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Human cardiac and bone marrow stromal cells exhibit distinctive properties related to their origin

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Aims Bone marrow mesenchymal stromal cell (BMStC) transplantation into the infarcted heart improves left ventricular function and cardiac remodelling. However, it has been suggested that tissue-specific cells may be better for cardiac repair than cells from other sources. The objective of the present work has been the comparison of *in vitro* and *in vivo* properties of adult human cardiac stromal cells (CStC) to those of syngeneic BMStC.

Methods and results Although CStC and BMStC exhibited a similar immunophenotype, their gene, microRNA, and protein expression profiles were remarkably different. Biologically, CStC, compared with BMStC, were less competent in acquiring the adipogenic and osteogenic phenotype but more efficiently expressed cardiovascular markers. When injected into the heart, in rat a model of chronic myocardial infarction, CStC persisted longer within the tissue, migrated into the scar, and differentiated into adult cardiomyocytes better than BMStC.

Conclusion Our findings demonstrate that although CStC and BMStC share a common stromal phenotype, CStC present cardiovascular-associated features and may represent an important cell source for more efficient cardiac repair.

Keywords Cardiac and bone marrow stromal cells • Cardiovascular differentiation • Cardiac regeneration • Myocardial infarction

1. Introduction

Stromal cells can be isolated from a variety of adult tissues and organs,[1] the most extensively characterized being those of bone marrow origin [bone marrow mesenchymal stromal cells (BMStC)].

Although BMStC are currently being evaluated for their capacity to regenerate both skeletal tissues and unrelated tissues, such as the heart,[2] up to now, poor attention has been given to the therapeutic potential of cardiac-specific stromal cells.

In this study, we investigated both *in vitro* and *in vivo* whether cardiac stromal cells (CStC) isolated from specimens of adult human auricles (CStC) were more oriented towards the cardiovascular phenotype compared with BMStC. Both cell types were obtained from the same patient in order to avoid possible confounding differences related to patient-specific variables including age, genetic background, and drugs taken at the time of sample collection. In spite of a remarkable similarity in growth, morphology, and immunophenotype, CStC and BMStC differed significantly in gene, microRNA (miR), and protein expression and exhibited tissue-specific responses to differentiating stimuli. Notably, CStC revealed higher efficiency in the acquisition of a cardiovascular phenotype both *in vitro* and following intramyocardial injection in a rat model of chronic myocardial

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infarction (MI). Furthermore, CStC persisted longer within the tissue, migrated into the scar, and differentiated into cardiomyocytes more efficiently than their bone marrow counterpart.

2. Methods

2.1 CStC and BMStC isolation and culture

Right auricle and sternal marrow were obtained from donor patients (see Supplementary material online, *Table S1*) undergoing cardiac surgery after Local Ethics Committee approval and signed informed consent in accordance with the declaration of Helsinki. BMStC were isolated from 5 mL of heparinized bone marrow aspirated from the sternum.[3] CStC were obtained from the same patient as described previously.[4] Briefly, the protocol[4] was adapted, increasing the pre-plating time from 2 h to overnight, to harmonize methods of harvesting for both cell population and to improve efficiency of CStC recovery. A detailed description is available in the Supplementary material online.

2.2 miR profiling

Comparative miR expression profiling was carried out by using TaqMan Low Density microRNA Assay (Applied Biosystem). All procedures were performed according to the manufacturer's instructions. Data were analysed using SDS 2.3 Applied Biosystem software and samples were normalized to the average median Ct value (DDCt = CtmiR − Ctmedian). The average BMStC's Ct median value was used as a control. Only miR with Ct < 35 (for BMStC or CStC), average DCt statistically different ($P < 0.05$), and DDCt > |3| (i.e. expression fold variation > 8 compared with BMStC) were arbitrarily considered biologically relevant.

2.3 Real-time reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from cells using TRIzol reagent and reversely transcribed using Superscript III reverse transcriptase (Invitrogen, Italy). cDNA was amplified by SYBR-GREEN (BIO-RAD, Italy) quantitative reverse transcription–polymerase chain reaction (qRT–PCR) in a BIO-RAD iQ5 Cycler. The sequences of primers are reported in Supplementary material online, *Table S11*. Relative expression was estimated using the DeltaCt (DCt) method. DCt for treatment and control groups was subjected to Student's *t*-test.[5] DCt = 25 was arbitrarily assigned to non-expressed genes. Fold changes in gene expression are reported in the Supplementary material online.

