Bone marrow-derived cells can acquire cardiac stem cells properties in damaged heart

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Abstract

Experimental data suggest that cell-based therapies may be useful for cardiac regeneration following ischaemic heart disease. Bone marrow (BM) cells have been reported to contribute to tissue repair after myocardial infarction (MI) by a variety of humoural and cellular mechanisms. However, there is no direct evidence, so far, that BM cells can generate cardiac stem cells (CSCs). To investigate whether BM cells contribute to repopulate the Kit^+ CSCs pool, we transplanted BM cells from transgenic mice, expressing green fluorescent protein under the control of Kit regulatory elements, into wild-type irradiated recipients. Following haematological reconstitution and MI, CSCs were cultured from cardiac explants to generate 'cardiospheres', a microtissue normally originating *in vitro* from CSCs. These were all green fluorescent (*i.e.* BM derived) and contained cells capable of initiating differentiation into cells expressing the cardiac marker Nkx2.5. These findings indicate that, at least in conditions of local acute cardiac damage, BM cells can home into the heart and give rise to cells that share properties of resident Kit^+ CSCs.

Keywords: cardiac stem cells • bone marrow transplantation • tissue regeneration • Kit^+ cells

Introduction

Ischemic heart diseases represent one of the major causes of morbidity and death, particularly in western societies. Two major cell therapeutic strategies for heart repopulation after cardiac damage have been proposed and are currently developed, based on the regenerative potential of either cardiac and/or extra-cardiac stem cells (CSCs). In the last years, the finding that the heart contains a reservoir of resident stem and progenitor cells opened new perspectives in the biology of cardiac regeneration, suggesting to explore experimental procedures aimed at *in vitro* expansion of CSCs, for *in vivo* transplantation. CSCs are positive for various stem/progenitor cell markers (Kit, Sca-1, Isl-1 and side population – SP – properties), propagate *in vitro* and develop features of heart cells after differentiation *in vitro* or *in vivo* [1–8]. Our own group has described a method of isolation and expansion of mouse and human heart adult stem cells (ASCs): these cells grow to form contractile cardiospheres (CSs), which include stem/progenitor cells of cardiac lineages and are able to improve cardiac function when transplanted into infarcted heart. [1] The regenerative

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effectiveness of such CSs, derived from percutaneous endomyocardial biopsy specimens, has been confirmed in more recent studies [8, 9]. However, the most used cellular source for experiments of cardiac repair and for clinical trials has been the bone marrow (BM), an easily accessible tissue which harbours different types of stem and progenitor cells, capable of contributing to the regeneration of multiple tissues [10–19]. A critical question is whether the endogenous CSC pool may be replenished by BM-derived stem cells. Heart cells derive from two waves of multipotent/bipotent embryonic stem progenitor cells [20–22] and mouse chimera experiments rule out any major (greater than 5%) subsequent contribution to the adult CSCs population from 'immigrant' non-CSCs [23], under physiological conditions. On the other hand, the presence of cardiac chimerism in patients receiving allogenic BM transplantation suggested that immigrant BM-derived cells could generate at least some cardiac cells [24]. It remains unclear whether these immigrant cells convert into myocytes directly or *via* a CSC intermediate cell capable of self-replication and long-term cardiac regeneration. If the latter is the case, exciting biological and therapeutic possibilities may arise. We addressed the question whether, following myocardial infarction (MI), BM cells contribute to myocardic tissue repair by the generation of a cell population with the functional features of CSCs. We combined two recent advancements made in our laboratories: (*i*) first, the possibility to grow *in vitro* mouse CSCs, which extensively proliferate, transiently express *Kit* and generate beating CSs, capable of further cardiac differentiation [1, 8]; (*ii*) second, the generation of transgenic mouse line (Kit/GFP [green fluorescent protein]) that efficiently expresses GFP in a variety of stem and progenitor Kit^+ cells, including BM and CSCs, under the control of mouse Kit regulatory DNA regions [25–28]. In the present work, we transplanted lethally irradiated mice with BM cells expressing the Kit/GFP transgene. Following BM reconstitution and induction of MI, cells from the explanted hearts were grown *in vitro*, to generate CS which, on the basis of GFP expression, might reveal a BM origin. Indeed, GFP^+ CSs were generated, which were capable of extensive proliferation. Furthermore, they contained a proportion of cells that spontaneously expressed cardiac markers and were able, upon transplantation, to engraft the heart of secondary wild-type (wt) mice infarcted.

