C-kit+ cardiac progenitors exhibit mesenchymal markers and preferential cardiovascular commitment

Elisa Gambini1,2, Giulio Pompilio1,2,3, Andrea Biondi4, Francesco Alamanni2,3, Maurizio C. Capogrossi5, Marco Agrifoglio2,3, and Maurizio Pesce1*

1Laboratorio di Biologia Vascolare e Medicina Rigenerativa, Via Parea 4, I-2018, Milan, Italy; 2Dipartimento di Scienze Cardiovascolari, Università di Milano, Italy; 3Dipartimento di Chirurgia Cardiovascolare, Centro Cardiologico Monzino-IRCCS, Italy; 4Centro Ricerca M. Tettamanti, Clinica Pediatrica Università degli Studi di Milano Bicocca, Italy; and 5Laboratorio di Patologia Vascolare, Istituto Dermopatico dell’Immacolata, IDI-IRCCS, Italy

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Aims

The heart contains c-kit+ progenitors that maintain cardiac homeostasis. Cardiac c-kit+ cells are multipotent and give rise to myocardial, endothelial and smooth muscle cells, both in vitro and in vivo. C-kit+ cells have been thoroughly investigated for their stem cell activity, susceptibility to stress conditions and ageing, as well as for their ability to repair the infarcted heart. Recently, expression of mesenchymal stem cell (MSC) markers and MSC differentiation potency have been reported in cardiac progenitor cells. Based on this evidence, we hypothesized that c-kit+ cells may have phenotypic and functional features in common with cardiac MSCs.

Methods and results

Culture of cells obtained from enzymatic dissociation of heart auricle fragments produced a fast-growing fibroblast-like population expressing mesenchymal markers. C-kit+ cells co-expressing MSC markers were identified in this population, sorted by flow cytometry and cultured in the presence or the absence of unselected cardiac cells from the same patients. Subsets of c-kit+ cells also co-expressed MSCs markers in vivo, as detected by immunofluorescence analysis of auricle tissue. Ex vivo expanded c-kit+ cells produced osteoblasts and adipocytes, although less preferentially than bone marrow-derived MSCs, possessed vascular smooth muscle cell features and were induced to differentiate into endothelium-like and cardiac-like cells.

Conclusion

In line with previous findings, our results indicate that c-kit+ cardiac progenitors are primitive stem cells endowed with multilineage differentiation ability. They further suggest a possible relationship between these cells and a heart-specific MSC population with cardiovascular commitment potential.

Keywords

C-kit • Cardiac progenitor • Cardiac MSC • Differentiation

1. Introduction

After the seminal discovery that the adult heart is a self-renewing organ, undergoing continuous replacement of myocytes throughout life,1–3 several candidate progenitor cells that sustain myocardial self-renewal have been found. These include side population (SP) myocardial cells, identified on the basis of their ability to extrude drugs by activity of MDR-1 gene product,4 progenitor cells characterized by expression of Sca-1/Sca-1-like antigens,5,6 cells derived from the so-called ‘cardiospheres’,7,8 Isl1+ cardioblasts,9,10 epicardial tissue stem cells11 and c-kit+ cardiac progenitor cells (CPCs).12–14

While the discovery of CPCs has raised expectations for the development of stem cell-based therapy of the heart, several issues still exist concerning an unambiguous definition of the phenotype of cardiac-derived stem cells.

Mesenchymal stem cells (MSCs) were first derived from the adherent component of the mononuclear cellular fraction isolated by density gradient centrifugation of crude bone marrow (BM), followed by culture in adhesive conditions. Compared with other stem cell types, MSCs cannot be uniquely distinguished by expression of tissue-specific markers. In fact, MSCs exist in the stromal compartment of virtually every tissue and are characterized by expression of unspecific...
antigen sets.\textsuperscript{15} Recently, minimal consensus criteria for identification of MSCs have been established; these are plastic adherence and formation of the so-called colony forming units-fibroblast (CFU-F) colonies, osteogenic and adipogenic differentiation ability, expression of CD29 (integrin-\( \beta_1 \)), CD44 (H-CAM), CD90 (Thy-1) and CD105 (Endoglin), and absence of hematopoietic (stem) cell markers such as CD34, CD45 and CD133.\textsuperscript{16}

