to form a monolayer of cells referred to as CDCs; these were passaged and divided 1:2 into new fibronectin-coated flasks as they became confluent. Cardiosphere-derived cells from the second passage were used in all experiments.

Characterization of cardiosphere-derived cells

The morphology of the cells at various stages of the culture protocol was monitored using phase-contrast microscopy and digital images were captured from a video camera. Cell surface markers were characterized by flow cytometry using the following antibodies—CD105-PE (R&D Systems), CD90-fluorescein isothiocyanate (FITC) (Abcam), and c-kit primary antibody (R&D Systems) followed by a FITC-labelled secondary antibody (polyclonal rabbit anti-goat IgG, Abcam). Appropriate isotype control antibodies were used as negative controls.

Short-tandem repeat testing

Polymerase chain reaction (PCR) amplification of 16 genetic loci was performed, followed by capillary electrophoresis to detect the fluorescently labelled PCR products. Each tested locus exhibited variation in the number of short-tandem nucleotide repeats (STRs) within the population. By combining the information about the number of STRs at each locus, for each allele, a unique genetic identity can be assigned to an individual, making this a prevalent technique in forensic science.[26–28] Short-tandem nucleotide repeat testing was performed on genomic DNA from three sources—the CDCs, the donor, and the cardiac transplant recipient. Comparison of STR patterns allowed determination of the proportion of CDC DNA originating from donor or recipient. Quantification of relative donor/recipient DNA was obtained for each informative STR marker by using the area under the curve of the capillary electrophoresis peak from the respective DNA sources.

The accuracy of the STR test was validated by making mock chimeric mixes of DNA from donor and from recipient, in three ratios, 5:95, 50:50, and 95:5, and constructing a standard curve based on STR analysis of genetic loci that allowed differentiation between donor and recipient DNA.

Fluorescence *in situ* hybridization

The presence of X and Y chromosomes in interphase CDCs was determined by fluorescence *in situ* hybridization (FISH) with probes specific for the centromeres of the X and Y chromosomes (Abbott Molecular, DXZ1, DYZ3). All available CDCs from each FISH slide were analysed for the presence or absence of the X and Y chromosomes. As a general control for accuracy of the method, FISH performed on CDCs derived from endomyocardial biopsies of non-transplanted patients with left ventricular dysfunction reproduced accurately the known gender and expected chromosome number (n = 18 subjects, with n = 20–30 CDCs counted by FISH per subject).

Quantitative real-time polymerase chain reaction

Quantitative relative real-time PCR was performed on CDC genomic DNA, using the Taqman method[29,30] (Taqman Universal Master Mix, ABI) on a real-time PCR instrument (7900HT Fast Real-Time PCR System, Applied Biosystems). Primers and probes for the human beta-globin (HBB) gene were based upon previously published oligonucleotides[31] (*Table 1*). Cardiosphere-derived cell DNA from two research participants was studied. Tissue typing data was used to identify a human leucocyte antigen (HLA)-A allele that distinguished donor from recipient. Subject 5 (*Table 2*) has HLA-A alleles 3 and 24, and their donor had HLA-A alleles 2 and 3. Thus HLA-A24 was used as a recipient-specific allele for this case. Subject 6 (*Table 2*) had a

Table 1 Primers and probes used for Taqman quantitative real-time polymerase chain reaction

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Probe (conjugated with 5′FAM and 3′TAMRA)
HBB	gtgcatctgactcctgaggaga	ccttgataccaacctgcccag	aaggtgaacgtggatgaagttggtgg
HLA-A24 (subject 5, recipient)	tgggacgaggagacagggaaag	gtcactcaccggcctcgctct	tactgcggatcgcgctccgctaccaa
HLA-A68 (subject 6, recipient)	agttcgtgcggttcgacagcga	ggtccccaggtccactcggtca	tgggaccggaacacacggaatgtgaa

HBB, haemoglobin B; HLA, human leucocyte antigen.