These results indicate that, following local tissue injury, a BM-derived population of Kit^+ cells with CSCs properties may be generated in the heart. We suggest that these cells are potentially capable of long-term regeneration and might contribute to cardiac repair.

Materials and methods

Mice

Kit/GFP and wild-type (BDF1) mice were kept according to institutional regulations. Kit/GFP transgenic mice (line 3) have been described [25].

Bone marrow transplantation and myocardial infarction

BDF1 female recipient mice, 8–12 weeks old, were lethally irradiated (830 rads, x-ray source) and tail-vein injected with 10^7 Kit/GFP BM cells. Controls were injected with cell-free medium. Engrafted animals were analysed 4–6 months after transplantation. To test the haematopoietic stem cell potential of CSs, we intravenously transplanted lethally irradiated female recipients with 5×10^5 or 10^6 Kit/GFP^+ suspended single cells derived from CSs, together with 5×10^4, 10^5 or 2×10^5 wild-type BM cells as support. Transplanted animals were subjected to MI by left anterior descending coronary artery (LAD) ligation [29] 4, 5, 6 and 9 months after transplantation; after further 2–3 weeks, they were killed and analysed as required.

Processing and isolation of cardiosphere forming cells

Murine tissue was derived from infarcted or non-infarcted hearts of Kit/GFP BM transplanted mice and processed as described [1]. Upon growth on poly-D-lysine-coated dishes, many cells remained adherent, while others progressively gave rise, after several days, to CSs, which detached from the dish, floating in the medium. CSs were individually collected and expanded as adherent monolayers as described [8]. When required for further analysis, secondary CSs were again obtained by growing them on poly-D-lysine coated dishes.

Immunohistochemistry and microscopy

CSs and cardiosphere-derived cells (CSDCs) were fixed in ethanol/methanol (4°C, 10 min.), washed twice with Ca^{++}/Mg^{++} free PBS and permeabilized (0.1% Triton X-100 and 1% bovine serum albumin [BSA]).Non-specific antibody-binding sites were blocked with 10% goat prior to incubation with primary antibodies: anticardiac troponin (TnI, Sigma, Milan, Italy), anticardiac-specific transcription factor Nkx2.5, anti-smooth muscle actin or anti-β galactosidase (β-Gal), (Chemicon, Milan, Italy).

After washing in PBS containing 0.1% Triton X-100 and 1% BSA, cells were incubated in Fitc-conjugated antimouse IgG or TRIC-conjugated anti-rabbit secondary antibody (Chemicon).

Confocal microscopy was performed with a Leica TCS DMIRE 2 (LCS lite software Leica, Leica, Milan, Italy), Vectashield mounting medium. No significant fluorescent signal was detectable with any of the secondary antibodies alone.

In vitro haematopoietic colony assays

Culture conditions for BM cells have been described [30]. For *in vitro* colony assay of CSDCs, 10^5 wild-type CSDCs were plated in 35 mm dishes containing 1 ml of the semisolid medium. Alternatively, single secondary CSs were dissociated and plated in 96 multiwell plates (1 CS/well containing ~0.1 ml of the semisolid medium).

PCR and RT-PCR

DNA was prepared from BM and from single or pooled CSs by standard methods and the GFP transgene was detected by PCR using

5′-ACATGAAGCAGCACGACTTC-3′ and 5′-TTGTGGCGGATCTTGAAGTT-3′ primers, and its identity confirmed by sequencing.

Total RNA was isolated from CS expanded cells and from a pool of CSs at days 3 and 8 after plating on poly-D-lysine, with the use of TriZol (Invitrogen), according to the manufacturer's instructions. Reverse transcription was performed on 2 μg starting RNA by M-MLV reverse transcriptase (Invitrogen, Milan, Italy) in a 20 μl reaction, and 2 μl of cDNA product were then subjected to PCR.

Conditions for the PCR on amplified cDNA template included enzyme activation at 95°C for 15 min., followed by 40 cycles (denaturation at 95°C for 15 sec., annealing at 58°C for 60 sec. and extension at 72°C for 60 sec.), with a final 4°C step. Cardiac genes were detected using the following primers: Nkx2.5-FW 5′-CAG TGG AGC TGG ACA AAG CC-3′; Nkx2.5-RV TAG CGA CGG TTC TGG AAC CA; Cardiac actin-FW TGA GAT GTC TCT CTC TCT CTT AG; Cardiac actin-RV ACA ATG ACT GAT GAG AGA TG.

