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RESEARCH ARTICLE

Mesenchymal stem cells or cardiac progenitors for cardiac repair? A comparative study

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Abstract In the past, clinical trials transplanting bone marrow–derived mononuclear cells reported a limited improvement in cardiac function. Therefore, the search for stem cells leading to more successful stem cell therapies continues. Good candidates are the so-called cardiac stem cells (CSCs). To date, there is no clear evidence to show if these cells are intrinsic stem cells from the heart or mobilized cells from bone marrow. In this study we performed a comparative study between human mesenchymal stem cells (hMSCs), purified c-kit$^+$ CSCs, and cardiosphere-derived cells (CDCs). Our results showed that hMSCs can be discriminated from CSCs by their differentiation capacity towards adipocytes and osteocytes and the expression of CD140b. On the other hand, cardiac progenitors display a greater cardiomyogenic differentiation capacity. Despite a different isolation protocol, no distinction could be made between c-kit$^+$ CSCs and CDCs, indicating that they probably derive from the same precursor or even are the same cells.

Keywords Mesenchymal stem cells · Cardiosphere-derived cells · c-kit$^+$ cardiac stem cells · Heart failure · Myocardial infarction · Differentiation · Coculture

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Introduction

Acute myocardial infarction (AMI) and cardiomyopathy are characterized by a considerable loss of cardiomyocytes (CMs) due to severe apoptosis and necrosis. As a consequence, the infarcted area is infiltrated by fibroblasts, resulting in the formation of noncontractile scar tissue. In addition, since adult CMs only display a very low mitotic index, the surviving CMs compensate for this loss of function by hypertrophy, which will eventually lead to the development of heart failure [1, 2]. Without taking heart transplantation into consideration, the current pharmacological and surgical therapies to treat patients after an AMI are inadequate since 1 year mortality remains very high. At the moment, stem cell therapy is considered to be a promising approach to functionally restore the injured heart. For that reason, several clinical trials have been conducted to improve cardiac function by intracoronary or intramyocardial injection of bone marrow–derived mononuclear cells (MNCs) [3–6]. However, all of these trials only demonstrated a limited improvement in cardiac function, which could be explained by the restricted cardiomyogenic differentiation potential of bone marrow–derived stem cells [7–9]. Recently, the first clinical trial transplanting a purified ex vivo expanded mesenchymal

stem cell population (MSCs) was performed. The results of this double-blind, placebo-controlled, randomized, dose-ranging phase I study were published by Hare and colleagues [10]. Stem cell–treated patients had improved outcomes with regard to left ventricular function and reduction of cardiac arrhythmias. Unfortunately, this study was unable to conclude that improved cardiac function was the consequence of cardiomyogenic differentiation of the transplanted cells. Furthermore other studies showed that improvement of cardiac function obtained by transplanting MSCs was the result of paracrine effects rather than cellular fusion or transdifferentiation [11, 12].

To further develop more successful stem cell therapies, the search for stem cells with greater cardiomyogenic differentiation potential continued. Over the past few years, evidence emerged that stem cells are present in adult mammalian hearts [13, 14]. These so-called cardiac stem cells (CSCs) are claimed to be actual stem cells residing in the heart and not bone marrow stem cells mobilized to the heart [15–17]. To date, two main methods have been described to isolate these CSCs: predominantly, isolation of CSCs based on the presence of the membrane receptor c-kit and secondly, by the outgrowth of cells after cardiosphere formation, named cardiosphere-derived cells (CDCs) [15, 16]. Although it is generally assumed that CSCs possess a greater myocardial differentiation potential compared to MSCs, some reports ascribe MSC properties to human CSCs. For instance, both cell types express common antigens, can differentiate into adipocytes or osteocytes, and their proliferation is activated through the Akt/GSK-β3 pathway [18, 19].

At this moment, still no clear evidence exists that the described hCSC populations are actual stem cells residing in the heart and not just migrated from the bone marrow. Furthermore, there is no consensus about the cardiomyogenic differentiation potential of MSCs, and reports describing the myogenic differentiation of CSCs have used various isolation and differentiation protocols, making it difficult to compare the results of these studies [8, 15, 16, 20]. Therefore, we performed a comparative study between hMSCs and hCSCs. In case of hCSCs, both CDCs and purified c-kit$^+$ cell populations were examined. Our results indicate an apparent phenotypical and functional difference between MSCs and cardiac progenitors. Furthermore, based on our results we advise exercising care in using "sphere formation" as a functional assay to identify stem cell properties.

Materials and methods

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the institutional review board and informed consent from each patient were obtained.

Isolation of MSCs

Human MSCs were isolated as previously described [7]. Briefly, bone marrow was aspirated from patients' sternum just before cardiac surgery and the MNC fraction was isolated by gradient-density centrifugation ($n = 12$). Cells were cultured in X-vivo 15 medium (Lonza) supplemented with 10% fetal calf serum (FCS; Hyclone) and 2% penicillin-streptomycin (pen-strep; Lonza).

Isolation of CDCs

CDC isolation was performed as previously described but with minor modifications [14, 15]. Human right atrial appendages were removed during routine cardiac surgery. The heart tissue was minced, washed with Dulbecco's phosphate-buffered saline (DPBS; Lonza), and digested three times for 5 min with Tryple LE select (Gibco). Fragments were placed on fibronectin-coated plates in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 1.5–2 weeks, cardiosphere-forming cells were isolated by pooling two washes of DPBS, one with Versene (Gibco) and one mild trypsinization with Tryple LE Select. Isolated cells were seeded in poly-D-lysine-coated culture plates (Beckton & Dickinson) at a density of 3×10^4 cells/ml in cardiosphere growth medium (CGM: 35% IMDM/65% DMEM:F12 (Lonza) supplemented with 3.5% FCS, 2% B-27 (Gibco), 100 μM dithiothreitol (DTT; Invitrogen), 10 ng/ml epidermal growth factor (Tebu-Bio), 20 ng/ml basic fibroblast growth factor (Tebu-Bio), 40 nM cardiotrophin-1 (Tebu-Bio), 40 nM thrombin (Tebu-Bio), and 2% pen-strep). After 5–7 days, spontaneously detached cardiospheres were isolated and replated in fibronectin-coated culture flasks. After attachment of the cardiospheres, monolayers of CDCs ($n = 12$) were generated in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep.

Isolation of c-kit$^+$ CSCs

Fragments of human right atrial appendages were removed during routine cardiac surgery and minced into 1–2 mm^3 cubes. Hereafter, tissue pieces were plated in fibronectin-coated plates in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 2 weeks, outgrowth cells were detached from the bottom of the culture plates by incubation with cell dissociation buffer (CDB; Gibco). Cells were incubated with a mouse anti-human c-kit-PE antibody (Miltenyi Biotec) for 30 min at 4°C. C-kit$^+$ cells were isolated by flow sorting with a FACSAria® (Becton & Dickinson). For each sorting experiment, quality control (QC) was performed on the calibration of the FACSAria®. This QC consists of sorting a multicolor bead population in

four separate populations. The purity of each purified bead population had to be at least 95% before continuing. If purity was insufficient, the flow sorter was recalibrated, and a new QC experiment was performed. Purified c-kit$^+$ cells were expanded on fibronectin-coated plates in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep ($n = 5$).

Cell cultures

MSC, CDC, and c-kit$^+$ CSC cultures were passaged each time they reached 80–85% confluency. Cell number was assessed by a Trypan Blue (Gibco) staining using a Fuchs-Rosenthal counting chamber. Hereafter, cells were seeded with a density of 5×10^3 cells/cm^2. The population doubling time of the different cell cultures, MSCs, CDCs, and c-kit$^+$ CSCs, was calculated by the algorithm provided by http://www.doubling-time.com [21]. For all experiments, MSC, CDC, and c-kit$^+$ CSC cultures between passage 4 and 7 were used.

Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes (NRCMs) were isolated from 3 to 4-day-old Wistar rats using the Worthington Neonatal Cardiomyocyte Isolation System Kit (Worthington Biochemical) according to the manufacturer's instructions. The NRCMs were cultured as previously described [7].

Flow cytometric characterization of expanded MSCs, CDCs, and c-kit$^+$ cells

At passage 4, a small aliquot of expanded cells was incubated for 30 min in the dark at room temperature with the human monoclonal antibodies shown in Table 1 and similarly conjugated isotype-matched control antibodies ($n = 3$ for each stem cell type). All antibodies were purchased from Becton & Dickinson except CD133-APC and CD133-Pe (Miltenyi Biotec), CD105-FITC (Serotec), TIE-2, VEGFR-2, and VEGFR-3 (R&D systems), and FGF (abcam). Cells were analyzed on a FACSAria® (Becton & Dickinson).