Preclinical studies have shown that BM-derived MSCs have a potential to repair the infarcted heart by promoting cardioprotection and neovascularization through a potent paracrine effect,\textsuperscript{17–19} but also by direct differentiation into cardiac myocytes.\textsuperscript{20–22} The advantage of MSCs over CPCs is the relative easiness and reproducibility of techniques to obtain and expand them in culture; a potential disadvantage of these cells compared with CPCs may arise from their commitment to differentiate into osteogenic cells, therefore raising the risk of unwanted bone formation in the heart.\textsuperscript{23–25} Despite this, the feasibility of using BM-derived MSCs for myocardial repair in patients has been demonstrated in a recent phase I clinical study\textsuperscript{26} where no side-effects of this kind were observed.

The existence of heart-specific MSCs has not yet been formally demonstrated. In contrast, results with cardiosphere-derived cells have suggested the existence of these cells based, for example, on expression of CD90 and CD105 markers.\textsuperscript{27,28} Furthermore, CPCs isolated from the human heart have been shown to differentiate into adipocytes and osteocytes in vitro.\textsuperscript{29} These results led us to hypothesize that c-kit\textsuperscript{+} cells may have phenotypic and functional features in common with cardiac-specific MSCs. In the present study, we therefore investigated expression of MSCs markers in c-kit\textsuperscript{+} cells in the human heart tissue and devised a protocol to culture cardiac cellular populations and isolate c-kit\textsuperscript{+} cells by high-throughput flow cytometry. Using this system, expression of cardiovascular and mesenchymal markers as well as multilineage differentiation were finally investigated in c-kit\textsuperscript{+} CPCs before and after ex vivo amplification steps.

### 2. Methods

#### 2.1 Ethics statement

The collection of auricle fragments and bone marrow specimens was performed after obtaining written informed consent from the patients. The study was approved by the Local Ethical Committee and review Board (approved on 4 August 2008; reference no CCFM C9/607 and CCFM C10/607), and was performed according to Italian national laws. Experimentation conformed to the principles outlined in the Declaration of Helsinki.

#### 2.2 Cell isolation, culture and differentiation

The procedure to obtain primary and secondary cultures of human CPCs from auricle fragments is adapted from a previously published method.\textsuperscript{13} The methods are described in detail in the Supplementary material online. To assess mesenchymal and cardiovascular in vitro commitment, CPCs were cultured in conditions known to induce adipogenic, osteogenic, endothelial and cardiac differentiation. The methods used to perform these experiments are detailed in the Supplementary material online.

#### 2.3 Flow cytometry

A FACSARia (Beckton-Dickinson, BD-Biosciences, San Jose, CA, USA) flow cytometer/cell sorter was used to identify and separate c-kit\textsuperscript{+} from c-kit\textsuperscript{–} cells. Cell sorting set-up and parameters are described in the Supplementary material online. Analysis of mesenchymal, endothelial, human leucocyte antigen (HLA) and hematopoietic markers was performed by multicolour analysis flow cytometry. Antibody panels and staining conditions are described in the Supplementary material online.

#### 2.4 Quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR)

Total RNA was purified from primary cells, c-kit\textsuperscript{+} cells immediately after sorting (T0) and transwell c-kit\textsuperscript{+}-derived cells at 4 passages after the beginning of the culture (P4) before and after culturing into pro-adipogenic, pro-osteogenic or cardiac inducing conditions. After reverse transcription, primers specific for cardiac, stem cells, differentiated mesenchymal cells and vascular cells were used to assess the cell phenotype of human CPCs by real-time PCR. Experimental procedures, conditions and a full list of primers used in these experiments are described in the Supplementary material online.