Table 2 Characteristics of the cardiac transplant recipients

Subject number	Age, years	Gender of the heart recipient	Gender of the heart donor	Time since transplant, days	Donor-recipient gender mismatch?
1	58	M	M	63	No
2	24	F	M	370	Yes
3	41	M	M	15	No
4	50	M	M	1541	No
5	71	M	M	612	No
6	61	F	M	16	Yes
7	75	M	M	21	No
8	65	M	M	93	No
9	62	M	M	22	No
10	61	M	M	2	No

HLA-A68 allele, which the donor did not have. Thus HLA-A68 was used as a recipient-specific allele. Sequence level data about these HLA-A alleles were determined from the online ImMunoGeneTics (IMGT)/HLA Database (http://www.ebi.ac.uk/imgt/hla/), part of the ImMunoGeneTics (IMGT) project. Knowledge of the sequence was used to design primers and probes (*Table 1*) to detect recipient cells, if present, in the CDCs.

Beta-globin was used as the internal control for quantitative PCR (qPCR) to confirm PCR amplification, with an expected relative abundance of 2:1 relative to the donor-specific HLA-A allele under investigation (diploid globin gene vs. heterozygous HLA-A alleles).

Recipient DNA was spiked into other human DNA samples to validate a log-linear relationship of delta Ct. Delta Ct was the difference in threshold cycle (Ct) of beta-globin to the recipient-specific DNA primer/probe set.

Statistical analysis

Data are presented as mean ± SD, except where indicated otherwise. The STR method for quantification of chimerism was validated by calculating the correlation co-efficient between artificial mixes of DNA, and the STR assay result. Throughout the studies, two-sided tests were used and a *P*-value of <0.05 was deemed significant. Statistical analyses were performed in SPSS version 17 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

The characteristics of the transplant recipients are shown in Table 2 ($n = 10$, age 56.8 ± 15.0 years, 8 of 10 subjects male.) All the transplanted hearts were from male donors. Mean time since cardiac transplantation was 283 ± 484 days (mean ± SD), with a range of 2–1541 days, a median of 57 days, and an interquartile range of 26–317 days.

Growth and characterization of cardiosphere-derived cells

The morphology and surface marker profile of the cultured cells were consistent with previous characterizations of CDCs.[10,13] *Figure 1* shows representatitive images of cell morphologies observed at each stage of CDC isolation and growth. The percentage of CDCs positive for various surface markers by flow cytometry was 84 ± 13% for CD105, a transforming growth factor beta receptor subunit; 24 ± 11% for CD90, otherwise known as Thy-1; and 8 ± 5% for c-kit, the stem cell factor receptor ($n = 5$, mean ± SD).

We next proceeded to investigate the cellular origin of CDCs.

Determination of cardiosphere-derived cell origin using short-tandem repeat testing

First, the CDCs underwent testing for short-tandem repeat polymorphisms at 16 genetic loci, which allowed DNA from the donor and recipient to be distinguished because of the distinctive allele patterns in DNA from each individual.

For validation of the test, artificial mixes of donor and recipient DNA were made in three ratios: 95:5, 50:50, and 5:95. *Figure 2* shows the results of these validation assays derived from 30

