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A Highly Sensitive and Accurate Method to Quantify Absolute Numbers of c-kit⁺ Cardiac Stem Cells Following Transplantation in Mice

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Abstract

Although transplantation of c-kit⁺ cardiac stem cells (CSCs) alleviates post-myocardial infarction left ventricular dysfunction, there are no reliable methods that enable measurement of the absolute number of CSCs that persist in the recipient heart. To overcome this limitation, we developed a highly sensitive and accurate method to quantify the absolute number of murine CSCs after transplantation. This method has two unique features: i) real-time PCR-based detection of a novel male-specific, multiple-copy gene, *Rbmy*, which significantly increases the sensitivity of detection of male donor cells in a female recipient, and ii) an internal standard, which permits quantification of the absolute number of CSCs as well as the total number of cells in the recipient organ. Female C57BL/6 mice underwent coronary occlusion and reperfusion; 2 days later, 10⁵ male mouse CSCs were injected intramyocardially. Tissues were analyzed by real-time PCR at serial time points. In the risk region, >75% of CSCs present at 5 min were lost in the ensuing 24 h; only 7.6±2.1% of the CSCs present at 5 min could still be found at 7 days after transplantation and only 2.8±0.5% (i.e., 1,224±230 cells/heart) at 35 days. Thus, even after direct intramyocardial injection, the total number of CSCs that remain in the murine heart is minimal (at 24 h, ~10% of the cells injected; at 35 days, ~1%). This new quantitative method of stem cell detection, which enables measurement of absolute cell number, should be useful to optimize cell-based therapies, not only for CSCs but also for other stem cells and other organs.

Keywords

Stem cells; Stem cell therapy; Myocardial infarction; Quantitative PCR

INTRODUCTION

Transplantation of stem cells is emerging as a potentially transformative strategy to ameliorate left ventricular (LV) remodeling and dysfunction after acute myocardial infarction (MI). However, a number of studies have reported that stem cells of various sources suffer low viability, and only few persist several weeks following transplantation into the injured myocardium [16, 19, 27–29, 33, 35]. For this reason, increasing survival and

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CONFLICT OF INTEREST STATEMENT

On behalf of all authors, the corresponding author states that there is no conflict of interest.

retention of transplanted stem cells in the heart constitutes one of the major challenges in the current field of cell therapy.

Development of effective stem cell therapies requires close and accurate monitoring of the number, distribution, and fate of the transplanted donor cells and correlation of these variables with changes in functional parameters. However, despite recent advances in imaging technology [38], it is often technically challenging to track or estimate the number of stem cells following transplantation. The majority of current methods to estimate or monitor the number of transplanted cells (which involve histological, *in vivo* imaging, or PCR-based techniques) permit only measurement of the relative changes in the transplanted cell number but do not provide information regarding the *absolute* number of cells present in a recipient organ of interest at a given time.

Here we report a highly sensitive and accurate method of quantifying the number of male mouse donor cells in a female recipient organ. This method has two unique features: i) quantitative real-time PCR detection of a novel male-specific, multiple-copy gene, *Rbmy*, which significantly increases the sensitivity of detection of male donor cells, and ii) use of an internal standard, which permits quantification of the total number of donor stem cells as well as the number of cells in the recipient organ. In the present study, we utilized this method to monitor the number of c-kit+/lin- cardiac stem cells (CSCs) in the female heart following intramyocardial injection in a mouse model of acute myocardial infarction.

MATERIALS AND METHODS

Animals

This study was performed in C57BL6/J female mice (age 11–12 weeks), purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in microisolator cages under specific pathogen-free conditions in a room with a temperature of 24 °C, 55–65% relative humidity, and a 12-h light–dark cycle. The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, Publication No. [NIH] 86–23) and with the guidelines of the Animal Care and Use Committee of the University of Louisville, School of Medicine (Louisville, KY).