Results

BM-derived cells are able to generate cardiospheres *in vitro*

We previously [1, 8] identified in human and murine hearts a CSCs population, based on the ability of cells derived from *in vitro* cardiac explants to efficiently proliferate and to generate floating CSs. CSs are a kind of microtissue, which spontaneously express cardiac markers in a minority of cells and occasionally show synchronous beating. They may undergo cardiac differentiation (*in vitro* and *in vivo*), and engraft the heart. The cell giving rise to the CS (CSCs) is proposed to be a Kit^+ precursor, based on immunofluorescence studies with anti-Kit antibodies and analyses of CSCs from Kit/GFP transgenic mice [1].

The Kit/GFP transgenic line expresses GFP from a construct driven by mouse Kit gene promoter and enhancer elements, linked to the GFP gene [25, 26] The transgene is appropriately expressed in several Kit^+ stem/ progenitor cell types, including primordial germ cells, early haematopoietic progenitors and stem cells (HSC) and CSCs [1, 25–28].

To investigate if BM-derived cells can give rise to CSCs, we transplanted total BM from Kit/GFP transgenic mice into lethally irradiated syngeneic non-transgenic mice (Fig. 1). After 5 months, a subset of the recipient mice were subjected to MI, and after three additional weeks, mice with or without MI were sacrificed. Full BM reconstitution, as assessed by haematological parameters (Fig. S1), was demonstrated.

In parallel, myocardial tissues were cultured as explants and after 1–4 weeks an adherent layer of fibroblast-like cells was formed and small, phase-bright cells migrated on top of it. These phase-bright cells were plated under conditions supporting the formation of CSs (Fig. 2). CS-forming cells were harvested at least four times at 6- to 10-day intervals from the same explant. Once formed, primary CSs (5–10/heart) were collected and amplified [1, 8], by growing them as adherent cells for 1 month, and subsequently transferred back onto poly-D-lysine-coated plates to generate large numbers of secondary CSs for analysis.

Efficient cell growth and CS formation was achieved in six of nine mice with MI, but not in mice not subjected to MI. During culture in fibronectin, very few cells expressed GFP, as expected [1]; however, all CSs that developed in poly-D-lysine (whether primary or secondary) contained the GFP transgene (as demonstrated by PCR) and progressively expressed GFP, marking a relatively large proportion of cells (Figs 2 and S2). At late stages of CS development, smaller numbers of GFP^+ cells persist, suggesting that the kit/GFP is down-regulated (see below).

Cells from Kit/GFP BM-derived CS express cardiac markers

CSs grow by expansion of a central core of immature cells, which initiate differentiation in the course of their displacement to the periphery of the sphere; this results in the appearance of a small number of cells positive for Nkx2.5, a transcription factor representing an early cardiac marker, and occasionally Troponin I, a latter cytoplasmic marker [1]. A very low level of *in vitro* differentiation of a type of Kit^+ CSC was also reported by Anversa's group [6]. A representative pool of about 120 CSs, derived from six different transplanted mice, were collected and individually stained with antibodies against Nkx2.5 or Troponin I to evaluate both antibody staining and GFP fluorescence. Confocal analysis shows cells that express GFP together with Nkx2.5 (Figs 3A, B and S2). The percentage of GFP^+ cells co-expressing Nkx2.5 is rather variable, ranging between 5% and 50% and depends on the developmental stage of the CSs. Conversely, a very low level of GFP, if any, is stained by anti-TnI-antibody (data not shown). This suggests that cells, which occasionally commit to cardiomyocyte differentiation (*i.e.* are $Nkx2.5^+$), progressively extinguish Kit/GFP expression, while up-regulating cardiac-specific genes. Interestingly, when a GFP^+ CS is dissociated and the resulting cells are replated in fibronectin, the kit/GFP gene is substantially down-regulated; however, if the cells are again grown on polylisine, GFP expression is reactivated (Fig. 3C). This is in agreement with previous findings by Messina *et al.* [1]. Thus, BM-derived cells present in the CS may express cardiac markers.