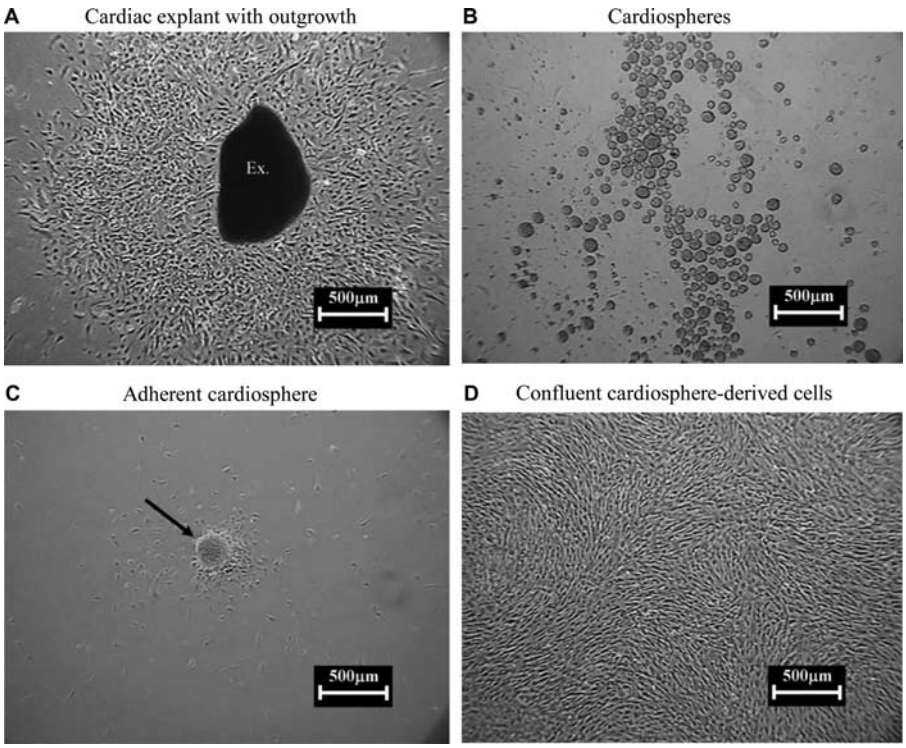

Figure 1 Representative cell morphology during each stage of the cardiosphere-derived cell protocol. (*A*) A cardiac biopsy explant (Ex.), with outgrowth of a monolayer of cells on fibronectin coated plastic. (*B*) The monolayer is harvested and placed into poly-D-lysine-coated plastic wells, resulting in the formation of floating clusters of cells known as cardiospheres. (*C*) The cardiospheres are plated back onto fibronectin-coated plastic, whereupon they adhere, flatten and spread once again as a monolayer (cardiosphere-derived cells). Black arrow indicates one cardiosphere. (*D*) Sheets of cardiosphere-derived cells become confluent. They are then harvested and passaged.

instructive STR tests in five different donor/recipient pairs, and in the three donor: recipient ratios. There was excellent correlation of the experimental DNA proportions with the STR assay result ($r^2 = 0.992$, $P < 0.00001$), indicating that this is an accurate test for quantification of the relative contribution of DNA from donor and recipient cells.

Figure 3 shows a typical result of an instructive STR allele, defined as an allele that allowed DNA from the two individuals to be distinguished. It demonstrates that the CDC DNA pattern exactly matches the donor DNA pattern. Of particular note, there is no recipient pattern DNA detectable within the CDCs. *Table 3* summarizes the STR results, indicating that for each donor/recipient pair there were four to eight instructive genetic loci. There was no convincing recipient DNA signal detected within CDC DNA for any of the instructive loci, for any of the 10 subjects. The lower detection limit of a minor allele within a chimeric DNA mixture is ~0.5–1.0%.[32]

Determination of cellular origin of cardiosphere-derived cells by fluorescence *in situ* hybridization

In the two cases in which a female patient received a heart transplant from a male donor, FISH was performed to label X and Y chromosomes. All available cells were analysed for each case, and *Figure 4* shows representative images. All examined CDCs (511 of 511, and 74 of 74 for research subjects #2 and #6, respectively) had an X and a Y chromosome, indicating male (donor) origin. Thus no CDCs of recipient origin could be detected by the FISH method either.

Determination of cellular origin of cardiosphere-derived cells by quantitative polymerase chain reaction of a recipient-specific HLA-A allele

The data presented to this point indicate that a very large majority, and perhaps all CDCs are cardiac in origin. Although we had failed to find any evidence of any recipient-origin DNA within CDCs, we aimed next to establish a lower limit on the number of recipient-origin cells that may be present within CDCs. A third method, qPCR, was employed to address this question in CDCs from subject 5 and subject 6. Recipient-specific primers and probe oligonucleotides were designed based on a recipient HLA-A allele. The beta-globin gene was used as an endogenous control for the PCR reaction. The primer/probe sets were validated for both subjects (*Figure 5A*). *Figure 5B* illustrates the results in subject 5. The recipient-specific primer/probes detected

HLA-A24 in recipient-derived DNA (positive control). Quantitative PCR for HLA-A24 detected no signal in donor-derived DNA even after 40 cycles (negative control). There was no qPCR signal for HLA-A24 in CDC-derived DNA, in concordance with other two methodologies. These qualitative findings were replicated for Subject 6, using HLA-A68 specific primers and probes; i.e. a recipient-specific qPCR signal was never detected in CDC DNA, even after 40 cycles.