Murine Model of Postinfarction LV Remodeling and Failure

The murine model of myocardial ischemia and reperfusion has been described in detail [15, 23]. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg i.p.) and ventilated using carefully selected parameters. The chest was opened through a midline sternotomy, and a nontraumatic balloon occluder was implanted around the mid-left anterior descending coronary artery using an 8-0 nylon suture. To prevent hypotension, blood from a donor mouse was given at serial times during surgery. Rectal temperature was carefully monitored and maintained between 36.7 and 37.3 °C throughout the experiment. In all groups, myocardial infarction (MI) was produced by a 60-min coronary occlusion followed by reperfusion. Mice were then allowed to recover for 5 min, 1, 7, and 35 days until euthanasia and tissue harvesting.

Transplantation of lin-/c-kit+ Mouse Cardiac Stem Cells

Isolation, characterization, and culture of lin-/c-kit+ mouse CSCs have been previously described [22]. Transplantation of mouse CSCs by intramyocardial injection has been previously described [7]. Briefly, at 48 h after MI, mice were reanesthetized and the chest reopened through a central thoracotomy. Lin-/c-kit+ CSCs (10^5 cells in 40 μ l PBS) were injected intramyocardially using a 30-gauge needle. A total of four injections (10 μ l each)

were made in the peri-infarct region in a circular pattern, at the border between infarcted and noninfarcted myocardium.

Isolation of Genomic DNA

After stem cell transplantation, recipient female mice were euthanized, and the following recipient organs were extracted at 5 min, 1 day, 7 days, and 35 days post-transplantation: heart, lung, liver, kidney, and spleen. Upon extraction, the heart was separated into four different regions: risk region (RR) including the border zone (anterior wall + septum), posterior left ventricular [LV] wall (nonischemic region), right ventricle (RV), and atria. Female human peripheral mononuclear cells (hPBMCs) were purchased from AllCells, LLC (Emeryville, CA). Aliquots of 10^5 hPBMCs (in 50 μ l PBS) were stored at -20°C until use. For isolation of genomic DNA, QIAamp DNA Mini Kit (Qiagen) was used according to the manufacturer's instructions with the following modifications. In order to achieve complete digestion, the tissue samples were placed in screw-cap tubes containing an excess volume of Buffer ATL (tissue lysis buffer; 400 μ l per 25 mg tissue) plus Proteinase K (40 μ l per 25 mg tissue). The internal standard (10^5 hPBMCs) was resuspended 50 μ l of the above tissue digestion solution and added to each tube (Fig. 1A). The samples were incubated overnight at 56°C on a nutating or rotatory mixer. After the overnight incubation, the samples were treated with RNase A (Qiagen) to prevent RNA contamination prior to proceeding to the column purification of genomic DNA. Control DNA samples, including hPBMC, mouse CSC, and female mouse DNA, were prepared in a similar manner. The DNA concentrations of the samples were measured using standard UV spectrophotometry. For cardiac tissues, the entire tissue segment was used for the DNA isolation. For extra-cardiac tissues, the whole organ was weighed and finely minced with a razor blade to create a relatively crude homogenate. Then, 25–50 mg of representative tissue sample from the minced organ was taken and processed for genomic DNA isolation and real-time PCR assay as described here. The total number of transplanted male CSCs in the entire organ was calculated based on the ratio of the weight of the processed tissue to the total organ weight.

