Online Tables

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Figure I. CSC preparation. A, Number and weight of cardiac biopsies. **B,** Time required for the initial expansion of small cardiac cells. **C,** Time required for the expansion of c-kit-positive mCSCs. **D,** Time required for the expansion of c-kit-positive KDR-positive vCSCs. Individual and average values are shown.

Methods

Myogenic (mCSCs) and Vasculogenic (vCSCs) Cardiac Stem Cells

Endomyocardial biopsies were obtained ex vivo from the right side of the interventricular septum of explanted hearts or from the apex of the left ventricle following LVAD implantation. Biopsies were collected with a standard clinical bioptome (7F Maxi-Curved, Argon) and were directed against the mid-right ventricular septum to mimic as much as possible the location of biopsies performed from a right internal jugular approach. The choice was made to obtain these specimens ex-vivo so as to 1) not interfere with the flow of clinical care involved in patients undergoing transplant often during off hours and 2) not put patients through additional procedures carrying additive risks such as arrhythmia and perforation. Two-five biopsies were harvested from each heart, pooled together, and subjected to enzymatic digestion in a solution containing collagenase.[1,2] Unfractionated small cardiac cells were grown and subsequently selected for c-kit with immunomagnetic beads (Miltenyi). C-kit-positive cells were then expanded in F12K medium supplemented with 10% fetal bovine serum to reach 5 x 10^6 cells. Cells were analyzed by FACS to evaluate their surface phenotype. C-kit-positive cells were also sorted for KDR with immunomagnetic beads to obtain vCSCs.

FACS Analysis

mCSCs and vCSCs were fixed in 4% paraformaldehyde for 15 minutes at room temperature and incubated with antibodies against epitopes of bone marrow hematopoietic and mesenchymal cells (Online Table II).[1-5] The fraction of c-kit-positive cells expressing KDR was also measured. In all cases, CSCs were incubated with the primary antibody for 45 minutes at 37°C. Flow cytometry was performed with FACSAria (Becton Dickinson), or Accuri C6 (Accuri Cytometers) instruments. Cellular debris and aggregates were gated out based on forward and side scatter. Gating on the signal of the nuclear stain DAPI was used to exclude additional artifacts. Isotype-matched negative controls were utilized to define the threshold for each specific signal and establish the appropriate gate. Data were analyzed with the instrument software.

Population Doubling Time (PDT), BrdU Labeling, Senescence and Apoptosis

mCSCs and vCSCs were plated at low density, 100 cells per cm^2. The number of cells per unit area was determined daily for 5-7 days.[1,2] PDT was computed by linear regression of $\log_2$ values of cell number. Only values in the exponential growth phase were used. To determine the fraction of cycling cells, BrdU (1 μg/ml) was added to the

medium for 12 hours. Cells were fixed in 4% paraformaldehyde and BrdU incorporation was determined by staining with a mouse monoclonal antibody (Roche) and confocal microscopy. BrdU positive and negative cells were considered cycling and non-cycling mCSCs or vCSCs, respectively. The fraction of cells that reached replicative senescence and irreversible growth arrest was evaluated by the expression of the senescence-associated protein $p16^{INK4a}$. Apoptosis was measured by TdT labeling (ApoAlert DNA Fragmentation Assay, Clontech).[1-3,5,6-8]

Telomere Length

Quantitative measurements of telomere length were determined by flow-FISH. Calibration of the flow cytometer, cell fixation, staining protocol and normalization were performed utilizing mouse lymphoma cells with known telomere length. Approximately 5 x 10^5 mCSCs or vCSCs and mouse lymphoma cells with long telomeres (L5178Y-R) were washed in hybridization buffer and re-suspended in hybridization solution containing formamide and 0.3 μg/ml FITC-conjugated PNA probe. Control samples were incubated in hybridization solution in the absence of PNA probe. Lymphoma cells were distinguished from hCSCs by immunolabeling with CD45 antibody. DNA content was established by propidium iodide staining. Cells were gated at G0/G1 based on DNA content and fluorescence intensity of telomeres was calculated.[9,10] All measurements were performed on the FACSCanto instrument (Becton Dickinson).