2.4 Flow cytometry

Cells were detached with 0.02% EDTA solution (Sigma-Aldrich), stained with FITC/PE-conjugated antibodies for 10 min and analysed using a FACSCalibur (Becton–Dickinson, Italy) equipped with Cell-Quest Software.

2.5 Capillary-like structure-forming assay on basement membrane extract

The tube-forming ability of the cells was tested by culture on Cultrex BME (Tema Ricerca, Italy).[6] Cells were seeded at a concentration of 2.5×10^4/well in EGM-2 or in complete medium; branching point number was evaluated after 3 and 7 h.

2.6 Collagen invasion assay

The assay was performed in 48-well chemotaxis chambers (Neuroprobe, USA) as described in Rossini *et al.*[4] by using 8 μm pore polycarbonate filters coated with 20 μg/mL rat-tail collagen (Roche, Italy). As chemoattractant, the complete medium was placed in the lower chamber. After 2 h incubation, cells migrated to the lower surface of the filter were stained with the Diff Quick kit (Neuroprobe) and counted at 10 times in six random fields.

2.7 Western blot

CStC and BMStC were lysed with Laemmli buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Proteins were resolved by SDS–PAGE, transferred onto nitrocellulose membrane (BIO-RAD), and incubated overnight at 4°C with 1 μg/mL rabbit anti-human whole molecule MMP-1 (Abcam, UK) or mouse anti-vimentin (Clone VIM 3B4, Dako, USA) and, subsequently, with anti-rabbit horseradish peroxidase-conjugated secondary antibody.

2.8 Animals and surgical procedures

The investigation was approved by the Veterinary Animal Care and Use Committee of the University of Parma and conformed to the National Ethical Guidelines (Italian Ministry of Health; D.L. 116, 27 January 1992) and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised in 1996).

MI was induced by ligation of the left coronary artery in 3-month-old male Wistar rats kept under anaesthesia. Three weeks after MI, 5×10^5 marrow or cardiac cells were co-injected with 200 ng/mL of hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1, PeproTech, USA), in three regions bordering the scar. All animals were immunosuppressed by cyclosporine-A (Neoral) treatment (5 mg/kg/day). After 3 additional weeks, haemodynamic measurements were performed by a micro-tip pressure transducer (SPC-320, Millar Instruments, USA, software package CHART B4.2). Thereafter, the rats were euthanized and the hearts perfusion-fixed for anatomy and immunofluorescence studies (see Supplementary material online, Methods).

2.9 Human cell tracking and immunofluorescence

2.9.1 Green fluorescent protein labelling

Cardiac and marrow cells were transduced *in vitro* by incubation with a lentiviral vector encoding green fluorescent protein (GFP).

2.9.2 Quantum Dot labelling

In some experiments, cells were labelled with Quantum Dots (QDots, Invitrogen).[7]

2.9.3 Genetic labelling

Human cells injected in the infarcted rat heart were also identified by fluorescence *in situ* hybridization (FISH) to detect human Y chromosome using specific CEP Y alpha-satellite III DNA probes (Vysis, Abbott Molecular, USA).

Images were acquired using a Leica DMI6000B microscope provided by Z-stack automation and deconvolution system. For some experiments, confocal microscope Zeiss AXIOVERT 200 M was used.

2.10 DNA content measurement

The cardiomyocyte nuclear DNA content was determined using a modification of previously described protocol.[8]

2.11 Immunofluorescence

Formalin-fixed hearts were embedded in paraffin and sectioned (5 μm thickness). Markers of interest were detected by incubation with specific unconjugated primary antibodies and fluorochrome-conjugated secondary antibody. Nuclei were counterstained by DAPI.

2.12 Enzyme-linked immunosorbent assays

IL-6 release was quantified in cell supernatants obtained from 24 h serum-free-cultured CStC and BMStC according to the manufacturer's instructions (R&D Systems, USA).

2.13 New vessel formation

The number of arterioles and capillaries was measured as described previously.[9]

2.14 Additional methods

An expanded Methods section is available as Supplementary material online.