Real-Time PCR

For each real-time PCR reaction, 100 ng of genomic DNA was amplified in a 20 μ l reaction using SYBR[®] Green PCR Master Mix and a StepOne Plus real-time PCR machine (Applied Biosystems). Each sample was run in triplicate and analyzed using three different sets of primers (*Rbmy*, *Actb*, and *HLA-A*) (Fig. 1C). The primers used in the present study are listed in Table 1. For quantification of male mouse genomic DNA in tissue samples, the *Rbmy* primer set #2 was used throughout the study. The PCR conditions were the following: 10 min at 95°C followed by 40 cycles of 2-step PCR (15 sec at 95°C and 1 min at 64°C). Melt curves were generated each time to validate the identity of the amplified products. The results were analyzed using StepOne Software v2.1 (Applied Biosystems). To generate hPBMC DNA standards, 30, 100, 300, 1,000, 3,000, or 10,000 pg of hPBMC genomic DNA was added to 100 ng female mouse genomic DNA. To generate male mouse DNA standards, 10, 30, 100, 300, 1,000, or 3,000 pg of male mouse CSC genomic DNA was added to 100 ng female mouse genomic DNA. Both sets of standards were analyzed by real-time PCR along with the samples, and the amount of target DNA (i.e., human or male mouse DNA) per 100 ng total DNA was plotted against the threshold cycle (C_T) value to obtain the standard curves.

Cell Number Calculations

The amount of target DNA (human or male mouse DNA) present in the PCR sample was calculated using the corresponding standard curve and divided by the amount of genomic DNA present per cell (i.e., 7.2 pg for mouse and 7.7 pg for human) to calculate the number of target cells present in the 100 ng PCR sample. The mouse genome contains

approximately 3.4×10^9 base pairs. For a diploid genome, there are 6.8×10^9 base pairs. The average MW of a double-strand DNA base pair is 645 Daltons, and 1 Dalton equals 1.65×10^{-24} g. Therefore, the DNA content of murine cells can be calculated as $6.8 \times 10^9 \times 645 \times 1.65 \times 10^{-24} = 7.2 \times 10^{-12}$ g = 7.2 pg DNA per diploid mouse cell. The DNA content per human diploid cell was calculated in a similar manner. The total number of cells present in each tissue was calculated using the following equation: $F = (f \times H) / h$, in which F, total number of (female) mouse cells in the tissue; f, number of mouse cells represented in the DNA sample; H, total number of human cells added to the tissue sample (i.e., 10^5); h, number of human cells represented in the DNA sample. The total number of male mouse CSCs present in each tissue was calculated using the following equation: $M = (m \times F) / f$, in which M, total number of male mouse cells in the tissue; m, number of mouse cells represented in the DNA sample. The above two equations can be merged into a simplified equation as the following: $M = (m \times H) / h$.

RESULTS

Rbmy is a Sensitive Marker of Male Mouse Genome

Male to female donor-recipient transplant pairs are commonly used in stem cell transplantation studies since the presence of the Y chromosome in donor cells can be detected either by PCR- or fluorescence *in situ* hybridization-based methods [8, 17, 33, 34]. This allows measurement of male donor cells engrafted in female recipient organs without having to genetically manipulate the donor cells prior to transplantation. Traditionally, the sex-determining factor, *SRY* gene on the Y chromosome [3], has been widely used to identify cells of male origin. Although *SRY* is specific to males only, it is present in a single copy in both human and mouse [14, 30, 31]. This may limit the sensitivity of assays that target *SRY*. Thus, we sought novel markers for male mouse genome that may potentially provide a higher sensitivity of detection of male mouse genomic DNA among female recipient DNA.

