

The proliferative potential of human cardiac stem cells was unaffected after a long-term cryopreservation of tissue blocks

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Background: Human c-kit-positive cardiac stem cells (CSCs) have been used to treat patients suffering from ischemic cardiomyopathy. This study aimed to investigate whether a long-term storage of cardiac tissues would influence the growth potential of the subsequently isolated CSCs.

Methods: A total of 34 fresh samples were obtained from various cardiac regions [right atrium (RA), left atrium (LA), and/or left ventricle (LV)] of 21 patients. From 12 of these patients, 18 samples kept frozen for ~2 years were employed to prepare and characterize the CSCs. After confirming the specificity of the cell sorting by c-kit immunolabeling, the growth rate (number of doublings per day), BrdU positivity, and colony forming unit (CFU) were measured in each CSC population; the values were compared among distinct cardiac regions as well as between fresh and frozen tissues from which CSCs were derived.

Results: Among independent measurements indicating growth potential, the growth rate and BrdU positivity remarkably correlated in freshly prepared CSCs. The cells obtained from every examined region displayed a high proliferative capacity with the growth rate of 0.48 ± 0.19 and the BrdU positivity of $15.0\% \pm 7.6\%$. The right atrial CSCs tended to show a greater growth than those in the other two areas. Similarly, the CSCs were isolated from tissue blocks, cryopreserved for ~2 years, and compared with CSCs derived from the fresh specimens of the same patients. Importantly, we were able to obtain and culture CSCs from every frozen material, and their proliferative potential, represented by the growth rate of 0.47 ± 0.22 and the BrdU positivity of $13.7\% \pm 7.9\%$, was not inferior to that of the freshly prepared cells.

Conclusions: The long-term cryopreservation of cardiac tissues did not affect the growth potential of the derivative CSCs. Our findings should expand the therapeutic applications of these cells over a longer time span.

Keywords: Adult stem cells; cell proliferation; c-kit receptor; cryopreservation; heart

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Introduction

The initial attempt to treat heart failure patients with autologous c-kit-positive cardiac stem cells (CSCs) (1) was extremely successful with the absence of major adverse events (2,3). Accordingly, it would be plausible to treat repeatedly, whenever necessary, by administering his/her own CSCs at a certain interval. These therapies were initially contrived to target adult patients with ischemic disorders, but the potential applications of CSCs may not be limited to this area. For instance, the recent progress of surgical interventions on congenital heart diseases, which affect 0.8% of live births, rescued >85% of pediatric patients but at the same time necessitated the treatment of spontaneously developed heart failure later in adulthood (4).

Based on these potential clinical demands, this research aimed to characterize human CSCs prepared from cryopreserved tissue samples. Specifically, we have analyzed the proliferative potential of freshly prepared CSCs and compared it to that of CSCs derived from surgical specimens stored in liquid nitrogen for about 2 years. Also, CSCs prepared from the right atrium (RA), which were employed for the clinical trial mentioned above, were compared to those obtained from the left atrium (LA) or left ventricle (LV) in the present study.

We hope that in the near future a piece of resected tissue can be frozen during cardiac operation and preserved in a “bank” as a back-up resource for later therapeutic use, without initiating the CSC isolation procedure immediately after the surgery.

Methods

Ethical concern

Every participant in this study provided a written consent. The entire research design, summarized in *Figure 1*, was viewed and approved by the Institutional Review Board of Sakakibara Heart Institute as well as that of Tokai University (#12I-18, #13I-27).

Tissue specimens

Tissue blocks of ~0.5 gram were obtained from RA and LV of patients (n=13 each) receiving left ventriculoplasty at Tokai University Hospital due to ischemic cardiomyopathy. The specimens of LA were harvested at the Sakakibara Heart Institute from patients (n=8) suffering mainly from valvular heart diseases coupled with refractory atrial

fibrillation. The LA tissues were aseptically packaged upon surgery and transferred to Tokai University. Each cardiac tissue block was divided into a few pieces, and the largest piece was freshly used while the rest was stored for 1.8 to 2.5 years.