#### 2.5 Immunofluorescence and confocal analysis

Transwell c-kit\textsuperscript{+}-derived cells at P4 were fixed or subcultured for 1–3 weeks into cardiac differentiation media; the cells were fixed using 4% paraformaldehyde in phosphate-buffered saline. After blocking with phosphate-buffered saline containing 5% serum and 0.3% Triton X-100 overnight at 4°C, cells were incubated with primary antibodies for \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) or cardiac markers. Auricle fragment tissue biopsies were fixed with 4% paraformaldehyde and further processed for tissue sectioning, immunofluorescence and confocal analysis. Further details are provided in the Supplementary material online.

#### 2.6 Cytokine, chemokine and growth factor detection

Bio-Plex assay (Bio-Rad Laboratories, Italy), a bead-based multiplex immunoassay, was used to quantify cytokines, chemokines and growth factors released into the culture supernatant conditioned for 24 h by similar amounts of primary cells at T0 and supporting stromal cells at P4. Supernatants were collected after 24 h of conditioning and analysed by Bio-Plex assay.

#### 2.7 Statistical analysis

Comparison of results was performed by using Student’s paired or unpaired t-tests using GraphPad statistical software. A value of \( P < 0.05 \) was chosen as the significance level for considering results different on a statistical basis.

### 3. Results

#### 3.1 Overlapping between c-kit\textsuperscript{+} CPC and MSC phenotypes in human atrial tissue

In the mouse and the human heart, c-kit\textsuperscript{+} CPCs have been defined as clonogenic cells arising from cell suspensions obtained by myocardium enzymatic digestion according to two methods based on (i) direct purification of c-kit\textsuperscript{+} cells from myocardial tissue primary cell suspensions using immunomagnetic beads\textsuperscript{12,13} or (ii) c-kit\textsuperscript{+} cells immuno-sorting from cells obtained by primary explant technique,\textsuperscript{13} followed by culture under clonal dilution. To maximize growth of stem cells before c-kit\textsuperscript{+} cell sorting, and in keeping with recent results showing derivation of cells endowed with cardiac repair ability directly from heart specimens,\textsuperscript{27} we plated for two passages the total cellular population obtained by enzymatic digestion of heart auricle fragments. This phase was designated the primary expansion period (Supplementary material online, Figure S1, Table S1). Auricle tissue fragments from patients undergoing
aorto-coronary bypass grafting were used according to previous results showing the presence of CPCs in the right atrium.\textsuperscript{8,30} Previous findings obtained with cardiosphere-derived cells from the mouse and the human heart demonstrated expression of MSCs markers in populations of CPCs.\textsuperscript{7,28,31} Therefore, also according to minimal consensus criteria for the definition of MSCs,\textsuperscript{15,16,32} an antibody panel recognizing MSC-specific markers was used to characterize the cell population grown during the primary expansion period. Flow cytometry analysis of these cells (Figure 1A and B) revealed high expression of MSC markers CD29, CD44, CD105, CD130 and CD200, intermediate expression of MSC marker CD90 and endothelial cell (EC) markers CD146 and KDR, and low expression of EC marker CD144. Primary cells were also investigated for c-kit expression and possible overlapping between c-kit\textsuperscript{+} cells and cells with MSC features, using multicolour flow cytometry analysis. The results revealed co-expression of CD105, CD29 and CD44 and, to a lesser extent, of CD90 in these cells (Figure 1C and D).

Co-expression of c-kit and MSC markers might be a consequence of cell differentiation during the primary expansion of the auricle fragment-derived cells. To assess this hypothesis, a four-colour confocal