One of the candidates was mouse *Rbmy* (RNA binding motif protein, Y-linked). Human *RBMY* belongs to a multi-copy gene family located in the AZF (azoospermia factor) regions of the Y chromosome [5, 24]. Database searches revealed that there are 5 members of the *Rbmy* gene family (i.e., *Rbmy1A1*, *Gm10256*, *Gm10352*, *AC132601.1*, and *AC163691.1*) in the mouse, all located in relatively close proximity on the Y chromosome and sharing nearly identical sequences (Ensembl; NCBI37) [11]. (The male-specific region of the human and mouse Y chromosomes contain other multiple-copy genes [31] which may be utilized for increasing the sensitivity of detection of male DNA or nucleus.) Of note, the *Rbmy* gene family shares significant sequence homology with its X chromosome counterpart, *RbmX* (RNA binding motif protein, X chromosome) [9]. In order to test its utility and specificity in detecting male mouse genomic DNA, two sets of primers were designed for mouse *Rbmy* and used to PCR amplify male and female mouse genomic DNA. Mouse *Sry*-specific primer sets were used as controls (Table 1). As expected, both *Sry* and *Rbmy* primers were specific for male mouse genomic DNA (Fig. 2B). However, PCR using *Rbmy*-specific primers conferred greater sensitivity for detection of male mouse genomic DNA compared with *Sry*-specific primers (Fig. 2A). Using *Rbmy* primers in quantitative PCR assays, we were able to detect as few as ~100 male cells in the female heart reproducibly (Fig. 3 and data not shown). This indicates that PCR-based detection of *Rbmy* is not only specific for male mouse DNA but also offers a highly sensitive method of detecting male donor stem cells in female recipient organs.

Development of a Novel Real-Time PCR Assay for Measuring the Absolute Number of Male Mouse Donor Cells in Female Recipients

One of the major problems of previously-established quantitative PCR-based approaches for measuring the number of donor cells is that they only allow measurement of the ratio of male donor cells to female recipient cells (which is often expressed as a percentage of male cells among female cells) [6, 13, 20, 37]. Although such data can be informative, the absolute number of male donor cells cannot be derived using this approach unless the total number of cells present in the tissue/sample is already known. In order to overcome this problem and allow accurate measurements of the absolute numbers of transplanted male stem cells in various female recipient organs, we developed a new method in which cells of another genotype are added to each tissue sample as an internal standard prior to genomic DNA isolation. For the internal standard, we chose normal diploid human peripheral blood mononuclear cells (hPBMCs), the presence of which can be detected by using a primer set specific for human genomic DNA (i.e., human *HLA-A* primers) (Table 1; Fig. 1A). Since the ratio of the internal standard (i.e., human DNA) to the mouse DNA should remain constant regardless of the efficiency of DNA isolation, the amount of human DNA in each sample can be used to calculate the total amount of mouse DNA present in the entire tissue, as well as the total amount of male donor DNA (Fig. 1). The amount of DNA of a particular genotype can then be converted to the number of cells by dividing it by the amount of genomic DNA present per cell (i.e., 7.7 pg for human and 7.2 pg for mouse cells) (See MATERIALS AND METHODS for details).

DNA standards were generated by adding varying amounts of human/hPBMC or male mouse/mouse CSC DNA (ranging from 10 pg to 10 ng) to 100 ng female mouse genomic DNA. Real-time PCR using human-specific *HLA-A* or male mouse-specific *Rbmy* primers was then performed to generate a standard curve for each genotype (Fig. 3A). In order to validate the assay, we analyzed female mouse liver tissue mixed with different numbers of male mouse splenocytes (ranging from 10^3 to 10^5 cells). Each tissue sample was added with 10^5 hPBMCs before being processed for genomic DNA isolation. Genomic DNA isolated from each sample was then analyzed by quantitative real-time PCR using *HLA-A* and *Rbmy* primer sets to quantify the amount of DNA of the corresponding genotype in the sample. The number of cells in each sample was calculated based on the amount of target DNA detected as described above. The result shows that the assay measures the number of male splenocytes in the tissue samples with a good accuracy, demonstrating the validity of the assay (Fig. 3B). A similar experiment using female tissues mixed with varying numbers of c-kit⁺ male mouse CSCs also confirmed the validity of the assay (Fig. 3B).