CSs have no haematopoietic potential

We also investigated whether haematopoietic progenitors, which express Kit, might be present within the population of GFP^+ cells of CSs. Firstly, we grew BM cells from wild-type untreated or transplanted-MI mice in the culture conditions used for CS formation. BM cells from two untreated and five transplanted-MI mice were plated in fibronectin-coated dishes and after 2 days transferred to poly-D-lysine-coated multiwell plates. The cultures, scored over a period of 1 month did not show any CS-like clones,

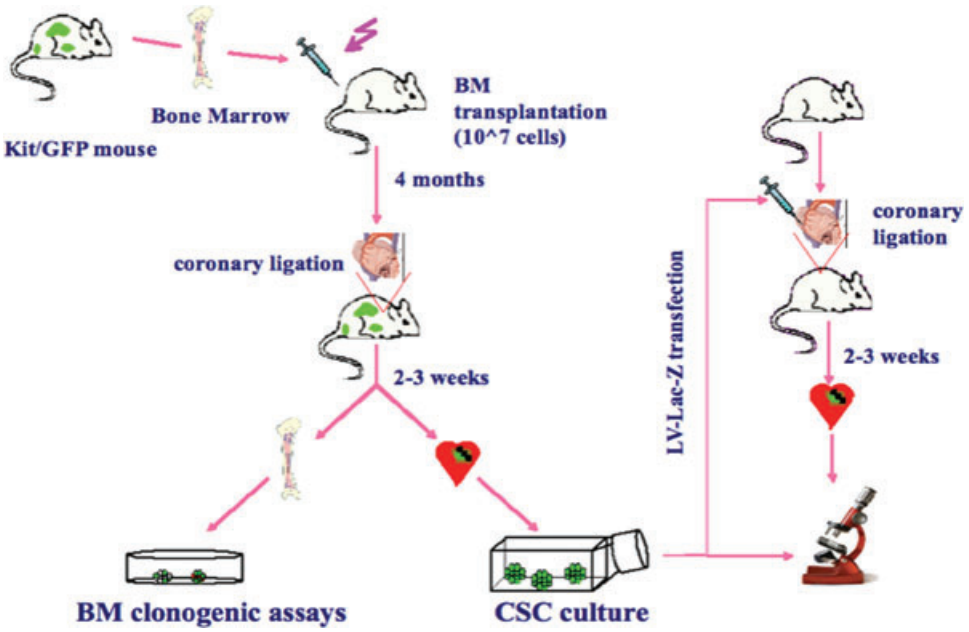

Fig. 1 Schematic representation of the experimental design.