3. Results

3.1 CStC and BMStC morphology, immunophenotype, and molecular marker expression

CStC and BMStC were obtained from male patients ($n = 15$; see Supplementary material online, *Table S1*). For the experiments described in this manuscript, only cells adherent to the plastic of culture dishes after 18 h plating were used. CStC and BMStC presented similar morphology (*Figure 1*) but different growth kinetic (see Supplementary material online, *Figure S1*). Both cell population expressed mesenchymal markers, i.e. CD105, CD73, CD29, CD44, and CD13, and were negative for CD34, CD45, CD14, and HLA-DR (*Figure 1*; see Supplementary material online, *Table S2*). CD90 expression was highly heterogeneous in CStC, as reported for putative mesenchymal cells obtained from sources other than bone marrow;[10] however, the expression of this marker was higher in BMStC than in CStC. Like BMStC, also CStC were positive for CD146, a known marker for pericytes.[11] Pericytes have been recently proposed to act as a reservoir of stromal cells in multiple adult human organs.[11] Notably, both BMStC and CStC exhibited: (i) low or negative expression for c-Kit, which is a marker of a resident stem cell population of the heart,[12] as well as for the endothelial markers CD31, VEGFR-2, and CD144 (see Supplementary material online, *Table S2*) and (ii) positivity for vimentin and for the human fibroblast surface protein (see Supplementary material online, *Figure S1*), both markers associated with human fibroblasts.[4,13] Finally, both CStC and BMStC exhibited clonogenic properties, although at low efficiency (CStC $= 0.6 \pm 0.2\%$; BMStC $= 1.0 \pm 0.2\%$). Importantly, CStC obtained by short time (2 h) adherence to plastic[4] were immunophenotypically indistinguishable from those obtained by 18 h adherence (see Supplementary material online, *Table S3*).

The comparison analysis of BMStC and CStC was refined by expression analysis of stemness-related and cardiac-specific genes performed by qRT–PCR (see Supplementary material online, *Figure S1*). We observed that a higher level of c-Kit, GATA4, GATA6, KLF5, and myosin light chain-2a (MYLC-2a) mRNA was detectable in CStC than in BMStC (see Supplementary material online, *Figure S1* and *Table S4*). The relative higher expression of c-Kit mRNA in CStC, in contrast to the very low expression of the protein (see Supplementary material online, *Table S2*), may be explained, considering the presence of post-transcriptional control levels. Remarkably, an independent series of experiments revealed that higher c-Kit protein expression could be achieved in CStC cultured in low serum (M. Vecellio, personal data not shown). Further experiments are necessary to elucidate this important aspect. CStC/BMStC also expressed comparable levels of Nanog, Oct-4, Sox-2, KLF4, and MDR-1 mRNAs; however, the presence of these transcripts is not directly related to the cell degree of stemness.[14] Finally, Nkx2.5, α-myosin heavy chain (α-MHC), α-sarcomeric actin (α-Sarc), and cardiac troponin-I (Tn-I) were either absent or present at very low level in both cell populations (see Supplementary material online, *Table S4*).

BMStC are known for their limited immunoreactivity.[15] CStC immunogenic properties, tested by performing one-way mixed lymphocyte reaction and FACS analyses, resulted as low as BMStC's (see Supplementary material online, *Figure S2*).

3.2 CStC and BMStC miR profiling

miR are short, non-coding single-stranded RNA molecules involved in gene regulation. Their role is emerging as a key determinant in cell differentiation and fate.[16] Expression profiling experiments (see Supplementary material online, *Table S5*) enabled us to identify a specific CStC miR signature, consisting of miR-126, -139, -146a, -184, -204, and -326 and miR-10a, -10b, -34b, -196a, -196b, and -615, respectively, up- and down-regulated compared with BMStC (*Figure 1*; see Supplementary material online, *Figure S3*). Interestingly, miR-126 is known for its pro-angiogenic action[17] and both miR-126 and -146a are highly expressed in proliferating cardiovascular precursors.[18] This miR signature did not appear significantly modulated by *in vitro* differentiation stimuli (see Supplementary material online, *Figure S4*).