Expression of pluripotency-associated genes

Expression of the following pluripotency-associated genes was examined: *Oct-4*, *Sox-2*, *DPPA-3*, *Tbx-3*, *Lin-28*, *C-myc*, and *Klf-4*. Primer sequences with annealing temperature (AT) and fragment size are listed in Table 2. Total RNA of cells (P4–P7) was isolated using the Rneasy Micro kit (Qiagen) following manufacturer's instructions. cDNA was synthesized using Superscript III and random hexamers (Invitrogen). PCR using *Taq* polymerase (Roche) was performed for 35 cycles consisting of 40 s at 95°C, 50 s at AT, and 1 min at 72°C, with a final extension step of 10 min at 72°C.

Table 1 Panels of antibodies used for flow cytometric characterization of ex vivo expanded cells

	FITC	PE	PerCP	APC	Pe-Cy7	APC-Cy7
Tube 1	CD2	CD13	CD19	CD5	CD10	CD45
Tube 2	CD4	CD38	CD3	CD29	–	CD45
Tube 3	CD15	CD44	–	CD55	CD56	CD45
Tube 4	CD16	CD33	CD117	HLA-DR	CD34	CD45
Tube 5	CD50	CD11b	CD117	VEGFR-3	CD34	CD45
Tube 6	CD71	CD109	CD14	CD90	CD34	CD45
Tube 7	CD90	CD140b	CD117	CD133	CD34	CD45
Tube 8	CD105	CD73	CD14	CD184	CD34	CD45
Tube 9	CD106	CD49c	CD117	CD90	CD34	CD45
Tube 10	CD31	CD133	CD14	TIE-2	CD34	CD45
Tube 11	CD144	CD133	CD14	VEGFR-2	CD34	CD45
Tube 12	FGF	EGFR				

FITC Fluorescein isothiocyanate, *PE* phycoerythrin, *PerCP* peridinyl chlorophylline, *APC* allophycocyanine, *Pe-cy7* phycoerythrin-Cy7, *APC-Cy7* allophycocyanine-Cy7

Adipogenic and osteogenic differentiation

Differentiation of MSCs, CDCs, and c-kit$^+$ CSCs into adipocytes and osteocytes ($n = 3$) was performed by using the Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems) according to the manufacturer's instructions. Cultures without adipogenic or osteogenic differentiation factors were used as negative control. To determine background staining in the immunofluorescence experiments, samples were incubated with the fluorochrome-conjugated secondary antibody only. Samples were judged positive if the staining intensity of the differentiated cells was higher than the background staining. Furthermore, the staining had to be associated with a clear nucleus (DAPI) and display some structural organization as shown by the instructions in the kit.

Proliferation assay

In order to confirm our flow cytometric data, 12.5×10^4 MSCs ($n = 3$), CDCs ($n = 2$), or c-kit$^+$ CSCs ($n = 2$) were incubated with 1 μg/ml CD140b and plated in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 48 h cells were detached and counted using a Fuchs-Rosenthal and Trypan Blue. The number of proliferative cells was assessed by incubating the cells with a ki-67-PE conjugated antibody (Beckton & Dickinson) according to the manufacturer's instructions.

Table 2 Primer sequences

Gene	Forward primer	Reverse primer	AT (°C)	bp
β-actin	5′-AGCGGGAAATCGTGCGTGACA-3′	5′-CCTGTAACAATGCATCTCATATTTGG-3′	56	791
OCT-4	5′-TCAGCCAAACGACCATCTGCCGCT-3′	5′-GAAGTGAGGGCTCCATAGCCTGG-3′	58	455
Sox-2	5′-AGATGGCCCAGGAGAACCCCAAGATG-3′	5′-ACCGAACCCATGGAGCCAAGAGCCA-3′	58	555
DPPA3	5′-GTTACTGGGCGGAGTTCGTA-3′	5′-TGAAGTGGCTTGGTGTCTTG-3′	50	167
Lin-28	5′-CCTTGTTCCCAACCTCCTAAG-3′	5′-CAGGTACAGGCTTTCCTACCC-3′	50	487
c-myc	5′-GTGCGTAAGGAAAAGTAAGG-3′	5′-AAGACTCAGCCAAGGTTG-3′	45	116
Klf-4	5′-CTGTTATGCACTGTGGTT-3′	5′-GTATGCAAAATACAAACTCC-3′	45	201
Tbx-3	5′-TTCCTACCTCACCGGGCG-3′	5′-CCGTTGGGAGGCAGCGT-3′	50	94
α-actinin	5′-CCTGCCTTCATGCCCTCCGA-3′	5′-TGCTCCACGCGGTCCTGGTG-3′	56	307
TnT	5′-AGAGGTGGTGGAAGAGTACGAG-3′	5′-GACGTCTCTCGATCCTGTCTTT-3′	56	406
MHC	5′-GAACACCAGCCTCATCAACC-3′	5′-AGGTTGGTGTTGGCTTGCTC-3′	53	522
TnI	5′-CCCTGCACCAGCCCCAATCAGA-3′	5′-CGAAGCCCAGCCCGGCCAACT-3′	60	232
Cx43	5′-CTTGGCGTGACTTCACTACTTTT-3′	5′-GCATTTTCACCTTACCATGCTCT-3′	53	490
Kv4.3	5′-CTTAAGACGATTGCAGGGAAGAT-3′	5′-CTTCTTGTGGATGGGTAGTTCTG-3′	53	473
α1c	5′-CTGGACAAGAACCAGCGACAGTGCG-3′	5′-ATCACGATCAGGAGGGCCACATAGGG-3′	60	562
Nkx2.5	5′-GCAGGTCAAGATCTGGTTCCAGA-3′	5′-GAGTGAATGCAAAATCCAGGGGAC-3′	56	550
GATA-4	5′-CCCCAATCTCGATATGTTTG-3′	5′-AGGAGCTGCTGGTGTCTTAG-3′	50	396

TnT Troponin T, *MHC* myosin heavy chain, *TnI* troponin I, *Cx43* connexin43, *AT* annealing temperature, *bp* base pairs

Production of GFP-containing lentiviruses and infection of cells

Cells were infected with a lentivirus expressing GFP under the control of a cytomegalovirus promoter (pRRL-CMV-GFP). For the viral production, pRRL-CMV-GFP was co-transfected with pMDLg-RRE, pRSV-REV, and pCMV-VSVG in HEK293-T cells, using EZ lentifect (MellGen Laboratories). All plasmids were kindly provided by Dr. R.C. Hoeben, University Medical Center, Leiden, The Netherlands [22]. Viral supernatant was harvested after 48, 60, and 72 h and snap frozen in liquid nitrogen. Before use, the titer of each viral stock was determined.

For lentiviral transduction, target cells were incubated with supernatant containing replication-defective lentiviruses supplemented with 8 μg/ml polybrene (Sigma). When passaged, transduction efficiency was analyzed by flow cytometry.

Cardiosphere forming assay

MSCs, c-kit$^+$ CSCs, primary cultures of adult human dermal mesenchymal cells, and primary cultures of myofibroblasts isolated from colon tissue (both kindly provided by the Laboratory of Experimental Cancer Research, University of Ghent, Belgium) were plated with a density of 3×10^4 cells/ml in CGM on poly-D-lysine coated plates. After 5–7 days the number of spheres was scored.

Cardiomyogenic differentiation potential

Monocultures

To test the myocardial differentiation potential in monoculture, stem cells were seeded with a density of 12.5×10^3 cells/cm^2 in X-vivo 15 medium supplemented with 10% FCS and 2% pen-strep. After 24 h the myogenic differentiation was stimulated by reducing serum contents to 2 or 0%, and 0, 1, or 3 ng/ml TGF-β was added throughout the entire culture time ($n = 3$).

Cocultures with NRCMs

To test whether the cardiomyogenic differentiation potential of the stem cells was stimulated by a cardiac microenvironment, cells were cocultured in the presence of NRCMs at a 1:3 ratio (stem cells:NRCMs) in X-vivo 15 medium with (2%) or without FCS and supplemented with 1 ng/ml TGF-β ($n = 3$). Prior to the coculture set-up, stem cells were labelled with GFP by lentiviral infection as described above.

Gene expression analysis

Total RNA was isolated using the Rneasy Micro kit (Qiagen). In monocultures, cells were lysed after 1 week by directly adding RLT buffer to the tissue culture well. In case of the cocultured stem cells, prior to cell lysis, GFP+ cells were purified by flow sorting using a FACSAria®.