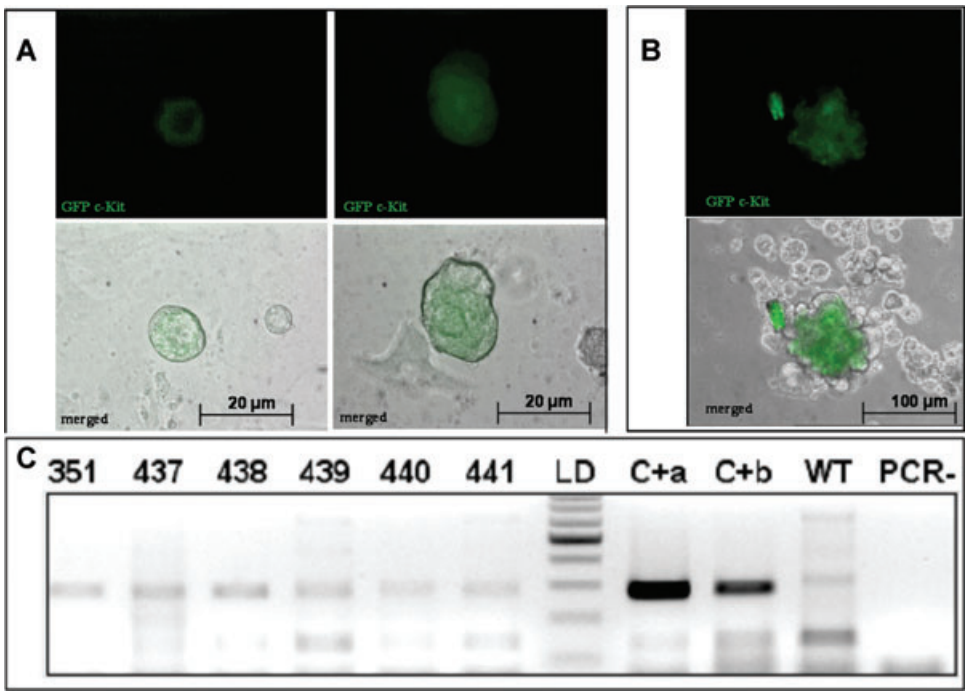

Fig. 2 Kit/GFP$^+$ CS and CS-forming-cells. (**A**) Kit/GFP$^+$ CS-forming cells were collected from the explant of infarcted heart 13 days after the beginning of the culture, and plated onto poly-D-lysine for 1 day to allow CS formation. Note the initial appearance of slightly fluorescent cells. (**B**) A CS at a more advanced stage of development shows transgenic Kit/GFP expressing cells at the centre of the sphere. (**C**) PCR analysis of Kit/GFP in genomic DNA extracted from single CSs. 351–441 indicate different transplanted-infarcted mice; LD, size-marker; C+a and C+b represent internal positive controls (genomic DNA from 10^7 and 10^6 Kit/GFP BM cells); WT, genomic DNA from 10^7 wild-type BM cells; PCR-, negative control on PCR mix. Product of amplification from sample 438 was confirmed by *in vitro* automatic sequencing (not shown).

but rather exhibited the morphological characteristics of mesenchymal cells (Fig. S3). Second, we plated CS cells, from either fibronectin- or poly-D-lysine-coated cultures, in clonogenic assays used for evaluation of haematopoietic progenitors. Cultures, scored at various time-points, did never reveal formation of haematopoietic colonies (not shown).

Finally, we analysed CSs to verify whether they might harbour haematopoietic stem cells, capable of long-term BM reconstitution.

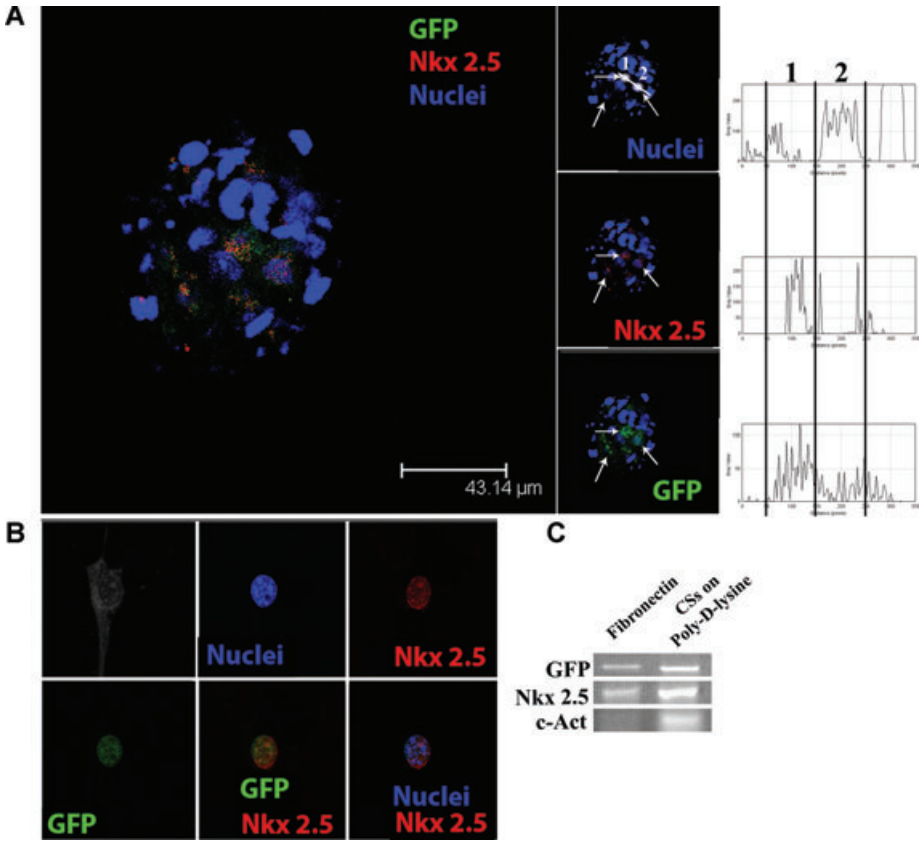

Fig. 3 CS and CSDCs phenotypes. Confocal analysis of a CS, derived from the infarcted heart of a lethally irradiated mouse transplanted with marrow cells of Kit/GFP transgenic mice (**A**) Merged image showing co-expression in some cells (arrows) of donor cell-derived GFP (green) and Nkx2.5 (an immature cardiac cell-specific transcription factor) (red); nuclei are stained blue by Hoechst dye. The figure is an average of 13 *z*-axis confocal sections. Single channel fluorescence intensity of some cells within the sphere is represented by the plots, depicting the fluorescence of cells traced by the white line. Co-localization is evident for the cells indicated as cell 1 and 2 whereas the third nucleus is expressing neither GFP nor Nkx2.5. (**B**) Single cell derived from CS dissociation (from a different CS), demonstrating nuclear co-localization of GFP and Nkx2.5. (**C**) RT-PCR of GFP, Nkx2.5 and cardiac actin from cells expanded on fibronectin from a single clonogenic CS, and later analysed as a monolayer on fibronectin (left) or as CSs (on polylisine).