3.3 CStC and BMStC differentiation properties *in vitro*

Experiments were performed to assess the ability of CStC and BMStC primary cultures to *in vitro* differentiate along different pathways. When cultured in osteogenic medium, only BMStC acquired a star-like osteoblast shape, expressed higher level of Osteopontin mRNA, and became positive for alkaline phosphatase (see Supplementary material online, *Figure S5* and *Table S6*). After 3 weeks in adipogenic medium, both BMStC and CStC stained positive for Oil-Red O (see Supplementary material online, *Figure S5*); however, the average lipid content was lower in CStC than in BMStC, as well as the expression of the adipogenic markers PPAR-γ2 and Adipsin mRNA (see Supplementary material online, *Figure S5* and *Table S6*).

After 3 weeks culture in the endothelium-specific medium EGM-2, CStC significantly up-regulated the expression of CD31, CD144, and VEGFR-2 (*Figure 2*), whereas BMStC remained negative. Additionally, in EGM-2, only CStC ability to develop capillary-like structures on Cultrex BME increased over eight-fold (*Figure 2*; see Supplementary material online, *Figure S6*), whereas BMStC response was unchanged.

CStC's and BMStC's cardiogenic differentiation potential was first examined by culturing cells in dexamethasone-based (DEX) medium for 3 weeks.[12] Under these conditions, BMStC up-regulated adipogenic genes (PPAR-γ2 and Adipsin), the osteogenic gene Osteopontin, and only GATA4 among the cardiovascular markers. The effect of DEX medium on CStC was different: these cells exhibited an increase in α-Sarc and α-SMA mRNA expression, whereas GATA4, PPAR-γ2, Adipsin, and Osteopontin were unchanged (see Supplementary material online, *Figure S7* and *Table S6*). It is noteworthy that in DEX medium, the expression of MYLC-2a, α-MHC, and Tn-I was not modulated in either cell type (see Supplementary material online, *Table S7*).

In an attempt to improve CStC cardiogenic potential, we tested a low serum medium supplemented with all-trans retinoic acid and phenylbutyrate (ATRA/PB medium[19]). Under these conditions, CStC sig-