The Number of Transplanted c-kit⁺ CSCs Declines Rapidly Following Intramyocardial Injection into Infarcted Heart

We then utilized the assay to examine the kinetics of stem cell survival and engraftment in the recipient heart following transplantation of CSCs. Female C57BL/6 mice underwent a 60-min coronary occlusion and reperfusion to induce acute myocardial infarction (MI), and two days later, 10^5 c-kit⁺/lin⁻ male mouse CSCs were injected intramyocardially along the border zone. The mice were allowed to recover for 5 min, 1 day, 7 days, or 35 days, and multiple organs were extracted at each time point. In order to monitor the accuracy of injection and detect potential migration of the injected cells to other cardiac regions, the heart was separated into the following four regions: risk region (RR) which included the border zone, posterior LV wall (nonischemic region), right ventricle (RV), and atria. In addition, lungs, liver, kidney, and spleen were also extracted from each recipient female to detect any possible cell migration or “leakage” of the transplanted cells to extracardiac tissues. Each tissue was added with 10^5 hPBMCs, processed for genomic DNA and analyzed

by real-time PCR as described above. DNA samples were also analyzed with *Actb* (β -actin)-specific primers to assess the relative amount of genomic DNA in each sample.

Surprisingly, only 43% of the CSCs injected into the infarcted heart were found at 5 min after injection (Fig. 4A; Table 2), suggesting that a large number of cells were lost almost immediately after the injection due to leakage out of the myocardium. Moreover, >75% of CSCs present in the risk region at 5 min were lost in the ensuing 24 h, and only $7.6 \pm 2.1\%$ of the CSCs present at 5 min could still be found in the recipient heart at 7 days after transplantation (Fig. 4A; Table 2). By 35 days, only $2.8 \pm 0.5\%$ of the cells present at 5 min (i.e., $1,224 \pm 257$ cells/heart; $1.2 \pm 0.2\%$ of total cells injected) were detected in the heart (Fig. 4A; Table 2). Although some transplanted donor cells were detected outside of the injected area (i.e., posterior LV, RV, and atria), the majority of the cells were found in the risk region throughout the observation period. The only extracardiac organ that harbored significant numbers of male donor cells was the lungs: at 1 day post-transplantation, approximately 8% of injected CSCs were found in the lungs (Fig. 4B). However, CSCs were no longer detectable in the lungs by 7 days (Fig. 4B).

DISCUSSION

Quantitative PCR has been used to assess donor cell engraftment in the recipient organ, especially in gender mismatched cell transplantation studies [6, 25, 33, 34]. Detection and quantification of donor cells in the transplanted tissue is performed by a highly sensitive PCR-based assay using a donor cell-specific marker DNA (e.g., *SRY* for male donors). In general, the standard curves are generated using DNA isolated from varying numbers of donor cells or varying amounts of donor cell DNA. Such method allows one to estimate the degree of donor-to-recipient “chimerism,” and the results are usually expressed as a number of donors in a unit number of recipients (e.g., 100 donor cells in every 100,000 recipient cells) or as a percentage of donor cells among recipient cells. The data obtained in this manner may provide sufficient information to assess the relative differences in the number of donor cells between different treatment groups. However, the absolute number of donor cells in the recipient organ cannot be estimated using such method. This problem arises from the fact that the sample DNA analyzed by quantitative PCR represents an unknown fraction of total tissue DNA and that the total theoretical amount of tissue DNA cannot be obtained without knowing the efficiency of DNA recovery. Some studies have claimed to have measured the absolute number of cells in the recipient organ by making an assumption that the total amount of DNA recovered from a tissue reflects the total number of cells in the tissue [25, 33]. However, this is a faulty assumption because the yield of DNA recovered from the tissue is always less than 100%.

In the present study, we overcame this problem by i) using the entire tissue for isolating genomic DNA and ii) introducing an internal standard into each tissue sample to measure the starting, total genomic DNA in the tissue (Fig. 1). The idea is that since the ratio of internal standard DNA to sample tissue DNA should remain constant regardless of the efficiency of DNA recovery, the amount of internal standard DNA detected by PCR can be used to back-calculate the starting amount of DNA in the tissue. Then, the total amount of recipient DNA in the tissue can be utilized to calculate the total number of donor cells based on the ratio of donor to recipient DNA obtained by quantitative PCR detection of donor-specific DNA elements (e.g., *Rbmy* or *Sry*) (see calculations in MATERIALS AND METHODS and Fig. 1). To validate the assay, we mixed known numbers of male mouse splenocytes or CSCs with female mouse tissue samples and tested if the assay accurately measures their numbers in each sample. The results showed that the assay indeed correctly measures the numbers of both male splenocytes and CSCs in tissue samples, demonstrating the validity of the assay (Fig. 3B). However, we acknowledge that another independent