**Figure 1** Characterization of human auricle primary cultured cells and co-expression of MSC markers with c-kit in primary cells at T0. (A and B) Expression MSC and EC markers in primary cells obtained from ex vivo amplification of auricle-derived cells. Except for CD90, all MSC markers were expressed in a cellular percentage higher than 98\% (n = 6). The EC markers were expressed at lower levels. (C) Plots on the left indicate the gating strategy adopted to analyse co-expression of MSC markers in c-kit\textsuperscript{+} cells at T0. The c-kit\textsuperscript{+} cells were recognized by staining with specific vs. isotype antibodies, after which the gated c-kit\textsuperscript{+} cells (c-kit\textsuperscript{+}/bright) were analysed for the expression of each MSC marker (plots on the right). (D) Bar graph shows quantification of the c-kit\textsuperscript{+}/CD29\textsuperscript{+}, c-kit\textsuperscript{+}/CD44\textsuperscript{+}, c-kit\textsuperscript{+}/CD90\textsuperscript{+} and c-kit\textsuperscript{+}/CD105\textsuperscript{+}. Apart from expression of CD90, all the other mesenchymal markers were expressed in percentages higher than 91\% in c-kit\textsuperscript{+} CPCs at the time of the initial sorting (n = 5).
Figure 2 Caption overleaf
sections with antibodies recognizing CD29 and CD105 MSC markers in conjunction with anti-c-kit and anti-cardiac marker α-sarcomeric actin (α-SA) antibodies (Figure 2). The results showed the presence of isolated or grouped c-kit+ cells co-expressing the two MSC markers. Notably, CD29 and CD105 expression was detected only in subsets of c-kit+/α-SA–/ cells within CPC niches, thus suggesting heterogeneity of CPCs population in vivo.

3.2 High-throughput sorting of c-kit+ cells and co-culture onto parental unselected cells allows the propagation of a CPC-enriched cellular population with MSC features

C-kit+ cells were isolated using a high-throughput flow cytometry-based cell sorting method (Figure 3A) and expanded for four consecutive passages using a transwell-based culture onto parental unselected primary cells, already used in a previous study from our laboratory (T0–P4 expansion period; Supplementary material online, Figure S1). The choice of this method was made in order to allow c-kit+ cells to grow in the presence of factors released by unselected cells but without physical contact with them (‘transwell’ conditions), and thus reveal potential paracrine effects of supporting cells on stem cell growth. As a control, cells cultured without supporting cells in transwell inserts were used (‘no transwell’ conditions).

To assess differences in the expression of MSC/EC markers in transwell vs. no transwell conditions, the percentages of c-kit+, MSC marker+, and EC marker+ cells were determined by flow cytometry. The results showed that either in the presence or in the absence of supporting cells, the percentage of c-kit+ cells was decreased (Figure 3B). However, in the presence of supporting cells, a higher percentage of c-kit+ cells was maintained, and the mean fluorescence intensity relative to c-kit antibody staining was significantly higher (Figure 3C). In these conditions, the absolute number of c-kit+ CPCs was increased by 26.44 ± 5.71-fold (mean ± SEM; n = 30; Supplementary material online, Table S2). Finally, expression of MSC and EC markers did not change (Figure 3D and E; Supplementary material online Figures S2, S3, S4).

The paracrine effect of heart-derived supporting cells was quantified by measuring the secretion of a cytokine array by Bio-Plex analysis. This revealed the expression of several factors which are likely to support growth of c-kit+ cells in coculture conditions. Interestingly, a number of these cytokines were upregulated from passage 1 to 4 (e.g. granulocyte-colony stimulating factor (G-CSF), interleukin-8, vascular endothelial growth factor and interleukin-6; Supplementary material online, Table S1), suggesting mutual induction between supporting cells and c-kit+ CPCs.

3.3 Phenotype stability of c-kit+ -derived cells

To assess the phenotype stability of CPCs during ex vivo expansion, qRT-PCR on cDNA obtained from primary cells, sorted c-kit+ cells at T0 and transwell-cultured c-kit+ -derived cells at P4 was performed using primers to amplify genes expressed in undifferentiated (c-kit and MDR-1) or early differentiating cardiac progenitors (GATA-4, Tbx-5 and Mlc2A). As shown in Figure 4A, T0 c-kit+ cells and transwell P4 c-kit+ -derived cells expressed c-kit at significantly higher levels compared with unselected primary cells; by contrast, the other genes were not changed (Figure 4A). To assess whether major changes occurred in the expression of cardiac genes in undifferentiated CPCs during the co-culture period, c-kit+ cells from the same patients were sorted at T0 and further resorted at P4, after which expression of the cardiac-specific genes was compared by qRT-PCR. Figure 4B shows the results of these experiments. The percentage of c-kit+ cells in sorting gate at P4 was similar to that of c-kit+ cells at T0; in addition, no differences were found in expression of cardiac genes in c-kit+ cells sorted at the two different culture steps. Taken together, these results suggest self-renewal and phenotype stability of CPCs over the time of the ex vivo amplification procedure.