Pools of CSs from Kit/GFP transgenic newborn mice, were injected into lethally irradiated recipients together with small numbers of wild-type BM cells as support. None of the reconstituted mice had CS-derived BM cells (Fig. S4), not even those transplanted with 10^6 CS cells, as assessed by PCR. We conclude that CSs do not seem to have haematopoietic potential.

Discussion

It was recently proposed that exogenous BM-derived cells can be introduced, or mobilized, into the heart, to regenerate cardiomyocytes and/or vascular endothelium, leading to functional heart improvement [10–14, 24, 31]. The mechanism for the beneficial effect of BM cells on the heart is controversial. Initial suggestions that BM *Kit*$^+$ cells may transdifferentiate into cardiomyocytes, independently of cell fusion [10, 31–33], were rejected by others, at least as far as haematopoietic cell types were considered [15, 16, 18, 34]. However, non-haematopoietic BM cells were reported to generate cardiomyocytes or cells with immunophenotypic properties of cardiac progenitors after homing to the heart [12, 35]; moreover, cardiomyocyte conversion of angioblastic lineages has been proposed as being obtainable by direct contact of cells with cardiomyocytes [36]. Finally, it was shown that relatively large numbers of BM cells home to the heart after MI and contribute to an improvement in cardiac function mainly through the release of angiogenic cytokines [19].

Here we show that small numbers of *Kit*/GFP-labelled BM cells, recruited to the heart following BM transplantation and cardiac infarction, are able to extensively proliferate *in vitro*, participating in the generation and growth of CSs. Cells from these CSs express the early cardiac marker Nkx2.5 *in vitro*. These data show that, under pathological conditions such as an infarction, BM-derived cells may give rise, in the heart, to a progeny that shares at least some of the properties of endogenous CSC.

BM-derived Kit/GFP$^+$ cells form *in vitro* CSs

The rationale for our experimental strategy is that resident CSCs are *Kit*$^+$ [6, 28] and that *Kit*/GFP$^+$ cells from the heart of *Kit*/GFP transgenic participate in cardiac cell generation and CSs formation *in vitro* [1]. Thus, if CS cells from mice transplanted with transgenic *Kit*/GFP marrow show GFP expression, they must derive from the BM; as Kit is a marker expressed in CSCs, GFP expression from these cells may represent the activation of a CSC

transcriptional program [1, 28]. In this view, BM cells from this transgenic line have been previously used to study cell trafficking: highly purified GFP$^+$ stem/progenitor cells from BM were recruited to muscle following local injury and participated in regeneration [37].

In our experiments, all CSs that grew from the hearts of transplanted (and infarcted mice) showed GFP fluorescence and in all CSs observed, a relatively large proportion of the cells were GFP$^+$ (Figs 2 and S2) indicating that endogenous CSCs were virtually absent in the heart of these mice; if any endogenous CSC had still been present, we should have seen at least some CS consisting entirely or predominantly of unlabelled cells.

Consistent with this result, explants from *Kit*/GFP BM transplanted mice that had not undergone MI, did not give rise to any CS. The lack of CS development from hearts of non-infarcted mice is not unexpected, for several reasons: first, the number or activity of CS-forming cells greatly declines post-natally, particularly in the mouse strain used for transplantation experiments (data not shown), and CS culture was carried out in mice at a relatively advanced age (greater than 8 months), due to the constraints of the experimental protocol; second, injury is required in adult mice to 'activate' stem/precursor cells, inducing them to regenerate cardiomyocytes [38]; third, it is conceivable that the irradiation necessary for BM transplantation may have damaged the endogenous CSC population and/or their niche, either directly or through irradiation-dependent anaemia and white blood cell deficiency.