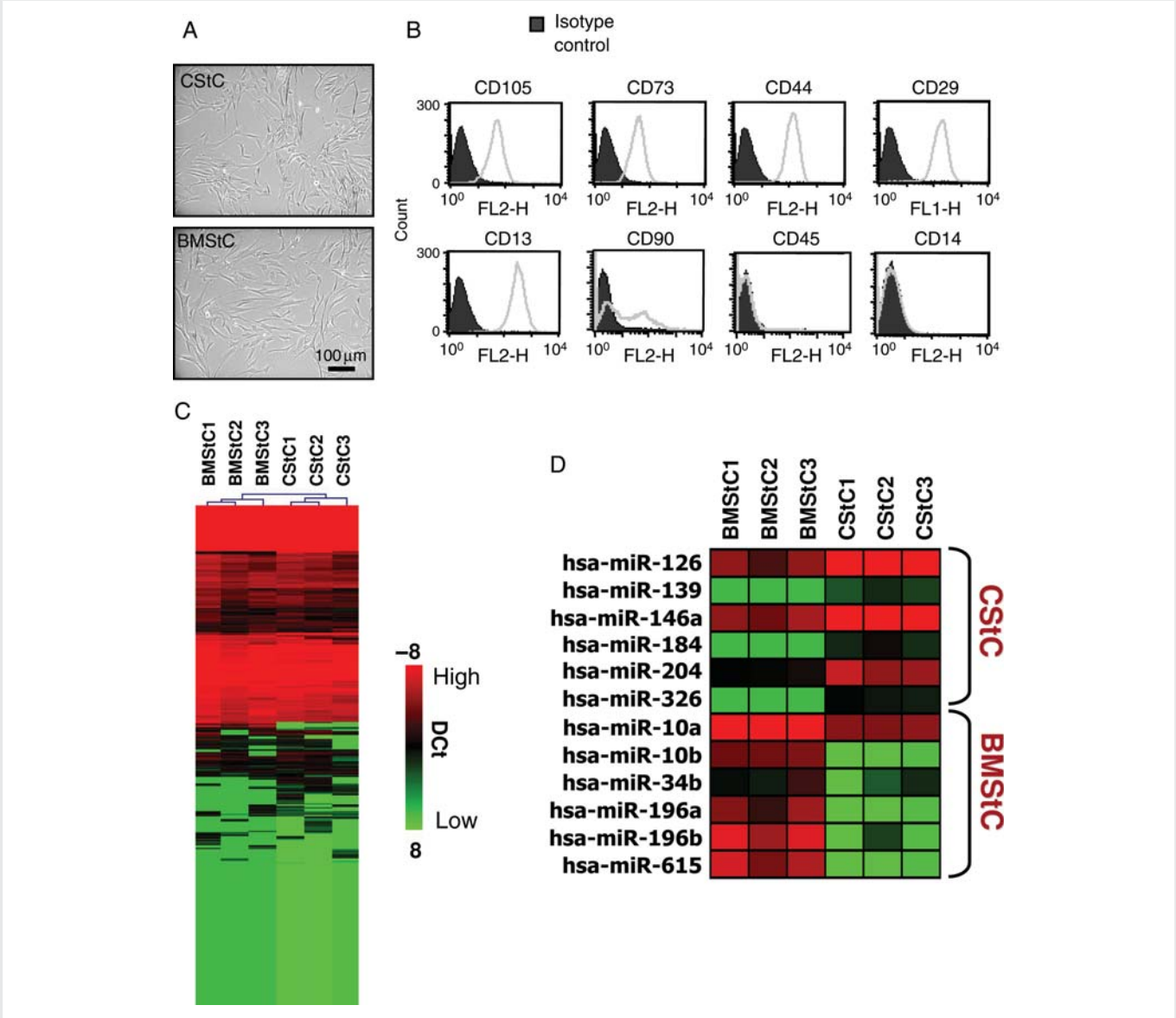

Figure 1 *In vitro* molecular characterization of CStC and BMStC. (*A*) CStC and BMStC were plastic adherent and fibroblast-like shaped. (*B*) Representative FACS analysis of CStC surface markers. (*C*) Comparison of miR expression profiles between CStC and BMStC. Hierarchical analysis divided the six cell population analysed (from the auricle and the bone marrow of three different patients) in two groups related to the tissue of origin. (*D*) Heat map representing average DCt of 12 miR differentially expressed between CStC and BMStC ($n = 3$).

nificantly up-regulated the expression of α-Sarc, α-MHC, Tn-I, and MYLC-2a, as well as α-SMA. In contrast, BMStC modestly up-regulated only GATA4 and MYLC-2a (*Figure 2*; see Supplementary material online, *Table S7*). Importantly, CStC did not up-regulate cardiac genes when cultured in low serum without ATRA and PB (data not shown).

Only CStC, but not BMStC grown in ATRA/PB, expressed the protein α-Sarc (*Figure 2*); however, the number of α-Sarc-positive cells was very low (≈0.05−0.1%) and no organized sarcomeric structures could be identified (*Figure 2*), suggesting that only a partial degree of differentiation could be achieved under these experimental conditions. Additionally, both untreated and ATRA/PB-treated cells lacked the expression of excitable properties (see Supplementary material online, Results and *Table S8*).

3.4 Effects of intramyocardial CStC and BMStC injection

The previous results indicate that CStC and BMStC exhibit properties related, at least in part, to the tissue of origin. In light of these observations, CStC and BMStC therapeutic potential was challenged *in vivo* in a rat model of chronic MI. Cells tagged with GFP and QDots (see Supplementary material online, *Figure S8*) were injected into the border zone of the infarcted left ventricular (LV) wall 3 weeks after coronary ligation. After 3 additional weeks, haemodynamic studies were performed and the heart excised for tissue section analysis. In agreement with previous works,[20] at the 3-week time point, the persistence within the cardiac tissue of both BMStC and CStC was significantly reduced. The persistence of both cell types in the infarcted