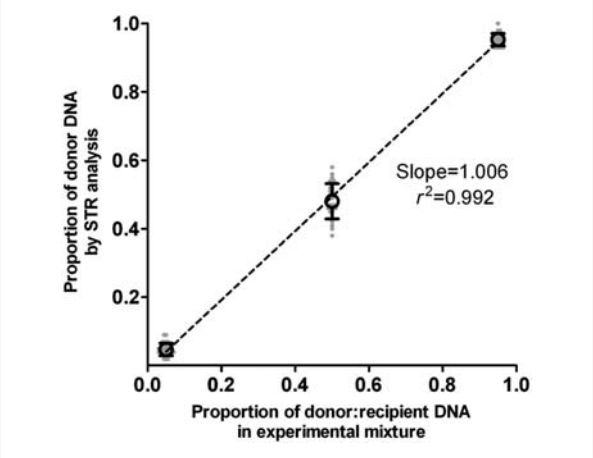

Figure 2 A spiking experiment validated the short-tandem repeat assay for determination of the proportion of DNA of interest within a DNA mixture. The proportion of DNA from donor, as measured by analysis of the proportional signals from short-tandem repeat polymorphism alleles in three artificial mixtures of donor: recipient DNA (5%:95%, 50%:50%, and 95%:5% ratios) was 4.8 ± 1.8, 48.0 ± 5.0, and 95.3 ± 1.8%, respectively (mean ± SD, $n = 30$ short-tandem nucleotide repeats in five different donor/recipient pairs, individual data points shown in grey). The actual proportion correlated closely with the assay result ($n = 90$ data points, $r^2 = 0.992$, $P < 0.00001$).

Based on (i) the PCR cycle at which the HBB gene amplification signal appeared when CDC DNA was used as template, (ii) the known relationship between the threshold cycles (Ct) of HBB and HLA-A reactions, and (iii) the absence of any HLA-A signal after 40 cycles, it was possible to calculate the lower limit of the proportion of recipient DNA within CDC DNA. By this methodology, a conservative estimate of the lower limit of any recipient DNA that may be present in CDC DNA was <1:1000. This places a lower limit on any extra-cardiac contribution to CDCs.

Discussion

We have demonstrated, by three independent molecular methods, that CDCs originate within the heart, with no demonstrable extracardiac contribution. All three methods used—short tandem repeat analysis, FISH for sex chromosomes, and qPCR of recipient-specific HLA-A alleles—resulted in a concordant conclusion. Thus, the evidence negates the hypothesis that a proportion of CDCs might originate from outside of the heart.

PCR–STR analysis is a reproducible and accurate method for determination of chimerism, and the relative contribution of each cell population, in the field of allogenic bone marrow transplantation.[33,34] The STR data presented here clearly indicate that the large majority of CDCs, if not all of them, originate from within cardiac tissue. Short-tandem nucleotide repeat methodology can only confidently detect minor cell populations of 0.5–5.0%,[32–35] although with rational selection of the polymorphisms used, the sensitivity in practice is thought to be at the lower end of this estimate. Therefore, although the current data convincingly demonstrate that CDCs are predominantly cardiac in origin, a small extra-cardiac contribution to CDCs, below this sensitivity threshold, could not be ruled out by the STR method. Hence, we also used qPCR, which established a lower limit of 0.1% as the maximum possible contribution of recipient cells to CDCs. This conclusion is further confirmed by the finding of no recipient cells among a total of 585 CDCs analysed by FISH (from two hearts). Remarkably, given the various complementary limitations