Infarction mobilizes and recruits into the heart a variety of BM cells [39]. Thus, we interpret our results to signify that, in CSC-deficient irradiated mice, following MI, the heart is repopulated, with BM cells. Some of them develop into *Kit*/GFP$^+$ cells resembling in their growth-properties endogenous CS-forming cells. A proportion of GFP$^+$ cells within CS do indeed express, at low levels, Nkx2.5 (Figs 3 and S2). Whether these BM cells could represent a potential source of endogenous CSCs will require further detailed examination of their functional properties. In this line, experiments are in progress to evaluate whether CS-derived cells obtained *in vitro* as described above are able to engraft damaged myocardium, generating new muscle fibres. Preliminary data suggest that cells from BM-derived CSs may engraft and give rise to cardiomyocytes and vascular cells *in vivo* (Fig. S5).

Biological and functional implications of BM-derived CSCs

We have shown that BM-derived cells can convert, upon migration to the heart, into cells that, when placed *in vitro*, can extensively replicate and give rise to precursors with CS-forming properties. Importantly, these observations differ in many respects from previous results of other investigators. It was initially proposed that direct implantation of BM cell populations into infarcted myocardium, or their mobilization from the BM into the circulation at the time of infarction, could lead to cardiomyocyte and/or vascular endothelium regeneration by transdifferentiation, independently of cell fusion [31, 33].

Whether or not these experiments are accepted, our results clearly differ from them, as we do not propose that BM cells directly transdifferentiate into myocardial cells, but rather suggest that they give rise to a 'stem cell' type that may persist in the heart, and participate in regenerative repair after MI.

A further link was recently established between endogenous cardiac repair, BM-derived cells and *Kit* function [19, 39]. Upon MI, *Kit*$^+$ cells from the BM are recruited to the heart, leading to neovasculogenesis and to formation of repair tissue *via* paracrine signalling pathways. These processes were strongly attenuated in Kit-mutant mice, causing precipitous cardiac failure. Although only a minority of the recruited cells persisted in the myocardium a month after the infarction, these experiments did not rule out the possibility that these cells may contribute to the pool of CSCs, and the authors concluded that their studies 'raise the possibility that the putative cardiac c-Kit$^+$ stem cells are in actuality not from the heart' [19]. In agreement with this suggestion, our study provides evidence of a cardiac population of BM-derived cells exhibiting some properties of resident CSCs and potentially useful for cardiac regeneration. Thus, the functional role of *Kit*$^+$ cells that we propose may be complementary to the non cell-autonomous function described by Fazel *et al.* [19]. Finally, a rare non-haematopoietic cell type (mesenchymal stem cell, MSC) in BM was also found to migrate to the heart upon infarction, and to give rise to a small number of cardiomyocytes [12], thus suggesting the potential usefulness of these stromal stem cells for cardiac cell therapy. In addition, BM-derived cells were shown to be able to migrate to the heart, generating cells with an immunophenotype corresponding to that of endogenous CSC [35]. Our results are consistent with these findings, and suggest that the reported [12] MSC to cardiomyocyte transition might occur *via* a cell showing the self renewing and differentiation features of CSCs.

A final issue concerns cell fusion, which was suggested to be the underlying cause of many transdifferentiation events previously reported [40, 41]. We cannot stringently rule out that cell fusion may have occurred in our experiments, but we think it is unlikely. The number of endogenous functional CSCs in the heart in the 8-month-old transplanted mice used in our experiments is very low, as indicated by our inability to grow any CSC from these hearts in the absence of MI (and thus of recruitment of exogenous cells to the heart), and a fusion event is therefore extremely improbable. It remains possible that exogenous *Kit*/GFP$^+$ cells have occasionally fused to endogenous myocardial cells; however, this event would explain only the appearance of labelled cardiac fibres, but not of mononucleated, extensively dividing cells, as we report here.

The nature of the BM cell responsible for the generation of CS in our experiments remains to be better clarified. It might be either haematopoietic or more likely, mesenchymal. Our results, however, provide evidence that CSs do not contain haematopoietic stem or progenitor cells as such: first, BM cells grown *in vitro* under conditions appropriate for CS formation do not give rise to any CS-like aggregate; second, even large numbers of cells derived from CSs are not capable of *in vitro* colony formation or *in vivo* BM reconstitution, upon transplantation. Thus, BM-derived cells that give rise to CSs are likely to be of a non-haematopoietic

cell type. In agreement with this, previous work [16] showed that haematopoietic-derived cells do not transform into skeletal or cardiac muscle. The precise identification of the BM cell type responsible for CS formation in this experimental protocol still requires rigorous investigation, and might be addressed by fractionation of Kit/GFP BM cells prior to transplantation.