To prevent RNA degradation, the flow sorter was cleaned with bleach, diethyl pyrocarbonate-treated (Fluka) solutions were used, and isolated cells were directly sorted in cooled (4°C) RLT buffer. After sorting, RNA quality was monitored using the Agilent bio-analyzer 2100 and the RNA 6000 Pico Chip Kit (both from Agilent Technologies). RT-PCR was performed as described above (see "Expression of pluripotency-associated genes"). Primers with AT and fragment size are listed in Table 2.

Immunofluorescence

Prior to the culture, glass cover-slips were sterilized and used as culture surface. After the coculture, cells were fixed in 4% paraformaldehyde for 20 min and subsequently permeabilized with 0.3% Triton X (Sigma-Aldrich) at room temperature. Cells were incubated overnight with rabbit antibodies against human cardiac troponin T (cTnT; 1:500; Abcam) or human cardiac troponin I (cTnI; 1:500; Chemicon) at 4°C. The next day, cells were incubated with a sheep anti-rabbit rhodamine-labelled secondary antibody (1:10) (Millipore) for 1 h at room temperature. Cells were visualized with the Axioplan 2 imaging fluorescence microscope (Zeiss).

Statistics

The data were analyzed using the SPSS® 15.0 software package (IBM, New York, USA). Continuous variables are displayed as means ± SD. Categorical data are presented as counts. Comparisons between groups were performed with the Kruskal-Wallis test, the Pearson's χ^2 test, or the Fisher's exact test as appropriate. Statistical significance was set at a P value <0.03.

Results

MSCs display different phenotypical and functional characteristics compared to c-kit$^+$ CSCs and CDCs

In order to obtain a homogenous MSC population, the MNC fraction was seeded in normal culture flasks, and already after 24 h, a small percentage of the cells was attached (Fig. 1A*a*). One week later, clear colonies of MSCs were visible (Fig. 1A*b*), which resulted in a homogenous MSC population as previously described (Fig. 1A*c*) [7]. For the isolation of cardiac progenitor cells, small fragments of right atrial appendages were placed in fibronectin-coated plates resulting in an outgrowth of cells. On top of this cell monolayer, clear phase bright cells appeared that have been described to be sphere-forming progenitor cells (Fig. 1B*a*) [14]. In order to isolate these cells, a mild trypsinization was performed, and the obtained cells formed cardiospheres after 5–7 days when cultured in CGM (Fig. 1B*b*). These spheres were harvested, replated, and when ex vivo expanded in X-vivo 15 medium, resulted in a monolayer of CDCs (Fig. 1B*c*). To obtain a pure culture of c-kit$^+$ CSCs, the explant outgrowth cells were harvested, and c-kit$^+$ cells (Fig. 1C*a*), negative for CD34 and CD45 (Fig. 1B*b*), were purified by flow sorting and subsequently ex vivo expanded (Fig. 1B*c*). To test the clonogenic character of these cells, a total of 960 cells were sorted with a density of 1 cell/well. After 2.5 weeks, 18 colonies were scored, showing a clonogenicity of 1.87%. These clones were flow cytometrically analyzed and showed an identical antigen expression profile to the general sorted c-kit$^+$ CSCs (data not shown).

When the growth characteristics of the different stem cell populations were analyzed in more detail, it turned out that, based on mean population doubling time, c-kit$^+$-sorted cells and CDCs grew faster, 2 ± 0.7 days (Fig. 2A*c*) and 5.24 ± 3.75 days, respectively (Fig. 2A*b*) compared to MSCs (7 ± 4.9 days, Fig. 2A*a*). Despite this, c-kit$^+$-sorted cells became senescent more rapidly, and most cultures even stopped growing already after passage 3. This senescence was not detected in the MSC and CDC cultures. When the population doubling time of MSCs, CDCs, and c-kit$^+$ CSCs was compared among patients, the growth pattern of the MSCs and CDCs was very variable from one patient to another (Fig. 2A*a, b*). The mean population doubling time, SD, and range of all cell types are shown in Fig. 2A*a–c*. These interpatient differences were not seen in the c-kit$^+$ CSC cultures (Fig. 2A*c*). These growth differences are not a consequence of clinical history of patients. As summarized in Table 3 no statistical difference between patient groups was obtained except for the parameter "peripheral vascular disease" ($P = 0.004$). Furthermore, the growth characteristics of MSCs and CDCs derived from the same patient were analyzed. As shown in Fig. 2A*d*, *e*, these results indicated that occasionally MSCs proliferated faster than the CDCs, while for other patients it was the other way around. Because of the limited amount of starting material, c-kit$^+$-sorted cells could not be included in this assay.

To investigate if cells still contain stem cell features when used in the differentiation experiment, we investigated the expression of several pluripotency-associated genes in the cultured MSCs, CDCs, and c-kit$^+$ CSCs. This analysis revealed that all three cell types still expressed *Oct-4*, *DPPA3*, *Lin-28*, *Tbx-3*, *C-myc*, and *Klf-4* (Fig. 2B), indicating that ex vivo expansion had no negative influence on the stem cell characteristics. No expression of *Sox-2* could be detected (Fig. 2B).

Light microscopic examination of ex vivo expanded MSCs (Fig. 1A*c*), CDCs (Fig. 1B*c*), and c-kit$^+$ CSCs

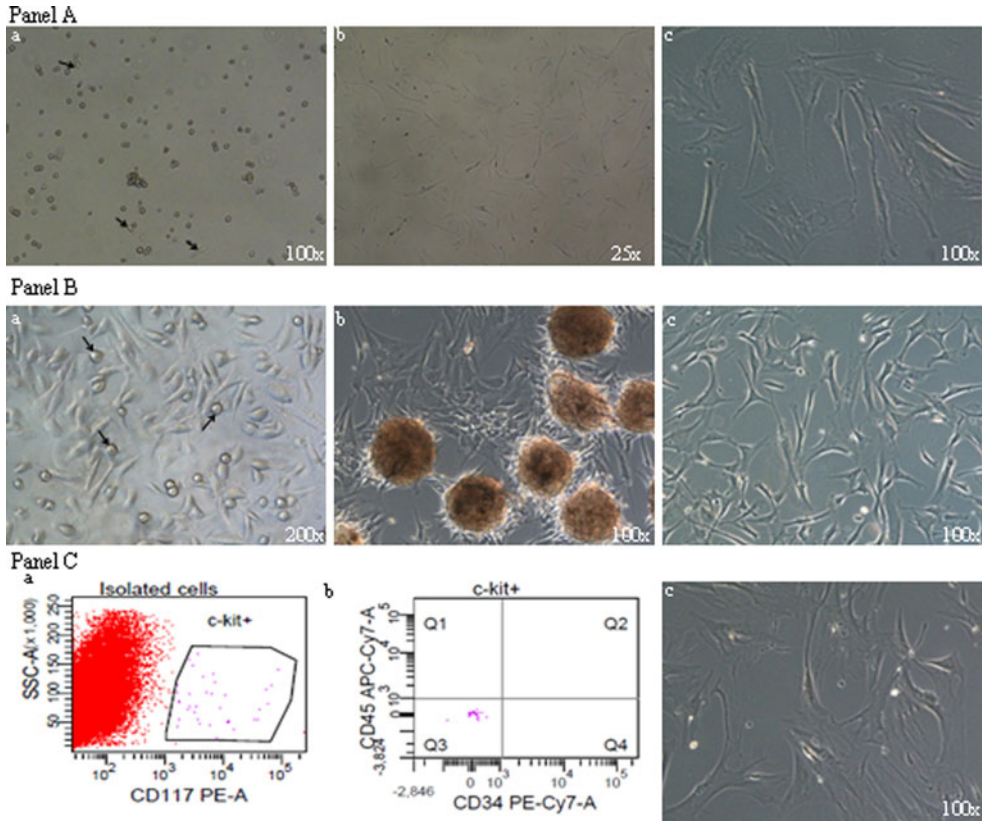

Fig. 1 Isolation of MSCs, CDCs, and c-kit$^+$ CSCs. **A** MSCs. After 24 h a small percentage of mononuclear cells attached and started to elongate (*a*; *arrows*). Within 1 week these cells started to grow colonies of MSCs (*b*). These colonies were further ex vivo expanded (*c*, P4). **B** CDCs. After 1.5–2 weeks phase bright cells (*a*) were harvested from the cardiac explant outgrowth and cultured in CGM. After 5–7 days, detached cardiospheres (*b*) were isolated and ex vivo expanded in a monolayer of CDCs (*c*, P4). **C** c-kit$^+$ CSCs. After 2 weeks the cardiac explant outgrowth was isolated and incubated with c-kit (CD117) (*a*). The c-kit$^+$ cells were CD34 and CD45 negative and judged as CSCs (*b*). These cells were isolated by flow sorting and ex vivo expanded (*c*, P4). Light microscopic analysis of the isolation and cultured MSCs (**A**), CDCs (**B**), and c-kit$^+$ CSCs (**C**) shows that these cells all display a very similar fibroblastic morphology