Telomerase Activity

The catalytic activity of telomerase was assessed by quantitative PCR.[11,12] Cells were homogenized in CHAPS buffer and centrifuged at 4°C. Two different protein concentrations, 0.5 and 1 μg, were employed for the assay. mCSC and vCSC extracts were incubated in a solution containing reverse transcriptase reaction mix and Taq polymerase (TRAPEZE RT Telomerase Detection Kit, Chemicon) at 30°C for 30 minutes. HeLa cells were used as positive control and serial dilutions of control template TSR8 were employed for quantitation. CHAPS buffer in the absence of protein lysates was used as negative control. PCR cycling conditions were as follows: 1 cycle of 95°C for 2.0 minutes; 40 cycles of 94°C for 15 seconds and 59°C for 60 seconds. Data were collected at the 59°C stage of each cycle.

Differentiation of mCSCs and vCSCs

mCSCs and vCSCs were exposed to dexamethasone, 10^{-8} M, for 5 days to induce commitment. Cells were fixed in 4% paraformaldehyde and the expression of proteins specific for myocytes, ECs and SMCs was determined by FACS analysis (see above). Cells were analyzed by FACSAria (Becton Dickinson).

Statistical Analysis

Results are presented as mean ± SD. Statistical significance with respect to previous results[1] was determined by Student's *t* test.[13] All *P* values are two-sided and values less than 0.05 were considered to be significant. For sampling, see Online Table III.

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Online Table I. Characteristics of the Patients

Age	Gender	EF (%)	Duration (y)	Etiology	Diabetes	Smoking	Hypertension	Hyperlipidemia
30	M	20	4	Non-ischemic*	yes	no	no	no
54	M	15	29	Non-ischemic**	no	yes	yes	yes
51	M	15	5	Ischemic**	no	yes	yes	no
67	M	15	21	Ischemic**	no	no	yes	yes
44	M	15	12	Non-ischemic**	no	no	no	no
55	M	35	17	Ischemic*	yes	yes	no	yes
23	F	20	2	Peripartum**	no	no	no	no
49	M	20	7	Non-ischemic**	no	yes	yes	yes
57	M	25	12	Ischemic*	no	yes	no	yes
50	M	20	24	Non-ischemic**	no	yes	yes	yes
67	M	65	14	Restrictive**	no	yes	no	yes
63	M	15	27	Non-ischemic**	no	yes	no	no
51	M	20	12	Non-ischemic**	no	yes	yes	yes
61	M	35	6	Ischemic*	yes	yes	yes	yes
53	F	20	6	Non-ischemic*	no	no	yes	yes
50	M	15	3	Non-ischemic*	no	no	yes	no
58	M	20	13	Sarcoidosis**	no	yes	no	yes
37	F	25	8	Non-ischemic**	no	no	no	no
54	M	15	11	Non-ischemic*	no	yes	yes	yes
55	M	20	14	Ischemic**	no	yes	yes	yes

EF: ejection fraction; *Indicates LVAD; **Indicate Explanted hearts.