In conclusion, we demonstrate generation of cells exhibiting CS-forming properties from BM-derived cells in the heart of irradiated mice subjected to cardiac damage. This may have not only induced the recruitment of cells to the heart, but also stimulated the conversion of some cells to CS-forming cells. Our experiments do not allow establishing whether irradiation-dependent cardiac damage or infarction is strictly necessary. These observations, however, provide the first evidence that cells with extensive proliferation potential (*i.e.* CSC-like cells) can be generated in the heart from exogenously derived cells, raising the possibility that these extracardiac cells might, potentially, be directed to replenish the CSC pool in damaged hearts.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Haematopoietic reconstitution of Kit/GFP bone marrow-transplanted mice.
The haematopoietic reconstitution of mice transplanted with Kit/GFP bone marrow was assessed after 4 months using clonogenic assays: (**A**) Haematopoietic precursors in donor and transplanted mice, (**B**) Percentage of reconstitution in the transplanted mice, as assessed by the percentage of fluorescent CFU-Mix colonies, (**C**) Example of fluorescent CFU-Mix colony (*left*, bright field; *right*, fluorescence).

Fig. S2 Kit/GFP and Nkx2.5 expression in different layers of a cardiosphere.
Confocal analysis of a CS, derived from the infarcted heart of a lethally irradiated mouse transplanted with marrow cells of Kit/GFP transgenic mice (**A**) Merged image showing co-expression in some cells (arrows) of donor cell-derived GFP (green) and Nkx2.5 (an immature cardiac cell-specific transcription factor) (red); nuclei are stained blue by Hoechst dye. The figure is an average of 13 *z*-axis confocal sections. Single channel fluorescence in different layers of the sphere is represented by histograms, depicting the fluorescence of each cell (**B**).

Fig. S3 *In vitro* bone marrow cells cultured in CS supporting medium. Bone marrow cells were cultured following the culture protocol used to obtain cardiospheres from heart explants. No sphere-like aggregates were obtained after culture in polylisine, while mesenchymal-like monolayers were observed (*left*, 100×; *right*, 200×).

Fig. S4 Analysis of CSC haematopoietic potential. (**A**) Schematic representation of the experimental procedure adopted to test the haematopoietic potential of CSC; (**B**) PCR on genomic DNA extracted from 10^7 bone marrow cells of each transplanted mouse 4 months after receiving a transplant of cardiosphere cells from the heart of Kit/GFP transgenic mice: none of the recipient animals (labelled from 422 to 436) showed the presence of Kit/GFP construct (C+, positive control; wt, untransplanted wt mouse).

Fig. S5 Engraftment and Differentiation of CSDCs. Confocal analyses of infarcted heart section of a wild-type mouse, 20 days after injection of β-galactosidase (β-Gal)-lentivirally labelled cells from GFP^+ cardiospheres obtained from hearts of Kit/GFP marrow-transplanted mice. (**A**) Low magnification picture of the CSDCs (identified with a primary antibody against nuclear β-galactosidase (red)) that are present in the transmural infarct, with cells expressing myosin heavy chain (MHC, green) in the regenerating area. (aI) higher magnification views of picture shown in panel a: co-localization of $β\text{-}Gal^+$ CSDCs and viable myocardium (arrows). (aII–aIV) Same picture showing the nuclear β-Gal expression, red (aIII), the corresponding Hoechst labelling (arrows) (aII) and (aIV) a merged image of the three fluorescent channels (MHC, green).
(**B**) nuclear β-Gal(green)-expressing CSDCs, double labelled for smooth muscle actin (red), are shown within the vessels walls (arrows) in the context of the infarcted area.
(bI–bIII) Single fluorescent channels view of cells indicated in panel b: the β-Gal-stained nuclei (bI, green) correspond to the Hoechst-labelling (blue) in the same cells (bII). In bIII, smooth muscle actin positive cells in the same field. bIV) merged image of the three fluorescent channels.
(c-cI) Haematoxylin/eosin and β-Gal (blue) hystochemical staining of criosections from one of the infarcted hearts, explanted 20 days after CSDCs injection: engraftment of the β-Gal (blue) stained CSDCs is shown. Blue-labelled nuclei are present within the haematoxylin and eosin stained area.

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