CSC isolation

CSCs were freshly isolated from the RA, LA, and LV as described previously (5). In brief, cardiac tissues were cut with scissors into pieces of ~1 mm in size. They were digested for 1 hour at 37 °C in 1–2 mg/mL of collagenase (Serva NB4) and further dissociated for 31 seconds in a gentleMACS C tube (Miltenyi Biotec) utilizing the preset program B_01. The resulting small cells were passed through a 40-µm filter and cultured in Ham's F12 Nutrient Mixture (HyClone) containing 10% fetal bovine serum (HyClone), 0.2 mM glutathione (Tanabe, Japan), 10 µg/L trafermin (Kaken, Japan), 5 U/L epoetin alfa (Kyowa Hakko Kirin, Japan), and antimicrobial agents (“growth medium”). Subsequently, cells positive for c-kit were sorted using the MACS separation system and CD117 MicroBead (Miltenyi Biotec) (1), following the manufacturer's instructions.

Cryopreservation of cardiac tissues

Upon cardiac operation at both hospitals, additional pieces of surgical specimens were immersed in the “freezing medium”, consisting of the growth medium supplemented with 10% dimethyl sulphoxide (DMSO, Sigma), in each cryogenic vial, respectively; they were housed in BICELL (Nihon Freezer Co., Ltd., Japan), a specialized container for cryopreservation of cells, and kept at –80 °C overnight. On the following day, the vials were transferred into boxes and kept in liquid nitrogen for ~2 years (range, 651 to 937 days). CSCs isolated from frozen RA, LA, and LV tissues (n=6 each), respectively, were analyzed and compared to the data obtained from the fresh samples of the same patients.

Immunocytochemistry for c-kit

An aliquot of CSCs were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and incubated overnight in a refrigerator with Rabbit Anti-Human CD117 (Dako) diluted 1:40 in PBS. After washing it with PBS, Rhodamine-conjugated Donkey Anti-Rabbit IgG (Jackson ImmunoResearch) at a concentration of 1:50 was applied, and the sample was incubated for an hour at 37 °C. Nuclei

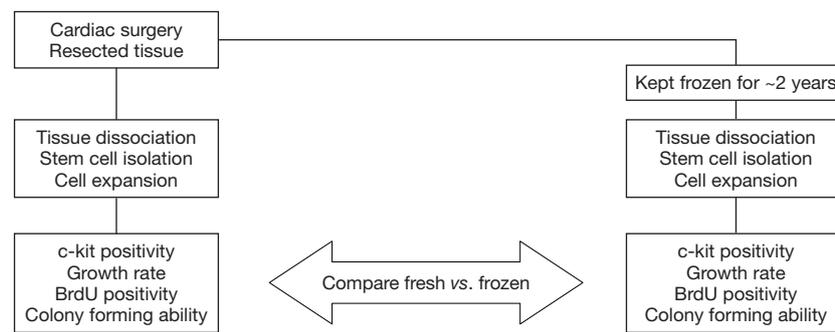


Figure 1 The scheme of the experimental design.

were stained using 4',6-diamidino-2-phenylindole (DAPI; Sigma), and Vectashield (Vector Labs) was mounted prior to the observation using a fluorescent microscope BZ-9000 (Keyence, Japan). At the same time, another group of cells was treated without the primary antibody and served as a negative control. The c-kit positivity was assessed utilizing the ImageJ software (available at: <http://imagej.nih.gov/ij/>).

Growth rate assay

Five thousand CSCs were seeded on a 35-mm dish equipped with a 2-mm grid. The cell number in each of the pre-determined nine squares was counted and summed up on 4 consecutive days. When the cell number increases from N_1 to N_2 in a given time interval (dT), $N_1 \times 2^{(dT/PDT)} = N_2$, in which PDT stands for population doubling time. Therefore, $dT/PDT = \log_2(N_2/N_1) = \log_2 N_2 - \log_2 N_1$. By plotting the time on the X-axis versus the cell number in a base-2 logarithmic scale on the Y-axis, $1/PDT = dY/dX$, i.e., the slope of the trend line. Finally, the growth rate is defined as the number of doublings that occur per unit of time, which is $1/PDT$.

BrdU immunostaining

On a 35-mm dish, 50,000 CSCs were seeded one day prior to the 1-hour incubation with a medium supplemented with $1 \times$ BrdU (Roche). Subsequently, the cells were immersed in a fixing solution containing 15 mM glycine and 70% ethanol (pH 2.0) and incubated for 30 minutes with Anti-BrdU (Roche) diluted in the Incubation Buffer (Roche). After washing the specimen, Anti-mouse IgG-FITC (Roche) was applied and kept at 37°C for half an hour, before adding DAPI. BrdU positivity was calculated as the ratio of BrdU-positive cells to the total number of cells.