(Fig. 1C*c*) showed that all three cell types displayed a very similar fibroblastic morphology at passage 4. In order to identify a parameter to distinguish among the three cell types, the antigen expression profile of these ex vivo expanded cells was assessed by flow cytometry, using a large series of antibodies (Table 1). These results revealed that the three ex vivo expanded cell types display only limited phenotypical differences. For instance, all cell types expressed the membrane antigens CD13 (Fig. 3A*a–c*), CD29 (Fig. 3A*d–f*), CD44 (Fig. 3A*g–i*), and CD73 (Fig. 3A*j–l*) and were uniformly negative for CD45 (Fig. 3B*g–i*), the hematopoietic stem cell markers CD34 (Fig. 3B*a–c*) and CD133 (Fig. 3B*d–f*), and the fibroblast growth factor (FGF) receptor (Fig. 3B*m–o*). Several other antigens (Table 1), mostly markers of the hematopoietic lineage, could not be detected on the membrane surface of the three cell types (data not shown). Strikingly, although we started from a pure c-kit$^+$ cell population for obtaining CSCs, ex vivo expansion of these cells resulted in the complete loss of c-kit expression (Fig. 3B*l*). Despite these large phenotypic similarities between MSCs and cardiac progenitors, we were able to detect some antigens in which these cells had different expression profiles. A first phenotypic difference is shown by the expression of CD90. While MSCs were uniformly positive for this antigen, CDCs as well as c-kit$^+$ CSCs showed only a partial expression (Fig. 3C*a–c*). A more apparent difference is shown by the expression of the endothelial growth factor receptor (EGFR) and CD140b. CDCs as well as c-kit$^+$ CSCs (Fig. 3C*e, f*) were weakly positive for EGFR in contrast to the MSCs (Fig. 3C*d*). Conversely, MSCs presented CD140b on their membrane, while this antigen could not be detected on CDCs or c-kit$^+$ CSCs (Fig. 3C*g–i*). This phenotypic difference was confirmed by the fact that antibody-blocking of the CD140b receptor inhibited the proliferation of MSCs. After blocking, the percentage of proliferative MSCs, as assessed by Ki-67 expression, decreased considerably from 34.3 ± 10.4 to 13.4 ± 5.2%. On the other hand, the percentage of proliferative CDCs and c-kit$^+$ CSCs was not affected by the antibody, 28.9 ± 8.7 versus

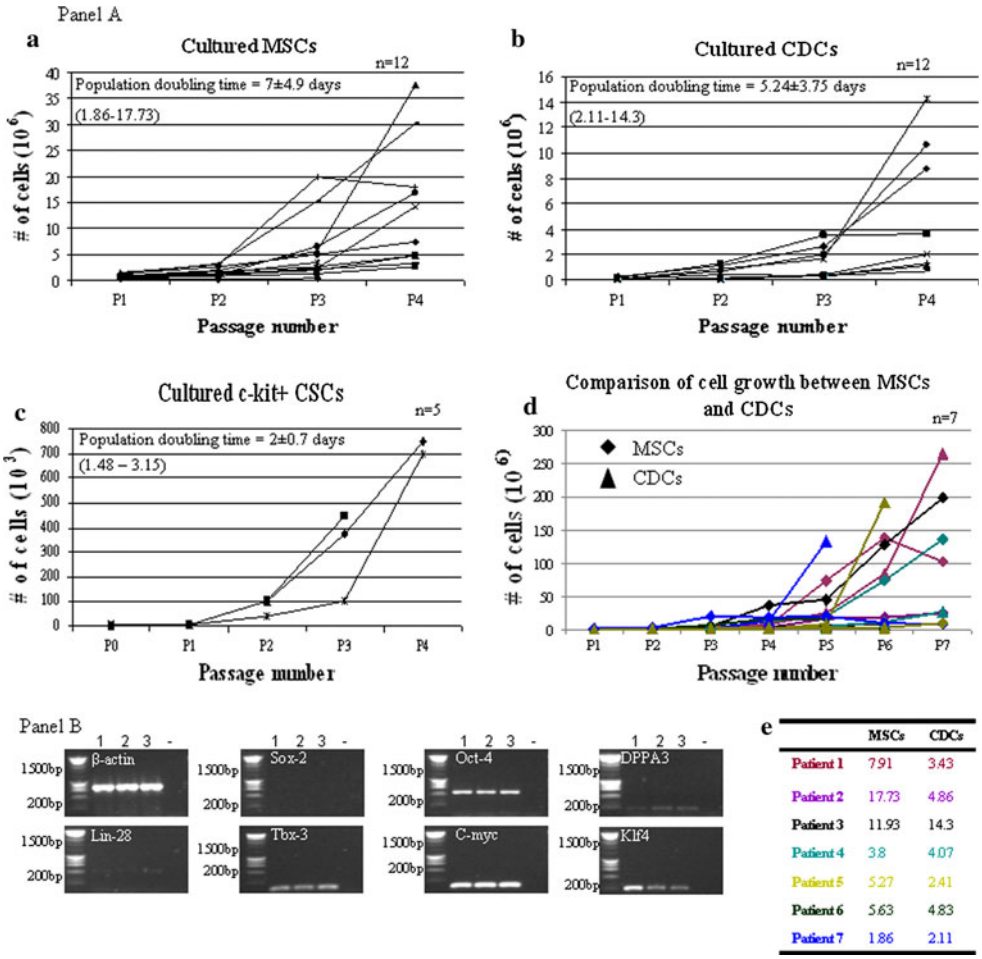

Fig. 2 Proliferation characteristics of MSCs, CDCs, and c-kit$^+$ CSCs and the expression of pluripotency-associated genes. **A** Growth pattern of MSCs (*a*), CDCs (*b*), and c-kit$^+$ CSCs (*c*). Growth pattern was more variable in MSCs and CDCs as compared to c-kit$^+$ CSCs, with an average population doubling time of 7 ± 4.9 days (range 1.86–17.73 days) in MSCs, 5.24 ± 3.75 days (range 2.11–14.3 days) in CDCs, and 2 ± 0.7 days (range 1.48–3.15 days) in c-kit$^+$ CSCs. Within patients (*d*), proliferation of MSCs could be faster or slower than CDCs (same patient indicated by *the same color*). The population doubling times of the MSCs and CDCs derived from these patients are indicated below the graph (*e*). Because of the limited amount of starting material, c-kit$^+$-sorted cells could not be included in this assay. **B** Expression of several pluripotency-associated genes. Ex vivo expanded MSCs (*lane 1*), CDCs (*lane 2*), and c-kit$^+$ CSCs (*lane 3*) uniformly express *Oct-4, DPPA3, Lin-28, Tbx-3, C-myc*, and *Klf4*. No expression of *Sox-2* could be detected. *B-actin* was used as internal control

29.1 ± 5.5% and 29.4 ± 9.5 versus 28.3 ± 5.7%, respectively (Fig. 3D*a*). This change in proliferation is reflected by the cell numbers as shown in Fig. 3D*b*.

Furthermore, we compared the adipogenic and osteogenic differentiation capacity of these cardiac progenitors and MSCs. It is well known that ex vivo expanded MSCs are able to differentiate into adipocytes, osteocytes, and chondrocytes [7, 23, 24]. Indeed after inducing differentiation down the adipogenic lineage, MSCs started to display a more round morphology and numerous vacuoles appeared. All phase bright vacuoles stained red after an oil-red-O treatment, indicating that they were triglyceride-filled fat droplets (Fig. 4a). This result was confirmed by immunofluorescent staining showing the presence of fatty acid binding protein-4 (Fig. 4b). Similarly, culturing MSCs in osteogenic-stimulating medium resulted in the formation of Ca^{2+} deposits, as determined by a positive Alizarin-red staining (Fig. 4c) and the presence of osteocalcin (Fig. 4d). In contrast, when c-kit$^+$ CSCs were stimulated to differentiate down to the adipogenic and osteogenic lineage, no signs of differentiation could be observed (Fig. 4e–h). Only negative background staining was obtained in the histochemical staining (Fig. 4e, g) and immunofluorescence (Fig. 4f, h). Similar results were obtained with CDCs (Fig. 4i–l).