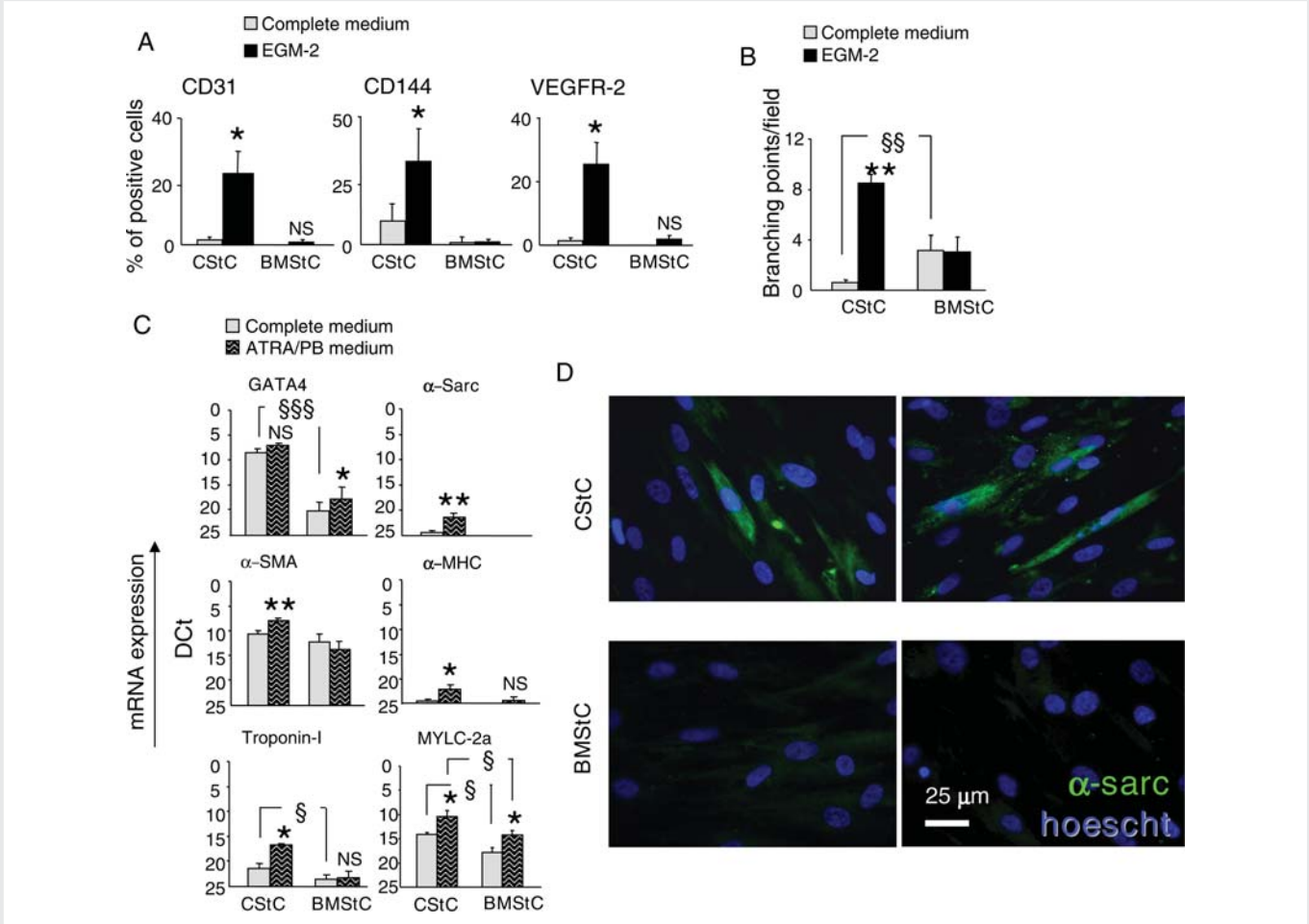

Figure 2 *In vitro* endothelial and cardiomyogenic differentiation. (*A*) After 3 weeks in EGM-2, only CStC acquired positivity for CD31, CD144, and VEGFR-2 ($n = 6$; $^{*}P < 0.05$). (*B*) Bar graph depicting average results for the tube formation assay on Cultrex ($n = 4$; $^{§§,**}P < 0.01$). (*C*) Cardiomyogenic differentiation was evaluated in a medium containing ATRA and PB, where CStC up-regulated the expression of α-Sarc and α-SMA transcripts as well as of α-MHC, Tn-I, and MYLC-2a. GATA4 and osteopontin expression was unvaried ($n = 12$). BMStC exhibited a modest enhancement in GATA4 and MYLC-2a expression, whereas α-Sarc, Tn-I, and α-MHC transcripts remained absent or low ($n = 12$; $^{§§§}P < 0.001$; $^{**}P < 0.01$; $^{§,*}P < 0.05$). (*D*) (i) In ATRA/PB medium, cells acquired an elongated shape and (ii) ~0.1% of CStC, but not BMStC, expressed the protein α-Sarc (FITC, green). Blue fluorescence identifies nuclei.