Colony forming unit (CFU) assay

For this purpose, 1,000 CSCs were plated on a 100-mm dish so that each cell would be well isolated from the others. A week later, the number of newly formed colonies on the entire dish was counted and recorded as the CFU per 1,000 cells; a colony was defined as a group of 10 cells or more within a high-power field, which went through more than three rounds of division during the 7-day observation period. In most cases, a colony held ~ 30 cells reflecting ~ 5 divisions.

Statistical analysis

Results are reported as the mean \pm standard deviation (SD). SPSS Statistics Version 24 (IBM) was used for the analysis. The normality of the measurements was evaluated by the Shapiro-Wilk test prior to the analyses. The comparison of the data among CSCs derived from the RA, LA, and LV tissues was performed using the Bonferroni method. For comparing the CSCs isolated from fresh and frozen tissue blocks of the same patient, the frozen-to-fresh ratio of the previously mentioned measurements was calculated; since the SDs of these values of freshly prepared CSCs were higher than 30% of the respective measurements, we considered any difference larger than 30% to be meaningful. P value of less than 0.05 was considered statistically significant.

Results

CSC preparation from fresh and frozen specimens

The clinical data of the participants and the nature of cardiac tissues are summarized in *Tables 1,2*, respectively. Following the primary cultivation of cardiac cells, CSCs

Table 1 Characteristic of the participants

Participants	Mean \pm SD	Range	Total count
Age	66.1 \pm 10.0	42.0–77.0	21
Male (%)	61.9	–	21
Smoke (%)	47.6	–	21
BMI	22.76 \pm 5.15	15.7–35.2	21
BNP (pg/mL)	529.0 \pm 573.0	10.1–1,857.4	12
NT-proBNP (pg/mL)	2,267.6 \pm 3,715.7	520.4–11,356.0	8
HbA1c (%)	6.42 \pm 0.99	5.3–9.2	21

SD, standard deviation; BMI, body mass index; BNP, brain natriuretic peptide.

Table 2 Characteristic of the tissue specimens

Tissue specimens	Mean \pm SD	Range	Number
Fresh RA (mg)	95.3 \pm 80.1	8–298	13
Fresh LA (mg)	1,467.9 \pm 731.0	408–2,380	8
Fresh LV (mg)	683.8 \pm 712.2	50–2,604	13
Frozen RA (mg)	56.7 \pm 63.5	10–181	6
Frozen LA (mg)	245.7 \pm 219.8	28–559	6
Frozen LV (mg)	233.5 \pm 166.9	35–486	6
Storage of RA (days)	668.2 \pm 26.4	651–720	6
Storage of LA (days)	797.0 \pm 109.7	666–937	6
Storage of LV (days)	676.3 \pm 28.6	651–720	6

SD, standard deviation; RA, right atrium; LA, left atrium; LV, left ventricle.

were sorted utilizing magnetic microbeads conjugated with an anti-c-kit antibody. The yielded proportion of CSCs was between 2% and 8% of the parental population in the majority of cases. Most importantly, we were able to isolate and culture over 1 million CSCs from both fresh and frozen tissues of all patients; this quantity was used for the preceding clinical trial (2,3). As represented in *Figure 2*, after immunostaining every sample, the positivity of c-kit was found to fall mostly in the range of 80% to 95%; there was no clear difference between the fresh and frozen samples used to prepare the CSCs.

Growth potential of freshly isolated CSCs

Cultured CSCs were analyzed during passages 4 to 7, where the cells seeded immediately after tissue dissociations were designated as passage 0. In order to estimate their growth potential, we employed three independent parameters: growth rate (number of doublings per day), BrdU positivity, and CFU (per 1,000 cells). *Figure 3* depicts the interrelationship of these values found in CSCs isolated from fresh tissues. The average values of all regions combined were 0.48 \pm 0.19 for the growth rate, 15.0% \pm 7.6% for the BrdU positivity, and 32.2 \pm 39.5 for the CFU. While the values of CFU were not normally distributed based on the Shapiro-Wilk test, the growth rate and BrdU positivity showed a strong correlation ($P < 0.001$, Pearson's coefficient 0.866) among the comparisons of these indicators. Accordingly, we focused on the growth rate and BrdU