method, such as histological assessment, would have greatly complemented the method of assay validation used in the current study. The present method offers a simple, highly sensitive, and accurate measurement of the absolute numbers of transplanted cells in the recipient organ, does not require any genetic manipulation of the donor cells, and can be applied to different stem cell populations and organ systems as long as there are donor cell-specific DNA elements that can be detected by PCR. Alternatively, any transgenes (e.g., EGFP) present in the donor cells can be used for their detection.

The current method has limitations. For example, it cannot distinguish between the cells originally injected and the cells formed by proliferation of the injected cells. It only measures the total number of donor and donor-derived cells and provides little information as to the kinetics of donor cell proliferation and death in the recipient tissue. Also, if transplanted stem cells undergo numerical or structural chromosomal abnormalities (e.g., binucleation and polyploidization) or cell fusion, the method would overestimate the actual number of engrafted cells. This problem would become more serious if a significant number of transplanted stem cells differentiate into adult cardiomyocytes, which are known to undergo binucleation and polyploidization [21, 36]. However, we have repeatedly observed that even when the transplanted cells express cardiomyocyte markers (e.g., α -sarcomeric actin), they do not acquire the phenotype of adult myocytes and do not exhibit binucleation [22, 32]. Regarding fusion, we have found no evidence that this phenomenon occurs after CSC transplantation. Previous papers indicate that fusion does not occur after CSCs are transplanted [1, 2]. Other factors that may limit the use of the current method include the fact that 1) it does not permit real-time monitoring of cell numbers in the same animal and 2) the entire tissue must be used to quantify the total number of cells, which does not allow parallel structural and histological studies.

One of the major problems associated with cell therapy is the low rate of donor cell survival and engraftment in the recipient heart [19, 27, 33, 35]. For example, in a mouse model of acute MI, Tang *et al.* reported that over 90% of the transplanted mesenchymal stem cells were lost during the first 24 h, and only 3.6% of the cells remained in the heart by 7 days post-transplantation [33]. Myocardial ischemia/reperfusion and infarction create a hostile environment for stem cells, because of the presence of inflammatory cells and cytokines/mediators, lack of extracellular matrix and supporting cells, and poor supply of oxygen and nutrients, all of which conspire to promote death of the grafted cells [12, 26]. Multiple factors and cell death pathways have been implicated in the poor survival of the grafted cells [18]. There are additional factors that may contribute significantly to the low retention of the transplanted cells in the recipient organ. Previous studies have suggested that a large proportion of cells injected into the heart are “washed out” by coronary blood flow and through the vascular system [10, 33]. Cell retention in the dead space of the syringe may also contribute to the rapid loss of transplanted cells soon after delivery [33]. The rapid loss of injected cells observed during the first 5 min of injection in the present study is consistent with the notion that physical loss or leakage of cells is one of the major factors limiting the efficiency of the cell delivery.

The present study provides important new information with respect to intramyocardial injection. The rapid loss of injected cells observed during the first 5 min after injection in the present study (>50% of the cells in the syringe) is consistent with the hypothesis that physical loss and/or leakage of cells are major factors limiting the efficiency of cell delivery. The near absence of donor cells in most organs other than the lungs at 5 min (Fig. 4B) suggests that the majority of the injected cells had leaked out the LV wall and into the chest cavity, rather than being released into the circulation. Because the injections were performed by a very experienced surgeon (YG), and because the same technique used here has been successfully used by us in several studies where transplantation of CSCs resulted in

substantial functional improvement [22, 32], it seems unlikely that the major initial loss of CSCs could be ascribed to inability to position the needle properly in the LV wall. However, in the mouse the LV wall is very thin (~ 1 mm), particularly after an infarction, and so it must be kept in mind that in this model intramyocardial injections are very challenging vis-a-vis other species in which the LV wall is thicker and the initial loss of cells would be expected to be smaller. Thus, it is unclear whether these findings can be extrapolated to other species.