3.4 Reduced mesenchymal differentiation of human heart-derived c-kit+ CPCs compared with canonical MSCs

The ability to differentiate into osteoblasts, adipocytes and chondroblasts is one of the major criteria to recognize cells with MSC phenotype. Transwell and no transwell c-kit+ -derived cells at P4 were then cultured in adipogenic and osteogenic differentiation conditions. Results showed similar morphological differentiation of these cells into adipocytes compared with BM-derived MSCs (Figure 5A), while osteocyte differentiation appeared unfavoured (Figure 5B). In addition, qRT-PCR experiments with primers amplifying key genes implicated in adipogenic (PPAR-y and Adipsin) or osteogenic differentiation (Osteopontin) revealed significantly lower expression levels compared with BM-derived MSCs cultured in pro-adipogenic and pro-osteogenic media, thus revealing reduced canonical MSC differentiation potency.

Figure 2 Confocal analysis of CD29 and CD105 in c-kit+ cells clusters in human auricle tissue. (A and B) Confocal images of CD29 staining (light blue fluorescence) in conjunction with c-kit (green fluorescence), α-SA (red fluorescence) and nuclear staining (blue fluorescence) in auricle tissue sections. The c-kit+ cells expressing the three markers (arrowheads), c-kit and α-SA (arrows), c-kit and CD29 (*) or c-kit alone (#) were observed in intramyocardial (A) or subepicardial positions (B). (C) Confocal Z-stack projection along the indicated x- and y-axes of c-kit, CD29 and α-SA staining of a large CPC niche. White and green dashed arrows indicate, respectively, a triple c-kit+/CD29+/α-SA+ cell and single c-kit+/CD29+/-α-SA– cell along the Z-stack projection of the x-axis, while red dashed arrows indicate a triple c-kit+/CD29+/-α-SA– cell along the Z-stack projection of the y-axis. (D–G) Confocal images of CD105 staining (light blue fluorescence) in conjunction with c-kit (green fluorescence), α-SA (red fluorescence) and nuclear staining (blue fluorescence) in auricle tissue sections. Clusters of c-kit+ cells are visible in intramyocardial and perivascular positions. Cells expressing the three markers (arrowheads), c-kit and α-SA (arrows), c-kit and CD105 (*) or c-kit alone (#) were found. Three different c-kit+ cells clusters are shown at low magnification (D, F and G). (E) The c-kit+ CPCs present the region enclosed into the dashed-line rectangle in D. Cm, cardiac myocytes; ep, epicardium; ar: arteriole.
Sorting procedure for c-kit^+ CPCs and comparison between transwell vs. no transwell culture conditions. (A) Sorting of c-kit^+ cells. The appropriate gating to separate c-kit^- from c-kit^- cells was established each time using allophycocyanin-conjugated isotype antibody. The purity of the c-kit^+ cells was checked by analysing sorted cells immediately after separation using flow cytometry (post-sort analysis) and immunofluorescence. (B and C) Co-culture in transwells onto unselected cells maintained expression of c-kit in a greater percentage of cells with a higher c-kit mean fluorescence intensity (MFI). (B) Analysis of cells from the same patient cultured in the two conditions. (C) Quantification of the percentage of c-kit^+ cells and log_{10} c-kit staining MFI in transwell vs. no transwell c-kit^- derived cells at P4. *P < 0.05 by Student's paired t-test (n ≥ 12). (D and E) Expression of MSC and EC markers in transwell vs. no transwell conditions reveals non-significant differences in the phenotype of c-kit^- derived cells at P4 (n = 5).
Figure 4 Phenotype stability of ex vivo amplified c-kit^+ CPCs. (A) qRT-PCR analysis of stem cell and cardiac marker expression in T0 c-kit^+ cells and transwell P4 c-kit^+-derived cells. The fold change in the expression of each gene was calculated by the $2^{-\Delta\Delta C_t}$ method and normalized to the expression level in primary cells (dotted line indicates $y = 1$ in each bar graph). The symbols *, # and $ indicate, respectively, $P$, $0.05 for the comparison, by Student's paired and unpaired $t$-tests, between the c-kit expression levels in T0 c-kit^+ vs. P4 transwell c-kit^+ -derived cells, or T0 c-kit^+ and P4 transwell c-kit^+-derived cells vs. primary cells ($n \geq 4$). (B) Sequential sorting and resorting of c-kit^+ cells at T0 and P4 after culture in transwells. Upper plots indicate gating adopted to sort c-kit^+ cells at the two stages; bar graph at the bottom left indicates no difference in the percentage of sorted c-kit^+ cells at these two stages ($n = 5$), while no differences were found in the expression of stem cells and cardiac markers (bottom right panel; data calculated by $2^{-\Delta\Delta C_t}$ method and normalized to the expression of each gene in sorted cells at T0; dotted line indicates $y = 1$ in each bar graph).
3.5 Multilineage cardiovascular commitment of P4 c-kit+ derived cells