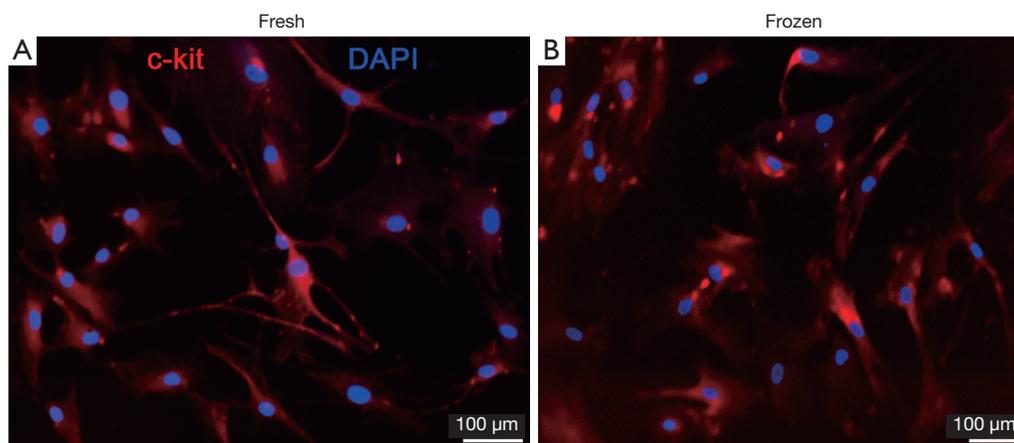


Figure 2 Human c-kit-positive cardiac stem cells (CSCs) prepared from fresh (A) and frozen (B) cardiac tissues. The specificity of the cell sorting was confirmed by the immunolabeling against the c-kit receptor (red). Nuclei were visualized using 4',6-diamidino-2-phenylindole (DAPI; blue).

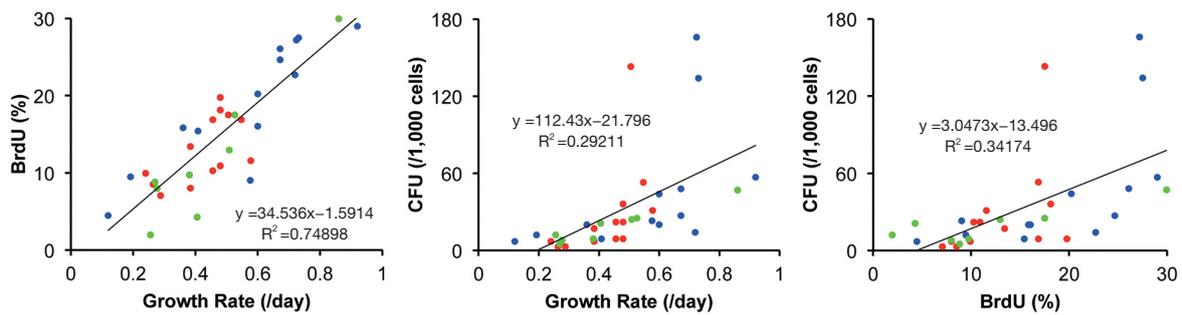


Figure 3 The growth rate, BrdU positivity, and colony forming unit (CFU) measured in freshly isolated human cardiac stem cells (CSCs) derived from the right atrium (RA; blue dots), left atrium (LA; green dots), and left ventricle (LV; red dots), respectively. The growth rate and BrdU positivity are strongly correlated ($P < 0.001$, Pearson's coefficient 0.866).

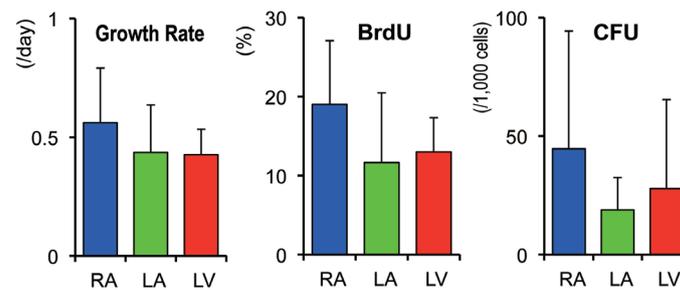


Figure 4 The comparison of the growth potential of human cardiac stem cells (CSCs) in each cardiac region. The growth rate and BrdU positivity of the CSCs derived from the right atrium (RA) tended to be greater than those from the left atrium (LA) and left ventricle (LV) without statistical significance.