The present study is the first to quantify the permanence of donor CSCs after transplantation. The results are qualitatively similar to those obtained with other stem cells, which have shown low initial retention and almost complete disappearance after a few weeks [19, 27, 33, 35]. The results are also consonant with our previous study in a rat model of MI [32], in which we found a very low persistence of transplanted cells (or their progeny) at 35 days after transplantation. The present investigation expands and strengthens that study by examining a different species and, most importantly, by providing the absolute number of cells that survive after transplantation.

In summary, our data indicate that, after intramyocardial injection, most murine CSCs are lost soon after transplantation, and that the total number of CSCs that remain in the heart is small (at 1 day, $\sim 10\%$ of the cells injected; at 35 days, $\sim 1\%$). The accurate and sensitive method presented in the current study should serve as an important tool for devising novel strategies to improve the engraftment of the stem cells and the efficacy of CSC therapies.

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Figure 1. Method for quantifying the absolute number of transplanted male donor cells in the female heart

A, When the recipient female tissue is processed for genomic DNA isolation, 10^5 human peripheral blood mononuclear cells (hPBMC) are added to the tissue and serve as the internal standard. **B**, The ratio of human/hPBMC DNA to the recipient mouse DNA in each sample remains constant regardless of the efficiency of DNA isolation. **C**, Each sample (100 ng) is analyzed by quantitative, real-time PCR for *HLA*, *Rbmy* and β -actin to quantify the amounts of human, male mouse, and total genomic DNA, respectively. **D**, Based on the standard curve generated for a human-specific marker, *HLA*, and the C_T (threshold cycle) value of each sample, the amount of human DNA is obtained and used to calculate the total, starting amount of the recipient mouse DNA as shown by the example. **E**, Based on the standard curve generated for a male mouse-specific marker *Rbmy* and the C_T value, the amount of male donor mouse DNA in each sample is obtained and used to calculate the total, starting amount of the male donor mouse DNA as shown by the example. Note that the standards may be prepared by adding varying amounts (e.g., 10 pg to 10 ng) of the genomic DNA of the target species or sex in a fixed amount (e.g., 100 ng) of the female recipient mouse DNA. The amount of DNA can be converted to the number of cells based on the amount of genomic DNA per cell (i.e., 7.2 pg per normal diploid mouse cell).

Figure 2. *Rbmy* is a highly sensitive and specific marker of male mouse DNA

A, *Rbmy*- or *Sry*-specific primer sets were used to PCR amplify varying amounts (0–3,000 pg) of male mouse genomic DNA under the same PCR conditions. Both primer sets for *Rbmy* conferred greater sensitivity of detection of male mouse DNA over *Sry* primers. **B**, Specificity of sex- and species-specific markers. Genomic DNA (100 ng) of indicated sex or genotype was PCR-amplified using primer sets for the indicated genes. Both *Sry* and *Rbmy* are specific for male mouse. *HLA-A* primers used in the present study specifically detected human genomic DNA only. PCR amplification for β -actin was used to confirm the presence of genomic DNA in each sample. hPBMC, human peripheral blood mononuclear cells; HUVEC, human umbilical cord endothelial cells.