Online Table II. List of Antibodies

Epitope	Manufacturer	Host Animal	Labeling
Surface Markers			
c-kit	DAKO	rabbit	direct, indirect, F, T
KDR	R&D Systems	mouse	indirect, T,Cy5
CD34	BD Pharmigen	mouse	direct, PE
CD45	BD Pharmigen	mouse	direct, APC
CD133	BD Pharmigen	mouse	direct, PE
Lineage Markers	BD Pharmigen	mouse	indirect, Cy5
CD90	BD Pharmigen	mouse	direct, PE
CD105	BD Pharmigen	mouse	direct, PE
CD31	Chemicon	rabbit	indirect, F,T
Transcription Factors			
GATA4	Santa Cruz	rabbit	direct, indirect, F, T
Nkx2.5	Santa Cruz	goat	direct, indirect, F, T
MEF2C	Santa Cruz	goat	indirect, F, T
GATA6	Santa Cruz	rabbit	indirect, F, T
Cytoplasmic Proteins			
α-sarcomeric actin	Sigma	mouse	indirect, F, T
von Willebrand factor	DAKO	rabbit	indirect, F, T, Cy5
α-smooth muscle actin	Sigma	mouse	indirect, F, T, Cy5
Other Epitopes			
$p16^{ink4a}$	Santa Cruz	mouse	direct, indirect, F, T
BrdU	Roche	mouse	indirect, F,Cy5

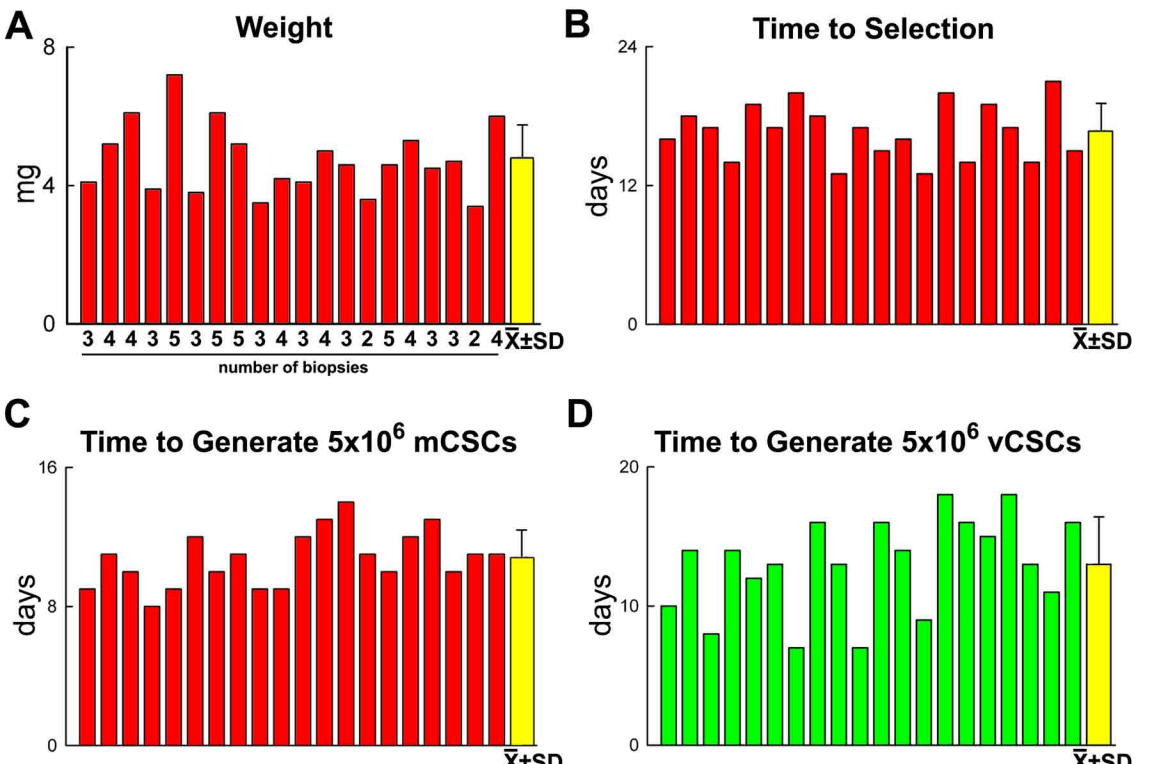
F: fluorescein isothiocyanate; T: tetramethyl rhodamine isothiocyanate; Cy5: cyanine 5.