Sphere formation is not a unique stem cell characteristic

Sphere formation has not only been described as a way to isolate cardiac progenitors, it is also considered to be a

Table 3 Patient characteristics

	MSC group ($n = 12$)	CDC group ($n = 12$)	c-kit group ($n = 5$)	P
Age (years)	67 ± 8.5	68.4 ± 7.1	58.4 ± 10.3	0.083
Male	6	5	5	0.079
Risk factors				
Weight	75.9 ± 18.8	79.3 ± 15;2	88.8 ± 10.7	0.452
Body mass index	27.3 ± 4.4	27.8 ± 4.5	27.8 ± 2.6	0.969
Last creatinin level preop	1.01 ± 0.4	1.1 ± 0.4	1.1 ± 0.3	0.637
Smoker	3	3	3	0.306
Family history of CAD	6	6	2	0.92
Diabetes	4	3	1	0.826
Hyperlipidemia	9	7	3	0.664
Renal dysfunction	2	4	1	0.617
Hypertension	10	9	2	0.184
Chronic lung disease	2	1	0	0.564
Peripheral vascular disease	0	1	3	0.004
Cerebrovascular disease	1	2	2	0.289
Pre-operative cardiac status				
Myocardial infarction	5	4	2	0.91
Congestive heart failure	5	5	0	0.204
Angina	8	6	4	0.465
Arrhythmia	1	1	1	0.738
Classification NYHA (I/II/III/IV)	0/3/7/2	0/4/6/2	1/0/4	0.253
Pre-operative medicine				
Beta-blockers	6	7	5	0.144
Nitrates PO	2	3	2	0.589
Nitrates IV	2	1	2	0.289
Diuretics	2	2	0	0.617
ACE inhibitors	6	5	1	0.519
Ca antagonists	1	1	0	0.8
Antiarrhythmias	2	2	1	0.984
Lipid lowering	8	6	2	0.539
Aspirin	11	10	2	0.051
Other antiplatelets	1	0	2	0.046
N vessels (0/1/2/3)	3/1/4/4	4/0/4/4	1/0/1/3	0.845
Surgical procedure				
CABG/valve/ablation/DOR	9/2/0/1	9/3/0/0	4/0/1/0	

MSC Mesenchymal stem cell, *CDC* cardiosphere-derived cell, *CAD* coronary artery disease, *NYHA* New York Heart Association, *CABG* coronary artery bypass graft

marker for stemness in several species, including rat, dog, and human [15, 25, 26]. Therefore, we wanted to compare the stem cell characteristics of human c-kit$^+$ CSCs and MSCs in their ability to form cardiospheres. As a negative control, primary cultures of adult human dermal mesenchymal cells and myofibroblasts from colon were cultured in CGM. Surprisingly, all cell types were able to form spheres when grown in these culture conditions (Fig. 5a–e). Furthermore, the number of spheres formed by these cells after 5 days was comparable. Out of 3×10^4 cells, MSCs formed 147 ± 10 spheres (Fig. 5a; $n = 3$), while c-kit$^+$ CSCs formed 102 ± 60 spheres (Fig. 5b; $n = 3$). CDCs, which can be considered as a positive control, formed around 117 ± 73 spheres (Fig. 5e). Similar results were obtained by the negative control cells: approximately 135 spheres were generated by human dermal mesenchymal cells (Fig. 5c) and 257 spheres by myofibroblasts (Fig. 5d). The size of the spheres was comparable for all different cell types (200 μM). We also noticed that the spheres of the myofibroblasts seemed to enlarge after 7 days.

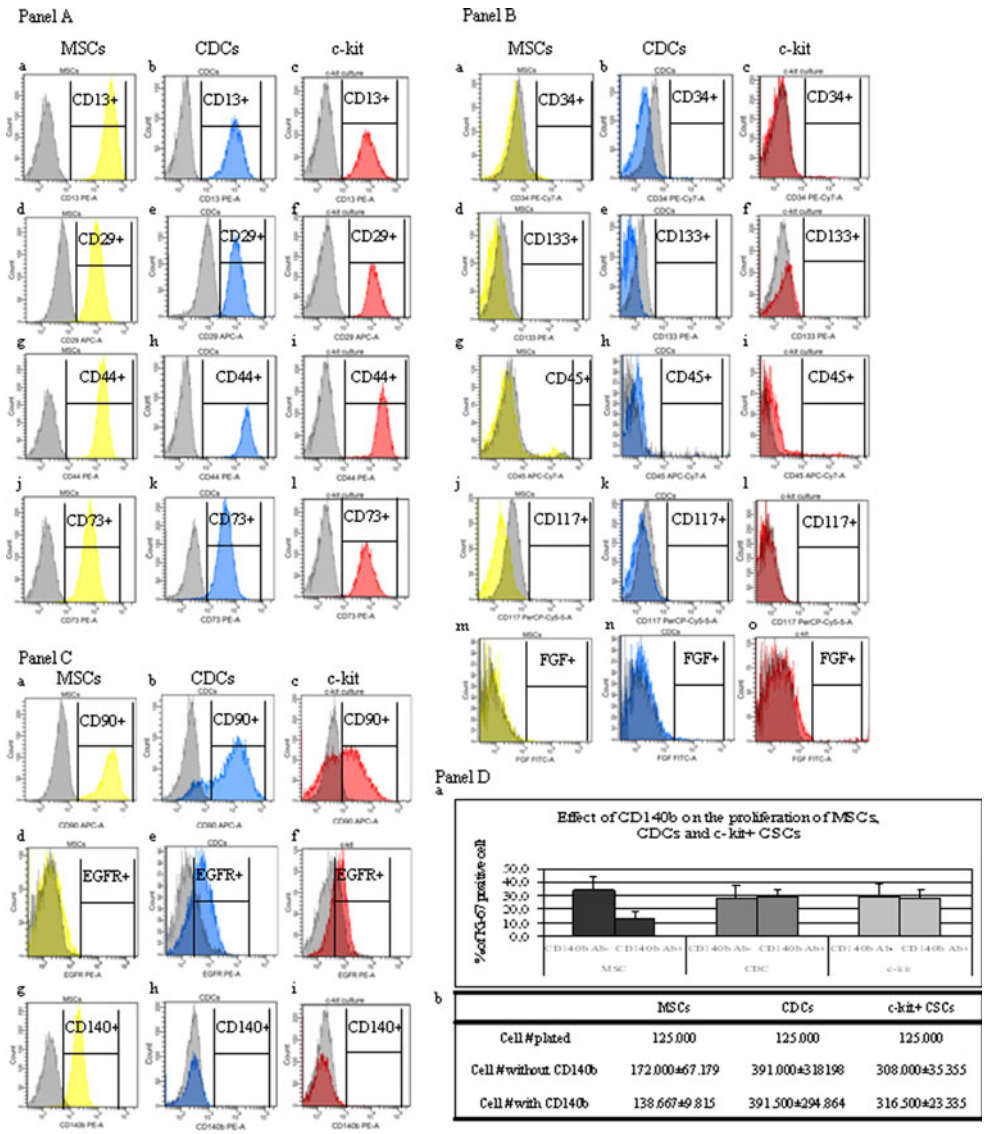

Fig. 3 Flow cytometric analysis of cultured MSCs, CDCs, and c-kit$^+$ CSCs. **A** MSCs (*yellow* population), as well as CDCs (*blue* population) and c-kit$^+$ CSCs (*red* population) express CD13 (*a–c*), CD29 (*d–f*), CD44 (*g–i*), and CD73 (*j–l*). The *gray* peak represents the isotype control. **B** All three cell types are uniformly negative for the hematopoietic markers CD34 (*a–c*) and CD133 (*d–f*), the leukocyte common antigen CD45 (*g–i*), c-kit (=CD117) (*j–l*), and fibroblast growth factor (FGF) receptor (*m–o*). The small peak seen in the CD45 panel of MSCs (*g*) is a consequence of nonspecific binding. **C** The first phenotypic difference between MSCs and both types of CSCs is shown by the expression of CD90. MSCs express this antigen uniformly on their membrane (*a*) while CDCs (*b*) and c-kit$^+$ CSCs (*c*) show only a partial expression of CD90. A more clear phenotypic difference is seen with the expression of the epidermal growth factor receptor (EGFR) and CD140b. CDCs (*e*) and c-kit$^+$ CSCs (*f*) both show weak positivity for the EGFR while this receptor is absent on the membrane of the MSCs (*d*). In contrast MSCs stain positive for CD140b (*g*), while the CDCs (*h*) and the c-kit$^+$ CSCs (*i*) do not express this receptor. **D** After the incubation with CD140b, the percentage of proliferative MSCs decreased significantly from 34.3 ± 10.4 to 13.4 ± 5.2%. However, the proliferation of CDCs and c-kit$^+$ CSCs was not affected (28.9 ± 8.7 vs. 29.1 ± 5.5% and 29.4 ± 9.5 vs. 28.3 ± 5.7%, respectively) (*a*). Cell counts associated with proliferation assay are shown in *b*