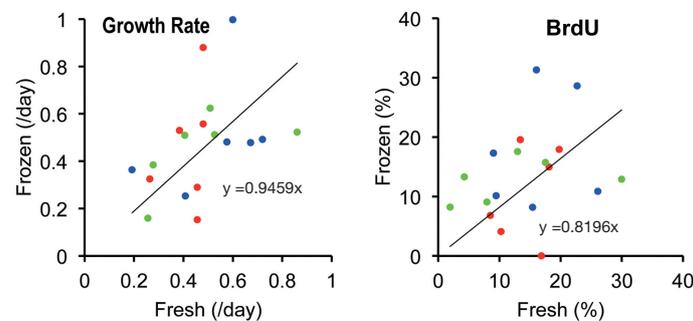


Figure 5 The growth rate and BrdU positivity of human cardiac stem cells (CSCs) isolated from cryopreserved tissues were comparable to those prepared freshly. Blue, green, and red colors represent the right atrium (RA), left atrium (LA), and left ventricle (LV), respectively, from which the CSCs were isolated.

positivity in the following analyses.

Growth potential of CSCs from different regions

The growth rate and BrdU positivity were calculated in

CSCs obtained from each cardiac region (*Figure 4*). As shown, CSCs of all three areas showed a great proliferative ability. Among them, the values of the right atrial samples tended to be higher than the other two groups, without a statistical significance. Note that the right atrial CSCs

and left ventricular CSCs were obtained from the same participants, while those from the LA were isolated from patients of different etiologies.

Difference in the growth capacity of CSCs from fresh and frozen tissues

We were able to successfully isolate and culture CSCs from all tissue specimens kept frozen for ~2 years. The subsequently measured growth rate and BrdU positivity were 0.47 ± 0.22 and $13.7\% \pm 7.9\%$, respectively (Figure 5), which were similar to the values of fresh tissues described above. In order to strictly compare CSCs obtained from these materials, the frozen-to-fresh ratio of each individual was calculated; the 95% confident intervals of the ratio of the growth rate and BrdU positivity were (0.830, 1.287) and (0.744, 1.785), respectively. As we preset a difference of 30% to be meaningful (see methods), the growth potential of the CSCs derived from frozen tissues was not inferior to that of freshly prepared cells. In other words, the long-term cryopreservation of specimens did not significantly decline the proliferative activity of the derivative CSCs.

Discussion

In our attempt, CSCs were successfully obtained from every surgical specimen that was kept frozen for ~2 years.

A former study depicted the maintained phenotypes of human mesenchymal stromal cells (MSCs) after 1-week-long cryopreservation of the parental bone marrow mononuclear cell (BM-MNC) population (6). Similarly, the cryopreservation of human BM-MNCs, for a period of 1 to 2 months, did not spoil the cellular function of the subsequently isolated MSCs (7). More recently, Shen *et al.* described the effectiveness of human MSC isolation from bone marrow cells that were cryopreserved for 23–25 years, although they compared these cells with freshly isolated MSCs from different individuals. The adherence rate of the preserved cells decreased in the initial culture; then surprisingly at the third passage, the MSCs derived from both groups' possessed similar features (8).

Subsequently, solid organs and connective tissues also became the focus of research; there are several former studies investigating the phenotypes of mesenchymal or stem/progenitor cells isolated from fresh and cryopreserved surgical specimens, respectively. The targeted tissues include deciduous teeth (9), dental follicles (10), striated muscles (11), adipose tissues (12), arterial walls (13),

umbilical cords (14), and testicular tissues (15). All these studies demonstrated the feasibility of cell isolation from frozen materials as well as the qualitative equivalence of such prepared cells compared to those freshly obtained.

To the best of our knowledge, however, there is no preceding research on the characteristics of stem/progenitor cells derived from the human heart, before and after 1-year-long cryopreservation. Our current study suggested that the growth potential of CSCs did not significantly decline after the prolonged storage of the original tissue in liquid nitrogen, which is consistent with the previous studies on various organs as mentioned above.

Regarding the comparison among different cardiac regions, the distinct etiology may have influenced the properties of CSCs; especially, the left ventricular samples taken during the left ventriculoplasty, involving infarcted and scarred myocardium, may contain more fibrous materials than the other specimens. In addition, accompanying medication and other external factors might have affected the outcome. These elements compose the limitations of this study.

Based on our observations, a resected specimen of any region of the heart could be kept frozen in the CSC freezing medium in an operation room to be used for therapeutic purposes in the future; specimens in the storage can be transferred to a tissue-processing facility for CSC isolation and preparation whenever necessary. The regenerative potential of CSCs isolated from frozen tissues may need to be examined *in vivo* by a future study, in order to complement and strengthen our findings.

Conclusions

Here we demonstrated that human CSCs in various cardiac regions preserved their proliferative potential following an extended storage of the tissues of origin.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The study was approved by the Institutional Review Board of Sakakibara Heart Institute as well as that of Tokai University (#12I-18, #13I-27) and written informed consent was obtained from all patients.

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