Cardiovascular commitment of c-kit+-derived cells was investigated by immunofluorescence, functional assays, flow cytometry and qRT-PCR. Vascular smooth muscle cells and endothelial phenotypes were first investigated by analysing the expression of α-SMA in primary cells, sorted T0 and transwell P4 c-kit+ derived cells (Figure 6A), and by plating transwell P4 c-kit+-derived cells onto matrigel to allow formation of tubular-like structures (Figure 6B). Subsequently, culture of transwell P4 c-kit+-derived cells into endothelial growing medium (EGM-2) and two different cardiac differentiation media was used to assess their ability to differentiate into mature endothelial cells and cardiac-like cells (Figure 6C and D).

Results of these experiments showed that transwell P4 c-kit+-derived cells were committed to smooth muscle cell phenotype and that they formed tubular structures in a comparable manner to human EC cells; in addition, culture in EGM-2 caused a significant reduction in c-kit+ cells and enhanced the expression of endothelial marker CD144 and, although not significantly, KDR, while CD146 was unchanged. Finally, differentiation into cardiogenic media induced the expression of mRNAs for the two cardiac genes α-myosin heavy chain (α-MHC) and α-SA, and of atrial natriuretic peptide, gap junction-associated protein connexin 43 and tropomyosin proteins. Taken together, these findings suggest that transwell cultured c-kit+ cells behave as multilineage cardiovascular progenitors, although they do not undergo a complete differentiation programme.

4. Discussion

4.1 MSC features of human c-kit+ CPCs: overlapping or heterogeneity?

In normal conditions, turnover of cardiac myocytes occurs with continuous replacement by progenitors producing new contractile cells throughout adult life. In the case of ischaemia, massive death of myocytes is not compensated by efficient replacement, but is associated to extensive fibrosis due to inflammation-related fibrovascular tissue formation. Although it is documented that lack of myocyte replacement after ischaemic insult is the consequence of enhanced stem cell death and/or stem cell pools exhaustion, it is not yet clear whether cross-talk exists between the stem cell compartment and the stromal component in the heart.