Table 3 Short-tandem repeat testing

Subject number	Informative genetic loci	Summary
1	D7S820, D2S1338, D8S1179, D21S11, and CSF1PO	Allele pattern of DNA from CDCs matched donor heart DNA, with no evidence of a recipient contribution
2	D16S539, D7S820, D8S1179, D2S1338, CSF1PO, TH01, and D21S11	As for subject 1
3	D8S1179, D2S1338, D13S317, vWA, D7S820, D21S11, and CSF1PO	As for subject 1
4	TH01, D18S51, D5S818, D8S1179, D2S1338, and D21S11	As for subject 1
5	D21S11, D19S433, D18S51, D7S820, TH01, and D16S539	As for subject 1
6	D13S317, D16S539, FGA, D19S433, D5S818, and D21S11	As for subject 1
7	D8S1179, D7S820, D13S317, TPOX, D16S539, D2S1338, D19S433 and vWA	As for subject 1
8	D8S1179, D18S51, D5S818, D16S539, D13S317, D2S1338, and vWA	As for subject 1
9	CSF1PO, D3S1358, D13S317, D7S820, D8S1179, D21S11, D16S539, and TH01	As for subject 1
10	D21S11, D3S1358, vWA, D16S539, and D2S1338	As for subject 1

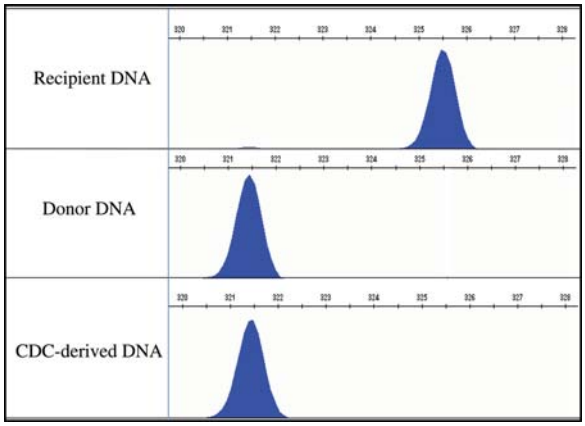

Figure 3 No DNA of recipient origin could be detected in cardiosphere-derived cell-derived DNA by analysis of short-tandem repeats. In this representative example, DNA from three sources (recipient, donor, and cardiosphere-derived cells) has been amplified for the CSF1PO genetic polymorphism locus. The vertical axis indicates the amount of polymerase chain reaction product. The horizontal axis indicates the size of the polymerase chain reaction product (heavier amplification products appearing to the right of smaller products). In this example both the donor and the recipient are homozygous at this locus, with different allele sizes. The smaller peak in donor cell DNA is a polymerase chain reaction artefact. Note that cardiosphere-derived cell DNA contains no detectable signal from recipient DNA.

of the various approaches, never did we find evidence of any recipient contribution to CDCs.

Given that recipient/donor chimerism of transplanted hearts is a well-documented phenomenon,[20–23] our data are notable for the fact that there was no detectable extra-cardiac contribution to CDCs by the STR, FISH, or qPCR methods, even when derived from a heart that had been transplanted more than 4 years previously (subject 6). This indicates the cells within the cardiac biopsy which give rise to CDCs do not significantly seed the heart from an extra-cardiac source, even over a period of several years.

The conclusions presented here only relate to the origin of CDCs, and not the origin of any of the various antigenically-purified subtypes of resident cardiac stem cells which have been described.[3–7,36] Nevertheless, the fact that CDCs are a heterogeneous mix of various populations of cells would favour the detection of mismatched cells within any given component population, unless such cells comprise an infinitesimal subfraction of CDCs.

A clinical implication of the current findings is that cardiac tissue will be required in order to grow CDCs for future clinical trials, as is the case with the ongoing CADUCEUS study, and for any eventual use of the cells as a treatment for heart disease. Less-invasive sources of tissue, such as peripheral blood or bone marrow, will not suffice as a starting material to grow this particular cell product.