Online Table III. Magnitude of Sampling

Parameter	N value	Aggregate sample size	Sample size (mean ± SD)
FACS Analysis			
c-kit	20	275,336 (1)	13,766 ± 5,795
KDR	20	138,234 (1)	6,911 ± 3,225
CD34	10	58,510 (1)	5,851 ± 3,424
CD45	10	56,686 (1)	5,668 ± 1,761
CD133	10	44,030 (1)	4,403 ± 1,641
Lineage markers	10	61,068 (1)	6,108 ± 2,393
CD90	10	54,563 (1)	5,456 ± 2,510
CD105	10	55,693 (1)	5,563 ± 1,665
Growth and Death			
mCSCs			
PDT	20	29,875 (2)	1,012 ± 39
BrdU	20	29,032 (2)	1,452 ± 349
TdT	20	27,598 (2)	1,380 ± 229
Telomere Length	20	123,088 (1)	6,154 ± 1307
Telomerase Activity	20	N/A	N/A
$p16^{INK4a}$	20	25,896 (2)	1,295 ± 253
vCSCs			
PDT	20	28,175 (2)	931 ± 89
BrdU	20	20,939 (2)	1,047 ± 163
TdT	20	20,785 (2)	1,039 ± 181
Telomere Length	20	93,805 (1)	4,690 ± 1346
Telomerase Activity	20	N/A	N/A
$p16^{INK4a}$	20	20,762 (2)	1,038 ± 285
Lineage Commitment			
mCSCs			
Baseline			
GATA4	8	56,972 (1)	7,121 ± 2,775
Nkx2.5	8	68,753 (1)	8,594 ± 2,104
MEF2C	8	79,668 (1)	9,958 ± 4,122
α-SA	8	59,177 (1)	7,397 ± 3,539
GATA6	8	67,577 (1)	8,447 ± 4,248
α-SMA	8	67,014 (1)	8,376 ± 3,841
CD31	8	46,439 (1)	5,804 ± 2,734
vWf	8	72,858 (1)	9,107 ± 4,458
Differentiated			
GATA4	8	81,303 (1)	10,162 ± 4,576
Nkx2.5	8	72,878 (1)	9,109 ± 4,632
MEF2C	8	69,679 (1)	8,747 ± 1,696
α-SA	8	82,497 (1)	10,312 ± 4,217

GATA6	8	92,320 [(1)]	11,577 ± 3,122
α-SMA	8	63,539 [(1)]	7,942 ± 2,728
CD31	8	70,110 [(1)]	8,763 ± 3,199
vWf	8	82,629 [(1)]	10,328 ± 2,959
vCSCs			
Baseline			
GATA4	8	74,705 [(1)]	9,338 ± 4,348
Nkx2.5	8	71,548 [(1)]	8,943 ± 3,287
MEF2C	8	77,449 [(1)]	9,681 ± 4,285
α-SA	8	56,505 [(1)]	8,072 ± 3,631
GATA6	8	70,779 [(1)]	8,847 ± 3,036
α-SMA	8	59,942 [(1)]	7,492 ± 1,501
CD31	8	55,592 [(1)]	6,949 ± 1,721
vWf	8	76,075 [(1)]	9,509 ± 3,004
Differentiated			
GATA4	8	69,312 [(1)]	8,664 ± 2,735
Nkx2.5	8	67,280 [(1)]	8,410 ± 2,524
MEF2C	8	67,204 [(1)]	8,400 ± 2,852
α-SA	8	73,213 [(1)]	9,151 ± 3,025
GATA6	8	77,198 [(1)]	9,649 ± 2,261
α-SMA	8	100,970 [(1)]	12,321 ± 5,861
CD31	8	61,147 [(1)]	7,643 ± 2,369
vWf	8	72,506 [(1)]	9,063 ± 2,453

[(1)] Number of cells analyzed by FACS; [(2)] Number of cells examined by confocal microscopy.



Online Figure I