Besides myocytes, vascular and progenitor cells, the adult heart contains stromal cells. Fibroblasts are the most abundant cardiac stromal cellular component, ranging from 30 to 70% (depending on species) of the total number of heart cells. The origin of cardiac fibroblasts is mesenchymal, and their presence in the heart is due to epithelium–mesenchyme transition of epicardium-derived progenitors or by direct differentiation of mesenchymal progenitors. The demonstration of a heart-specific mesenchymal cell population has not been formally provided. However, the expression of mesenchymal markers in heart-derived clonogenic and non-clonogenic progenitors has already been documented. For example, expression of MSC markers was reported in clonally amplified mouse Sca-1+ CPCs (M. Pesce et al., unpublished...
Figure 6  Cardiovascular commitment of P4 c-kit⁺-derived cells. (A) Expression of α-SMA in T0 c-kit⁺ and transwell c-kit⁺-derived cells at P4. Immunofluorescence micrographs of transwell c-kit⁺-derived cells at P4 show α-SMA staining in cells with different morphologies, as follows: small cells (*) expressing low or intermediate α-SMA levels, larger cells expressing higher α-SMA levels (arrows) and cells showing partially polymerized α-SMA in the cytoplasm (arrowheads). The bar graph on the right indicates the fold change expression of α-SMA mRNA in T0 and c-kit⁺-derived cells at P4. Data are calculated by the 2⁻¹ΔΔCt method and normalized to the expression of each gene in primary amplified cells (dotted line indicates y = 1 in the bar graph). *P < 0.05 by Student’s unpaired t-test for the comparison of α-SMA expression levels in T0 and c-kit⁺-derived cells at P4 compared with primary cells (n = 4). (B) Formation of tubular-like structures by P4 c-kit⁺-derived cells and human umbilical vein endothelial cells (HUVECs) onto Cultrex basement membrane. Micrographs at the top show the morphology of these structures. Bar graphs show the quantification of these structures per microscopic field (left) and the number of branching points between them (right). No statistically significant differences were found (n = 3). (C) Expression of EC markers in transwell c-kit⁺ cells at P4 and after replating these cells into EGM-2. Histogram plots show the fluorescence profile of each antigen after culture into EGM-2. Insets show the reference fluorescence plots obtained by staining cells using isotype antibodies. Bar graph on the right shows the comparison between c-kit⁺, CD144⁺, CD146⁺ and VEGFR-2/KDR⁺ cells before and after culturing P4
4.2 C-kit⁻ CPC amplification ensures self-renewal but does not completely prevent differentiation

Self-renewal of c-kit⁻ CPCs in cardiac stem cell niches is promoted by paracrine factors released by supporting cells. For example, it has been reported that Notch ligands regulate the fate/proliferation of CPCs in vivo and in vitro. In the present study, we adopted a

Table 1  Expression of growth factors and cytokines in unselected cells compared with supporting stromal cells recovered from the transwell’s lower chambers. Table indicates the expression of each cytokine in ng/ml/10⁵ cells/24 h and the results of statistical comparison of cytokine yield by the two cell types by Student’s t-test.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Primary culture cells (n = 4)</th>
<th>P4 c-kit⁻ supporting cells (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiopoietin-2</td>
<td>0.148 ± 0.049</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Follistatin</td>
<td>0.734 ± 0.231</td>
<td>1.29 ± 0.13</td>
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<tr>
<td>G-CSF (32)</td>
<td>0.003 ± 0.0006</td>
<td>23.77 ± 6.77⁺</td>
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<tr>
<td>HGF</td>
<td>0.069 ± 0.029</td>
<td>1.36 ± 0.73</td>
</tr>
<tr>
<td>Interleukin-8</td>
<td>0.295 ± 0.023</td>
<td>30.84 ± 5.21⁺</td>
</tr>
<tr>
<td>Leptin</td>
<td>0.017 ± 0.001</td>
<td>0.15 ± 0.02⁺</td>
</tr>
<tr>
<td>PEGF-BB</td>
<td>0.012 ± 0.001</td>
<td>0.43 ± 0.04⁺</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>0.183 ± 0.016</td>
<td>4.51 ± 0.39⁺</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>0.101 ± 0.025</td>
<td>8.16 ± 1.57⁺</td>
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<td>0.0007 ± 0.0004</td>
<td>0.4 ± 0.08⁺</td>
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<td>0.51 ± 0.17</td>
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<tr>
<td>SCF</td>
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<td>0.0025 ± 0.0016</td>
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<tr>
<td>Interleukin-6</td>
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<td>35.66 ± 3.93⁺</td>
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<td>GM-CSF</td>
<td>0.001 ± 0.0003</td>
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<td>Interferon-γ</td>
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<td>Interleukin-2</td>
<td>&lt;0.0001</td>
<td>0.029 ± 0.003⁺</td>
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<tr>
<td>Interleukin-4</td>
<td>&lt;0.0001</td>
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<tr>
<td>Tumour necrosis factor-α</td>
<td>&lt;0.0001</td>
<td>0.019 ± 0.002⁺</td>
</tr>
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</table>