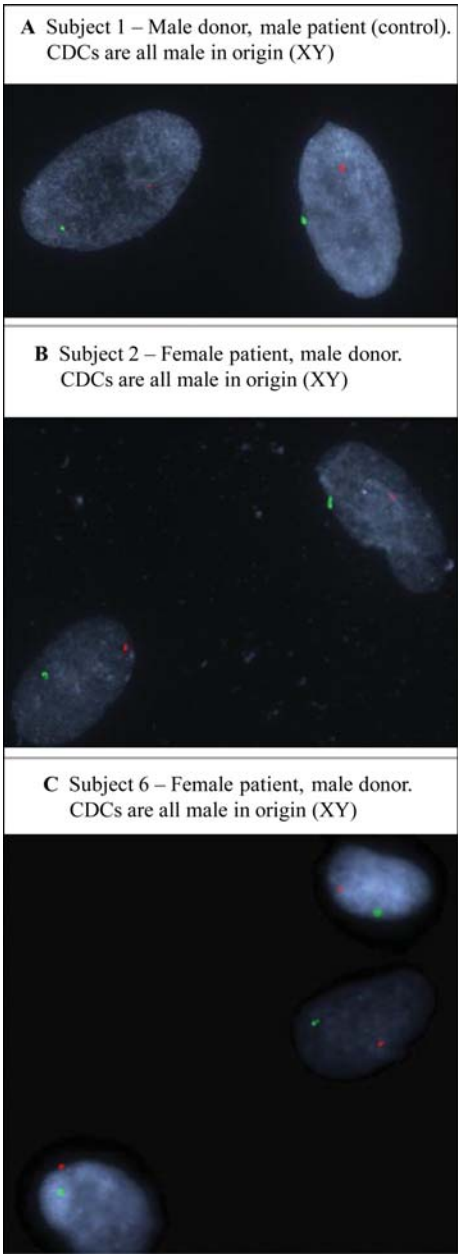

Figure 4 No cells of recipient origin could be detected by fluorescence *in situ* hybridization of X and Y chromosomes in cardiosphere-derived cells. X is stained red, Y is stained green. Representative cells shown. (*A*) Subject #1, a male subject, with a male donor, was used as a positive control specimen. Five hundred of 500 cells examined were male in origin (XY), as expected. (*B*) Subject #2 was a female subject, with a male cardiac donor. Five hundred and eleven of 511 cardiosphere-derived cells examined were male in origin (XY). (*C*) Subject #6 was a female subject, with a male cardiac donor. Seventy-four of 74 cardiosphere-derived cells examined were male in origin (XY).

The current data add to our knowledge base regarding CDCs. Much has already been established about these cells including their surface markers,[10] single cell electrophysiology,[10] growth