areas was improved by the addition of 200 ng/mL HGF and IGF-1 growth factors (GF) to the cell preparations immediately before injection in the infarcted ventricular wall.[21] The positive effect of GF on the early engraftment of the injected cells was also documented. In this condition, a 7- and 6.4-fold increase in QDot signals was detected 24 h after injection of CStC and BMStC, respectively. However, QDot signal associated with CStC was significantly higher than that obtained from BMStC (*Figure 3*). Notably, in standard culture conditions, both cell types expressed similar transcript levels of GF receptors, namely c-Met and IGF-1R[21] (see Supplementary material online, *Figure S9*). Therefore, it is conceivable that both cell populations could be activated by GF pre-treatment. All subsequent experiments were performed in the presence of GF. Three weeks after cell injection, QDots associated with BMStC were organized in localized clusters predominantly distributed in the border zone, whereas CStC appeared uniformly distributed in infarcted and peri-infarcted regions (*Figure 3*). This finding is in agreement with the secretome analysis, revealing that CStC, compared with BMStC, exhibited higher metalloproteinase activity (*Figure 3*) and higher expression of other proteins involved in cell migration and extracellular matrix turnover (see Supplementary material online, *Table S9* and Results). Accordingly, *in vitro* CStC presented a higher invasive capacity than BMStC (*Figure 3*).

Haemodynamic studies demonstrated that CStC, but not BMStC, improved cardiac performance compared with PBS and GF alone (*Figure 4*); specifically, the increase in LV end-diastolic pressure (LVEDP) and the decrease in $+dP/dt$ and $-dP/dt$ that follow MI were partially prevented by CStC, but not by BMStC, injection. Both cell types appeared to increase LV systolic pressure (LVSP) when compared with PBS and GF, but these positive trends did not achieve statistical significance. Interestingly, LV chamber volume exhibited a two-fold increase after MI; GF, BMStC, and CStC were all efficient in reducing dilation compared with saline injection; however, the beneficial effect of CStC on LV remodelling was significantly higher than that of BMStC and GF (*Figure 4*). Further, the infarcted wall was thicker in CStC- than in BMStC-, GF-, and