Figure 3. Validation of the quantitative PCR assay for detection of male donor stem cells

A, Standard curves for quantification of human DNA (internal standard) and male mouse DNA. Varying amounts (ranging from 10–10,000 pg) of human (hPBMC) or male mouse (c-kit⁺ mouse CSC) genomic DNA were mixed with 100 ng female mouse genomic DNA and PCR-amplified (“real-time”) using genotype-specific primer sets (*HLA-A* for human and *Rbmy* [set #2] for male mouse) in the presence of SYBR Green. The threshold cycle (C_T) was plotted against the amount of target DNA present in the sample. **B**, Measurement of the number of male mouse splenocytes mixed in female mouse liver. Normal diploid male mouse splenocytes were freshly isolated, counted using a hemacytometer, and added to approximately 25 mg of female mouse liver in varying numbers, along with the internal standard (i.e., 10⁵ hPBMCs). Genomic DNA isolated from these was analyzed by quantitative PCR as described. Note that the assay faithfully measures the number of male cells with a good accuracy.

Figure 4. Stem cell engraftment after transplantation of c-kit⁺ CSCs into infarcted female mouse hearts

A, Female mice with acute MI were intramyocardially injected with 10^5 c-kit⁺/lin⁻ murine CSCs. At 5 min, 1 day, 7 days, and 35 days post-transplantation, the heart was extracted and separated into four different regions: risk region (RR) including the border zone, nonischemic zone (NIZ; posterior LV wall), right ventricle (RV), and atria. Each tissue sample was added with the internal standard (i.e., 10^5 hPBMCs) before processing it for genomic DNA isolation and analyzed by quantitative PCR as described. n=5 for the 5 min and 35 days groups and n=4 for the remaining time points. The error bars represent the SEM. **B**, Number of transplanted stem cells found in extra-cardiac organs. N.D., not detected.

Table 1

Primers used for the quantitative PCR assays

Primer Name	Primer Sequence	Reference
<i>Rbmy</i> fwd 1	5'-GACAAGAAGTGCTTCCACCA-3'	Present study
<i>Rbmy</i> rev 1	5'-CAGCCCATCCTTAGGTGAAT-3'	Present study
<i>Rbmy</i> fwd 2 *	5'-GATTCCATGAGGCACCATCT-3'	Present study
<i>Rbmy</i> rev 2 *	5'-ATGGTTCTCCTCTTCCACCA-3'	Present study
<i>Sry</i> fwd 1	5'-ATGGAGGGCCATGTCAAG-3'	Present study
<i>Sry</i> rev 1	5'-CCAACTTGTGCCTCTCACC-3'	Present study
<i>Sry</i> fwd 2	5'-GGTGAGAGGCACAAGTTGG-3'	Present study
<i>Sry</i> rev 2	5'-ATCTCTGTGCCTCCTGAAA-3'	Present study
<i>Actb</i> (β-Actin) fwd **	5'-AGACTTCGAGCAGGAGATGG-3'	Present study
<i>Actb</i> (β-Actin) rev **	5'-CAGGCAGCTCATAGCTCTTCT-3'	Present study
<i>HLA-A</i> fwd	5'-GCTCAGTTCCAGTTGCTTG-3'	[4]
<i>HLA-A</i> rev	5'-GCAGTGAGCCAAGATTGCAC-3'	[4]

*, Used for quantification of male mouse genomic DNA in the present study

**, For both human and mouse genes

Table 2
Number of c-kit+ CSCs found in the recipient heart following intramyocardial injection

	Heart				
	Total	RR	NIZ	RV	Atria
5 min (n=5)	43,084 ± 17,273	40,627 ± 17,379	1,436 ± 585	756 ± 370	265 ± 144
24 hours (n=4)	9,678 ± 3,140	4,671 ± 1,342	4,012 ± 1,912	441 ± 191	554 ± 480
7 days (n=4)	3,279 ± 800	2,521 ± 380	317 ± 258	309 ± 124	132 ± 81
35 days (n=5)	1,224 ± 257	1,068 ± 349	208 ± 195	63 ± 63	0

The values represent mean ± SEM. RR, risk region; NIZ, nonischemic zone; RV, right ventricle.