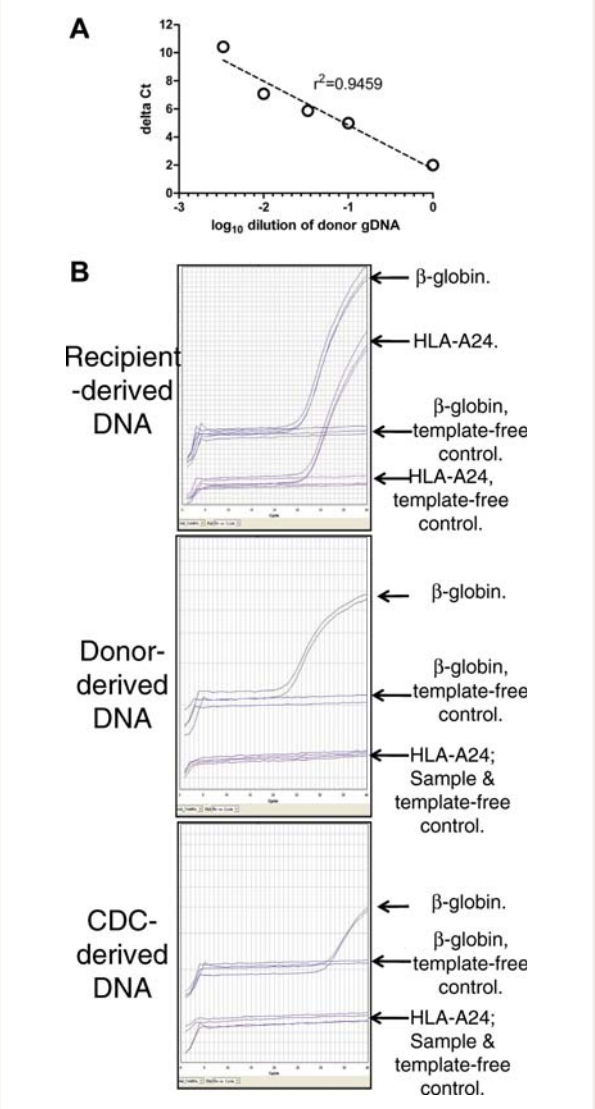

Figure 5 No DNA of recipient origin could be detected in cardiosphere-derived cell DNA by quantitative polymerase chain reaction using recipient-specific probes. (*A*) Primers and probes for HLA-A24 were validated by serial dilutions of recipient-derived DNA from subject 5. Delta Ct refers to the difference in the threshold cycle between beta-globin and HLA-A24 amplification reactions. The HLA-A68 primers and probes were similarly validated for subject 6. (*B*) Illustrative real-time polymerase chain reaction readouts for beta-globin and for HLA-A24 using recipient-, donor- and cardiosphere-derived cell-derived genomic DNA from subject 5. The real-time polymerase chain reaction curves indicate that the primers and probes work well using recipient- DNA as template (positive control, upper panel), but that no HLA-A24 DNA signal was detected using donor-derived (middle panel) or cardiosphere-derived cell-derived (lower panel) DNA as template.

characteristics,[10,13] clonogenicity and multilineage potential,[10,13] and ability to mediate functional improvement in large[14,15] and small[10,12] animal models of cardiac dysfunction. The current findings have clarified that CDCs originate within the heart, thereby clearly differentiating CDCs from other non-cardiac cell types currently under investigation (e.g. bone marrow-derived mesenchymal[37–39] and mononuclear cells[40] and endothelial progenitor cells[41]).

Limitations

Chemotactic mediators recruit blood-derived cells to the heart (and peripheral tissues for that matter) in response to ischaemia or injury. The transplanted hearts studied in this paper were not known to be affected by ischaemia based on standard clinical criteria. In the setting of acute and/or chronic myocardial ischaemia, the heart may become enriched with blood-derived cells;[8] in such a situation, unlike that explored here, it is conceivable that some cardiosphere-derived cells might end up being of non-cardiac origin.

The 10 individuals studied in this paper were taking immunosuppressive agents to suppress rejection. We chose to study such patients for two reasons: (i) The presence of genetic differences between donor and recipient allowed determination of the origin of the cells, and (ii) the patients were undergoing clinically-indicated cardiac biopsy, enabling the ethical harvesting of tissue. Although there is no reason to suspect it (indeed, the opposite has been reported[20–23]), we cannot rule out the possibility that immunosuppression may inhibit blood-derived seeding of the heart, in which case our findings might not apply to immunocompetent individuals.

In addition, the CDCs in these studies were derived from relatively small endocardial biopsy specimens. Although efforts were made to sample various parts of the right ventricle, it is remotely possible that different results might have been obtained from other regions of the myocardium (e.g. from the left ventricle). Thus, we cannot categorically conclude that our results are generalizable to the whole heart, although prior work has revealed no major differences among cardiac-derived cells harvested from diverse regions of the heart.[12]

Finally, it must be acknowledged that, for two of the three molecular methods used in this paper—sex chromosome FISH, and qPCR of HLA alleles—the conclusions are based on samples from only two research subjects with appropriately mismatched donor/recipient pairs.

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Conflict of interest: E.M. holds founder's equity in Capricor, Inc. R.R.S. is an employee of Capricor, Inc.

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