CDCs and c-kit$^+$ CSCs display a greater myocardial differentiation potential compared to MSCs

To determine which cell type has the best capability to differentiate into cardiomyocytes, the cardiomyogenic differentiation potential of all three cell types was tested by setting up monocultures in medium with low serum concentration (0 or 2%). Since the cardiomyogenic-inducing characteristics of TGF-β have been described in some reports [17, 27], different concentrations of TGF-β were used as additional cardiomyogenic stimulus. Differentiation was assessed by analyzing the expression of cardiac-specific genes by RT-PCR (Fig. 6A). Regardless of the differentiation medium used, all three stem cell types

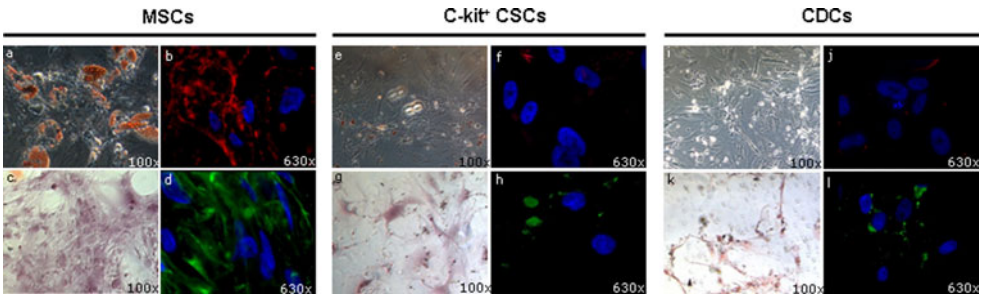

Fig. 4a–l Multilineage differentiation assay. Bone marrow–derived MSCs were able to differentiate into both adipocytes and osteocytes. The adipogenic differentiation is clearly shown by the presence of oil-red-O-positive lipid droplets (**a**). Immunofluorescent staining for fatty-acid-binding protein 4 (FABP-4) shows the presence of this protein in all cells (**b**). Furthermore, the osteogenic differentiation of these cells is shown by a clear positive Alizarin-red staining (**c**) and an apparent positive immunofluorescence for the detection of osteocalcin (**d**). As opposed to MSCs, c-kit$^+$ CSCs (**e**–**h**) and CDCs (**i**–**l**) failed to differentiate either into adipocytes or osteocytes, and cells had a senescent-like morphology. Only negative background staining of oil-red-O (**e**, **i**) and Alizarin-red (**g**, **k**) was obtained. Immunofluorescent analysis for FABP-4 (**f**, **j**) and osteocalcin (**h**, **l**) showed a similar staining pattern as in the negative control (only secondary antibody) and therefore these were judged negative

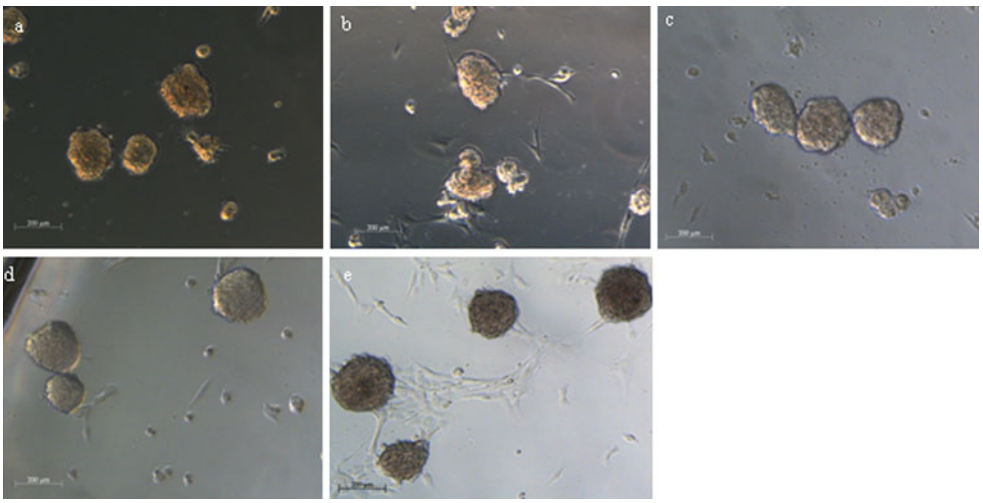

Fig. 5a–e Cardiosphere-forming assay. All five cell types, MSCs (**a**), c-kit$^+$ CSCs (**b**), cultured dermal mesenchymal cells (**c**), myofibroblasts (**d**), and CDCs (**e**) were able to form spheres. The number of spheres formed was comparable among the different cell types. The size of the spheres derived from all cell types was around 200 μM

expressed *α-actinin*, the gap-junction protein *connexin43* (*Cx43*), the voltage-gated potassium channel *Kv4.3*, and the L-type Ca^{2+}-channel *α1c* subunit. In contrast to MSCs, both types of CSCs expressed *troponin T* (*TnT*) and *GATA-4*, a transcription factor activating the expression of sarcomeric genes such as *troponin C* and α- and *β-myosin heavy chain* (*MHC*), in all conditions [28, 29]. TGF-*β* only influenced the myocardial differentiation of MSCs by inducing *TnT* expression. However it failed to activate the expression of *GATA-4*. Despite these promising results, all three stem cell types failed to express *Nkx2.5* and some important structural genes such as *MHC* and *troponin I* (*TnI*) (Fig. 6A). *β-actin* was used as an internal control.

To investigate if a cardiac microenvironment is required for an enhanced cardiomyocyte differentiation, the three types of stem cells were cultured on a layer of contractile NRCMs in low serum concentrations. Since TGF-*β* improved cardiac differentiation of MSCs in monoculture, this agent was also used in the cocultures. All types of cocultured stem cells showed an identical gene expression profile as shown by RT-PCR (Fig. 6B). As in the monocultures, cells expressed *α-actinin, TnT*, *Cx43*, *Kv4.3*, *α1c*, and *β-actin* (internal control). The presence of NRCMs stimulated the expression of *MHC* in all three cell types. Furthermore, expression of *GATA-4* was induced in MSCs after 1 week. Although culturing stem cells in a cardiac microenvironment improved myocardial differentiation, no expression of *Nkx2.5* or *TnI* could be detected. To control whether cell fusion occurred during the coculture, cells giving a positive signal in the FITC channel were sorted out and spun down on microscope slides. The nuclei were stained with DAPI, and cells were analyzed under an Axioplan 2 imaging fluorescence microscope. This analysis revealed that all sorted cells were mononucleated and labelled with GFP (data not shown).