*Indicates P < 0.05 by unpaired t-test. G(M)-CSF, granulocyte (monocyte)-colony stimulating factor; HGF, hepatocyte growth factor; PDGF-BB, platelet-derived growth factor-BB; LIF, leukaemia inhibitory factor; SDF-1α, stromal-derived factor-1α; SCF, stem cell factor.

human cardiosphere-derived cells, human Sca-1-like cells and epicardial CD34+ cells. Interestingly, in some of these reports, c-kit was found in these cells, while in others co-expression of c-kit and MSC markers was not observed or not investigated. Work performed in mammalian embryos has shown a possible heterogeneity of CPC populations. For example, postnatal cardioblasts characterized by expression of transcription factor Isl1 do not express c-kit. Conversely, progenitors residing in the neonatal heart expressing enhanced green fluorescent protein controlled by c-kit regulatory sequences were not found to express Isl1. This may result, in the adult, in the different MSC differentiation potency of c-kit+ cells. Endorsed with cardiac, vascular and mesenchymal differentiation with a role of c-kit as a stem cell marker defining multilineage progenitors, this finding, according to recent results, is consistent with role of c-kit as a stem cell marker defining multilineage progenitors endowed with cardiac, vascular and mesenchymal differentiation ability.

Another important finding from the present study concerns the different MSC differentiation potency of c-kit+ derived CPCs compared with BM-derived MSCs. In fact, as shown in Figure 5, morphological differentiation of c-kit+ derived CPCs compared with BM-derived CPCs was similar, while expression of pro-adipogenic and pro-osteogenic genes was strongly reduced, suggesting functional diversity of cells bearing similar MSC immunophenotypes. In line with this, our group has recently found that cardiac-derived MSCs have a higher propensity to repair the infarcted heart compared with BM-derived MSCs, and characterized the molecular signature underlying this relevant difference. Mesenchymal features in human heart progenitors: Sca-1-like BM-derived MSCs, and characterized the molecular signature under which this relevant difference. This finding, according to recent results, is consistent with role of c-kit as a stem cell marker defining multilineage progenitors endowed with cardiac, vascular and mesenchymal differentiation ability.

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transwell co-culture system to amplify high-throughput sorted c-kit+ CPCs in the presence or the absence of supporting cells coming from the same tissue specimen. Strikingly, in either set of conditions, the percentage of c-kit+ cells was reduced. However c-kit expression was less dramatically downregulated in transwell vs. no transwell cultured cells (Figure 3); in addition, the transwell conditions allowed maintainance of a constant amount of c-kit*bright cells at T0 and P4 culture stages (Figure 4). Taken together, these findings suggest that the presence of parental primary cells supports, although only in part, self-renewal of human CPCs, and that it creates a microenvironment where asymmetric division of these cells appears to be maintained over time.

In an attempt to clarify the molecular mechanisms underlying the reduced c-kit downregulation in c-kit+ CPCs cultured in transwells, it was found that supporting unselected cells secrete an array of pro-angiogenic/pro-inflammatory cytokines. Interestingly, the quantification of factors released in conditioned medium by supporting stromal cells showed that the production of cytokines by these cells was enhanced at the end of the culture. The most upregulated cytokines in these cells at P4 were G-CSF, interleukin-8, vascular endothelial growth factor and interleukin-6 (Table 1). These data, also in line with recent observations by Hatzistergos and co-workers using direct co-culture of endomyocardial biopsies onto BM-derived MSCs layers, identify the interplay between cardiac progenitors and surrounding cells as a key mechanism to establish a permissive micro-environment for stem cell propagation, and represent a first step towards the definition of a supporting cell-free culture medium able to maintain high levels of c-kit+ cells in culture without the need for clonal amplification. How this microenvironment interacts with cellular machinery responsible for c-kit+ CPC renewal vs. multilineage differentiation is a matter for future molecular investigations.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

Note added in proof: A study from our laboratory reporting full characterization of cardiac specific mesenchymal cells was accepted for publication in Cardiovascular Research at the time of proofreading of the present article. Please refer to A. Rossini et al.

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