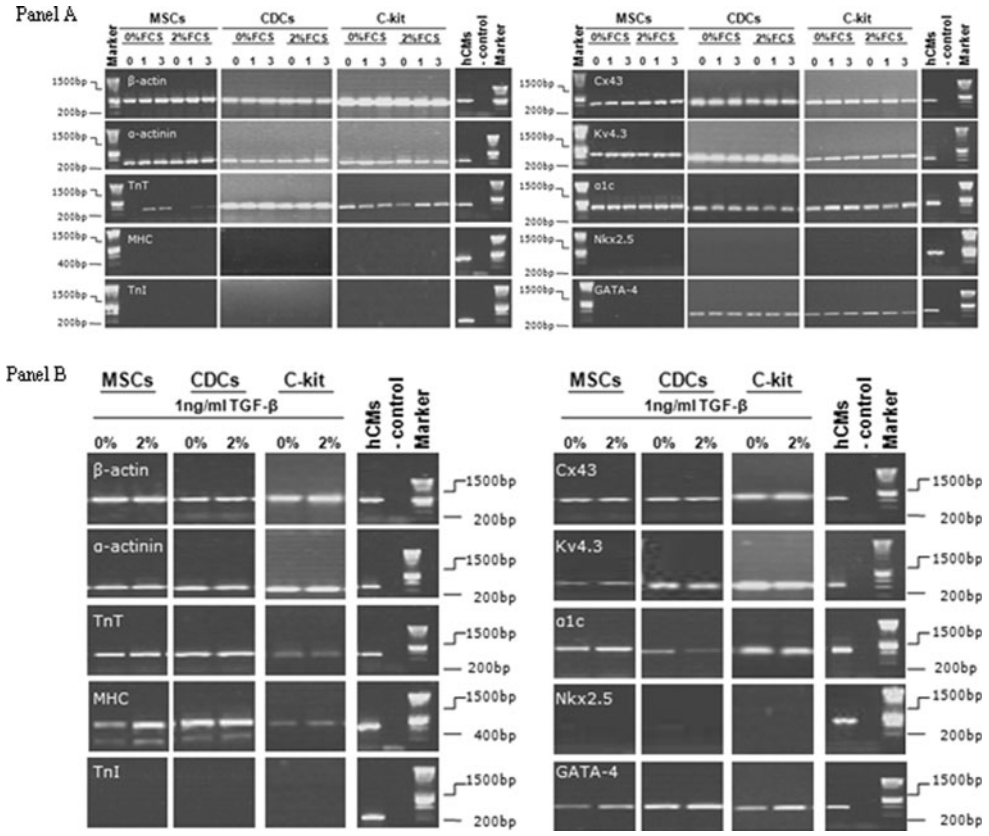

Fig. 6A, B Myocardial differentiation of MSCs, CDCs, and c-kit$^+$ CSCs assessed by RT-PCR. **A** Monoculture. Gene expression of monocultured MSCs, CDCs, and c-kit$^+$ CSCs in absence or presence (2%) of FCS and TGF-β (concentrations are indicated as 0, 1, or 3 ng/ml). Expression of *β-actin* (internal control), *α-actinin*, *Cx43*, *Kv4.3*, and *α1c* could be detected in all conditions. Regardless of the differentiation medium used, CDCs and c-kit$^+$ CSCs also expressed *TnT* and *GATA-4* while MSCs only expressed *TnT* under the influence of TGF-*β*. However, no expression of *GATA-4* could be detected in these monocultured MSCs. The expression of *MHC*, *TnI*, and *Nkx2.5* could not be detected in any condition. **B** Coculture. All cell types expressed *β-actin*, *α-actinin*, *TnT, MHC*, *Cx43*, *Kv4.3*, *α1c*, and *GATA-4*. No expression of *TnI* or *Nkx2.5* could be detected in any condition. Human CMs (hCMs) were used as positive control

Since the transcription of genes is no guarantee that the corresponding protein is present in an organized fashion or even expressed at all, immunofluorescence was performed to detect cTnT and cTnI. This revealed that despite the expression of *TnT* at the RNA level, no cTnT could be detected in any condition of monocultured stem cells (data not shown). In coculture conditions however, both types of CSCs expressed this protein in an organized manner showing sarcomeric structures already after 1 week (Fig. 7A*e–l*). In accordance with previous results, MSCs still failed to express cTnT in coculture (Fig. 7A*a–d*) [7]. Concurrent with the RT-PCR results, all three cell types failed to express cTnI in monoculture (data not shown) as well as in coculture (Fig. 7B*a–l*).

Discussion

After an AMI, the patient's cardiac function can be severely diminished. At present a range of pharmacological and surgical treatments are used to treat these patients. However, these therapies only delay remodeling of the heart and eventually heart failure will develop. Regenerative stem cell therapy is a promising approach to repair the injured heart after an MI. Over the last years a variety of clinical trials, transplanting the bone marrow–derived MNC fraction in acute or chronic infarcted patients, have been performed [3–6]. All of these studies reported no or only a limited improvement in left ventricular function. One possible explanation could be the very low percentage of stem cells present in the bone marrow–derived MNC fraction. In order to augment the effect of stem cell therapy, one approach could be to transplant a more enriched stem cell population. However, the ideal stem cell to be used in this setting has yet to be determined. For example, CD133$^+$-purified hematopoietic stem cells (HSCs) have already been used in clinical trials but showed only limited improvement of cardiac function [30, 31]. Another candidate to augment the regenerative effect of stem cell transplantation is the MSC. Early studies reported the

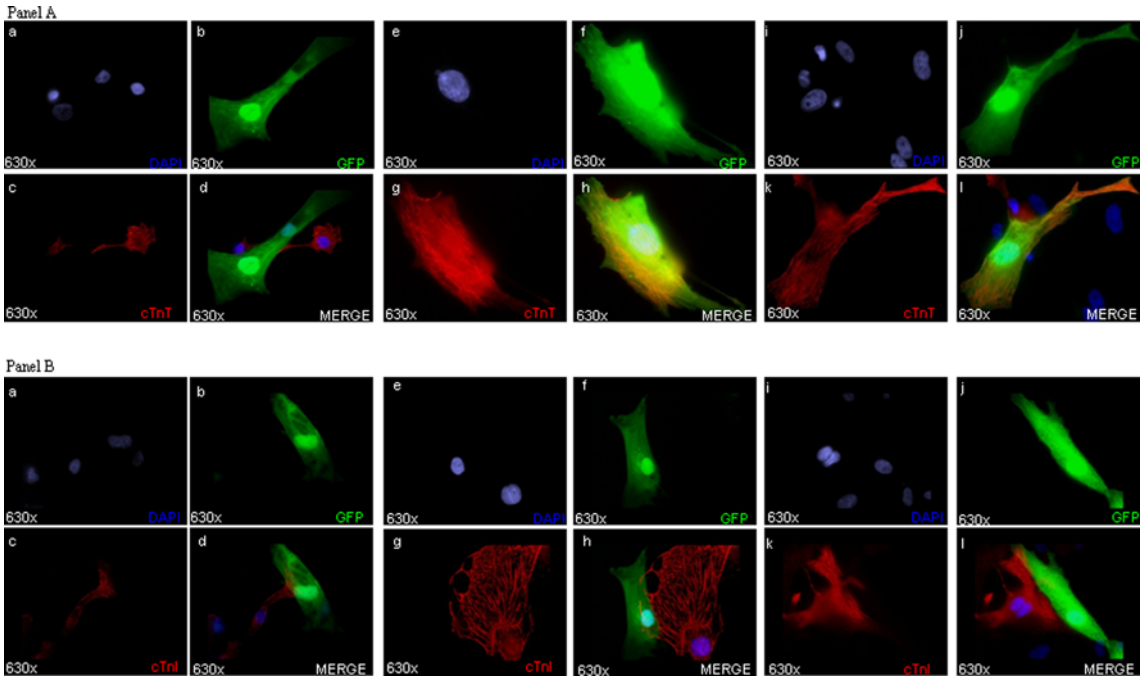

Fig. 7 Myocardial differentiation of MSCs, CDCs, and c-kit$^+$ CSCs assessed by immunofluorescence. **A** cTnT. Cocultured GFP-transfected MSCs (*green*) (*b*, *d*) do not express cardiac TnT (*red*). Both types of CSCs, CDCs (*f*, *h*) and c-kit$^+$ CSCs (*j*, *l*), do express cTnT. Cell nuclei are stained with DAPI (*blue*). **B** cTnI. All three cell types, MSCs (*a–d*), CDCs (*e–h*), and c-kit$^+$ CSCs (*i–l*), failed to express cardiac troponin I (cTnI). Nuclei are stained with DAPI (*blue*)

cardiomyogenic differentiation of rat MSCs after treatment with 5-azacytidine, bone morphogenetic protein 2, or fibroblast growth factor-4 [32–34]. However, in a recent study we have shown that the incubation of human MSCs with 5-azacytidine or dimethylsulfoxide does not influence cardiomyogenic differentiation of these cells [7]. The inability of these cells to differentiate down the cardiomyogenic lineage was shown by the failure to express *MHC*, *TnI*, and *α-actinin* by RT-PCR. Also no sarcomeric organization could be detected by immunofluorescence or electron microscopy. In the present study we showed that TGF-*β* enhances the myocardial differentiation of bone marrow–derived MSCs by the expression of *TnT* in monoculture and *MHC* in coculture. Furthermore, cocultured MSCs expressed the transcription factor *GATA-4*, but no expression of *Nkx2.5*, which is normally activated by binding of *GATA-4* at the Nkx2.5 promoter region, could be detected [35]. The failure to induce this expression could be either due to the short coculture time or simply to the fact that no functional protein is present. Indeed, the improved cardiac differentiation of MSCs at the RNA level was not associated with the corresponding proteins. For instance, cTnT could not be detected by immunofluorescence. Based on these results we can conclude that bone marrow–derived MSCs still only display a limited cardiomyogenic differentiation potential and are probably not the ideal stem cell type to regenerate the injured heart. Therefore, the myocardial differentiation capacity of other cell types is currently under investigation.

Increasing evidence emerges that the heart is not a static organ but contains some intrinsic regenerative capacity through the proliferation of adult cardiomyocytes and differentiating CSCs [1, 13, 14, 16]. These CSCs can be isolated, ex vivo expanded, and are able to integrate in the myocardium to improve cardiac function [15, 16]. The isolation of the adult hCSCs is either based on the expression of the membrane antigen c-kit or by their ability to form cardiospheres. To date, no clear evidence exists as to whether these cells are indeed intrinsic stem cells from the heart. Several studies ascribe MSC properties to hCSCs and vice versa [18, 19], but phenotypical and functional comparison between these two CSC types and bone marrow–derived MSCs had not yet been reported. In our study, phenotypic characterization showed that all three cell types were uniformly positive for CD13, CD29, CD44, and CD73 and negative for CD34, CD45, and CD133, indicating that the markers normally used to characterize MSCs are also present on human CSCs. Although at the moment of isolation CSCs were uniformly positive for c-kit, during ex vivo expansion they lost their c-kit expression. These data are in contradiction with earlier reports stating that after expansion these cells still express the stem cell factor receptor c-kit [15, 16]. On the other hand, in line with our results Tateishi et al. [19] also

reported a low c-kit expression in clonally derived CSCs. The reason for this discrepancy could be explained by the internalization of the c-kit receptor during the ex vivo expansion. Jahn et al. [36] reported that this receptor can be internalized upon activation by stem cell factor. This phenomenon is not only described for the c-kit receptor, Hendrikx et al. also noticed that overnight incubation of bone marrow–derived MNCs in Teflon bags was accompanied by a decrease in CD34 expression (personal communication). In addition, it is well known that the c-kit receptor is susceptible to trypsinization. Although several products and protocols have been tested for harvesting the cells, we were never able to detect the c-kit receptor on the membrane.

Strikingly, thorough flow cytometric analysis revealed that MSCs can be discriminated from cardiac progenitor cells by the expression of CD90, EGFR, and CD140b on their membrane. In case of CD90 expression, MSCs stain uniformly positive for this antigen, while CD90 expression of the CDCs and c-kit$^+$ CSCs is only partial. This expression pattern is in accordance with previously published data [7, 15, 16] but makes it limited to use as a differential marker. A more clear distinction could be made by the expression profile of EGFR and CD140b. CD140b is a tyrosine kinase receptor for platelet-derived growth factor-beta polypeptide (PDGFRbeta) and is mostly used as a marker for cells of mesenchymal origin [37, 38]. PDGFRbeta activates PI-3, which in turn is part of the AKT pathway, known to be involved in MSC proliferation [39]. Tateishi et al. [19] reported that proliferation of hCSCs was also mediated via the AKT pathway and therefore resembled MSCs. However, the receptor mediating this activation had not been identified. Our results showed that CD140b antibody inhibited only the proliferation of MSCs but did not have any influence on hCSC proliferation. The AKT pathway can be activated by all kinds of receptors such as insulin-like growth factor-1 receptor or EGFR [39, 40]. Indeed, Tateishi et al. stimulated CSC proliferation using EGF/basic FGF, indicating that the AKT pathway in CSCs is probably activated by the EGF and bFGF receptors. Since our data show that the ex vivo expanded CDCs and c-kit$^+$ CSCs only express the EGFR and not the FGF receptor, it is most likely that the proliferation of the CDCs and c-kit$^+$ CSCs is regulated by the EGFR.

Besides this phenotypic difference, a clear functional distinction could be made between the MSCs and both types of CSCs. In accordance with previously published data, ex vivo expanded MSCs were able to differentiate into adipocytes and osteocytes [7, 23, 24], while both populations of CSCs could not. The failure of CSCs to differentiate into adipocytes and osteocytes is in contradiction with recently published data by Itzhaki-Alfia et al. [18]. These apparently conflicting results can be explained by the heterogeneous population that was used for their multilineage differentiation assay. Only 22% of their total cell population expressed c-kit. Therefore it can not be excluded that contaminating cells such as MSCs are responsible for the positive differentiation results. In the present study, adipogenic and osteogenic differentiation was induced on a purified homogeneous c-kit$^+$ CSC population. Our results indicate that cardiac progenitor cells behave differently from MSCs, therefore suggesting they are a different cell population. However, a true specific cardiac progenitor marker still has to be identified.

It is known that CDCs as well as c-kit$^+$ CSCs are able to differentiate into functional cardiomyocytes [15, 16]. Indeed, in the present study CSCs clearly displayed a greater myocardial differentiation capacity compared to MSCs. As opposed to MSCs, CDCs as well as c-kit$^+$ CSCs already expressed *TnT* and *GATA-4* in monoculture although these differences in gene expression profile disappeared after coculture. All three cell types expressed *α-actinin, TnT*, *MHC*, *Cx43*, *Kv4.3*, *α1c,* and *GATA-4*. No expression of *TnI* or *Nkx2.5* could be detected under any conditions. The lack of Nkx2.5 expression in CDCs and c-kit$^+$ CSCs had no negative influence on their myocardial differentiation capacity. As shown at the protein level, MSCs failed to express cTnT after 1 week of coculture, while CDCs and c-kit$^+$ CSCs expressed this protein in an organized sarcomeric fashion. Even coculturing the three stem cell populations for 3 weeks did not improve the myocardial differentiation (data not shown). Despite the expression of cTnT in CDCs and c-kit$^+$ CSCs, no expression of cTnI could be detected. Although cTnT and cTnI are family members located in the same region of the chromosome, expression of these genes is independently regulated. Probably, in contrast to cTnT, key transcription factors responsible for cTnI expression are not upregulated during differentiation. cTnI is known to be regulated by transcription factors such as *GATA-4*, *GATA-5*, and *GATA-6*, and *Nkx2.5*. Since the expression of *Nkx2.5* was not detected, probably cTnI expression can not take place. Still, these data indicate that both types of CSCs have a better cardiomyogenic differentiation capacity and are therefore better candidates for cardiac regenerative therapy. However, these data are based on in vitro experiments only. The in vivo behavior of these cells should be studied in the larger animal model because in rodents, the hydrodynamics as well as the heart rate, is totally different when compared to humans. Most of the positive myocardial differentiation data involving MSCs have been obtained after transplantation of these cells in rats or mice [34, 41]. However, when MSCs were transplanted in the chronic infarction swine model, these cells differentiated into vascular smooth muscle cells or endothelial cells but, in accordance with the results presented here, not into cardiomyocytes [42].

Despite the fact that CDCs and c-kit$^+$ CSCs were obtained by two different isolation protocols, no phenotypic or functional distinction could be made between these two types of CSCs. Both cell types expressed the same membrane antigens as assessed by flow cytometry, and they both failed to differentiate into adipocytes and osteocytes. Furthermore, in mono- as well as in coculture these cells displayed the same gene and protein expression profile. Based on these data we hypothesize that CDCs and c-kit$^+$ CSCs are derived from the same ancestor cell or even just are the same cells. As shown by Andersen et al. the phase bright cells, assumed to be cardiosphere-forming cells [14], are actually CD45+ and unable to form cardiospheres [43]. Previously we have also shown that CD45+ cells present in outgrowth cells from heart fragments are negative for c-kit [44]. Isolation of these phase bright cells is based on a method of mild trypsinization that can not exclude accidental contamination of adherent explant outgrowth cells [14]. All these data support the theory that cardiospheres are probably generated by "contaminating" c-kit$^+$ CSCs coming from the explant outgrowth. As a consequence, the cardiosphere-forming cells and c-kit$^+$ CSCs could be similar or at least originate from the same precursor. Furthermore, one should be careful using sphere formation as a functional assay to discriminate between cell types. In this study we showed that not only cardiac progenitor cells are able to form spheres. Cells unrelated to heart tissue such as bone marrow–derived MSCs and even primary differentiated cells such as human dermal mesenchymal cells and myofibroblasts from colon were able to grow into spheres when cultured in cardiosphere-specific medium. Accordingly, in a recent publication Andersen et al. [43] showed that murine cardiospheres are not a source of cardiac progenitor cells but arise by aggregation of multiple cells instead of clonal cells. Therefore, care should be taken to use sphere formation as a stand-alone technique to characterize the stemness of a certain isolated cell population. Other assays determining stem cell characteristics, such as clonogenicity and the expression of pluripotency-associated genes, should also be performed in parallel. Despite this, sphere formation can be very valuable to isolate CSCs and for use as a culturing system [15, 45, 46]. Indeed, spheres resemble a 3D environment, which can be more beneficial in keeping cells undifferentiated during culture.

The data presented here suggest that a pool of stem cells is present in the human adult heart. This CSC population is phenotypically and functionally distinct from MSCs. CSCs display a greater cardiomyogenic differentiation potential compared to bone marrow–derived MSCs. If cardiomyogenic differentiation is the aim in regenerative cardiac therapy, CSCs would be the preferred choice. However, researchers should use their discretion to decide which isolation protocol best fits their expertise and infrastructure. We have shown that despite a difference in isolation protocol two well described types of CSCs are identical in phenotype and functional properties. This could imply that these cells, irrespective of the isolation method used, are generated by the same ancestor cell or even are the same cells. We suggest that the pool of stem cells present in the human adult heart is further characterized and that its phenotypical and functional properties be well documented before these cells are used in future clinical trials.

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