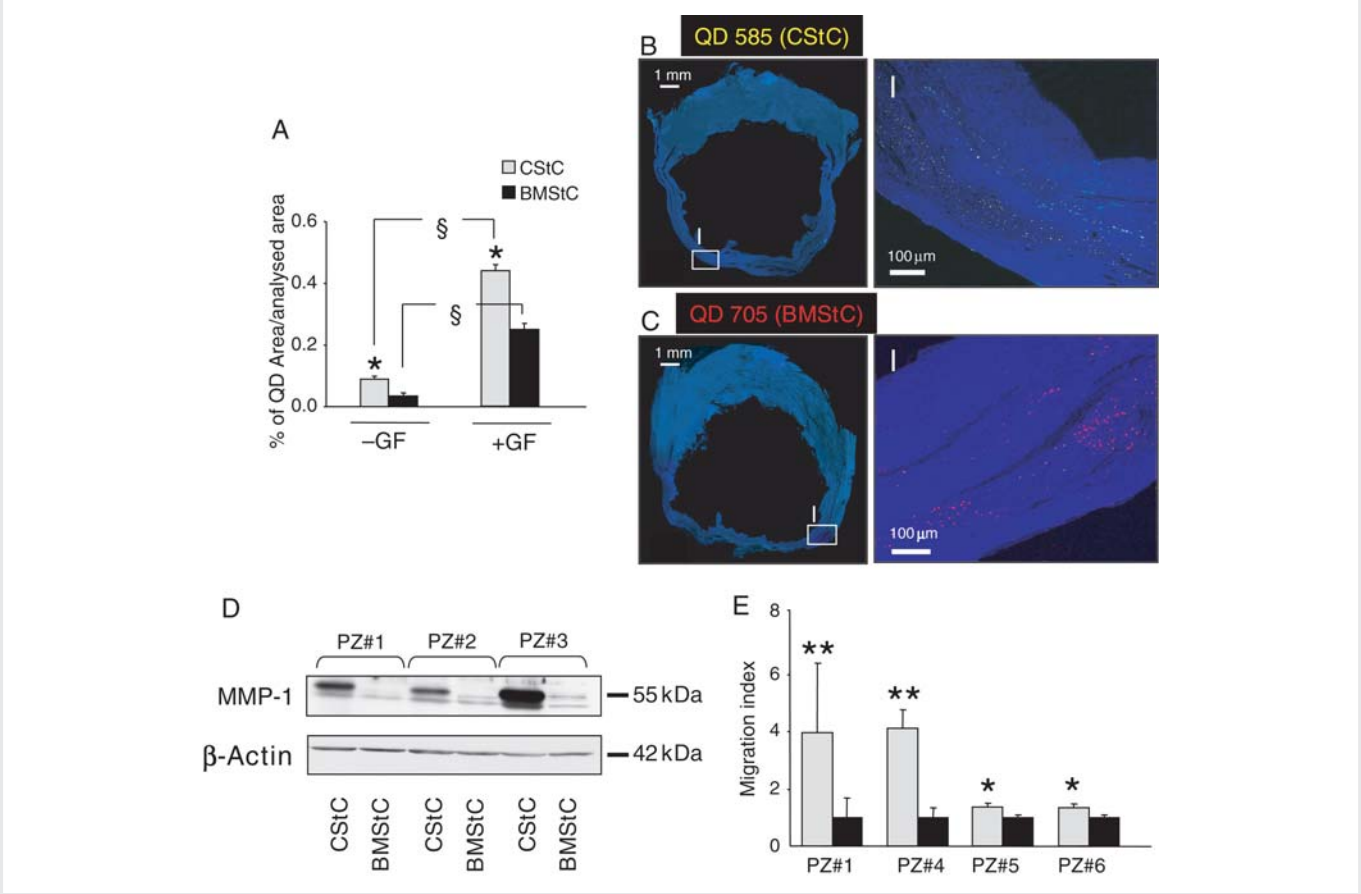

Figure 3 QDot labelled cell engraftment. (*A*) Bar graph indicates the LV fractional area occupied by QDots both in the absence (−GF) and in the presence (+GF) of supplementary growth factors ($n = 3$; $^{*}P < 0.05$ for CStC vs. BMStC; $^{§}P < 0.05$ vs. ±GF). (*B* and *C*) LV unstained cross-sections of paraffin-embedded hearts injected with CStC and BMStC, respectively, and analysed under UV-light to quantify the area occupied by QDots. The areas included in the rectangles are shown at higher magnification. Only CStC-QDots distributed uniformly within the scar and the area proximal to the border zone (*B*), whereas BMStC-QDots remained organized in clusters and were typically reduced within the scar (*C*). (*D*) Western blot confirming that MMP-1 expression was enhanced in CStC compared with BMStC (upper) from three different patients (PZ). (*E*) Also CStC invasive ability was higher than BMStC's. The data are expressed as the fold increase in the number of migrated CStC relative to the number of migrated BMStC (migration index, $n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$).

PBS-treated hearts (*Figure 4*). The results obtained transplanting BMStC in the border zone of rat chronic MI are in keeping with prior reports describing only a modest increase in cardiac function and remodelling following injection of marrow-derived cells.[3,22]

Importantly, before injection, CStC were evaluated for genomic stability at the chromosomal level and no specific alterations were found (see Supplementary material online, *Figure S10* and *Table S10*), suggesting that these cells may represent a potential therapeutic tool with an elevated level of biological stability and safety.

3.5 CStC and BMStC *in vivo* cardiomyogenic differentiation

To assess CStC and BMStC *in vivo* differentiation into cardiomyocytes, we identified cells positive for GFP (GFP^{POS}), human chromosome Y (HC^{POS}), and α-Sarc (α-$Sarc^{POS}$; *Figure 5*). Remarkably, about 25% of HC^{POS}/GFP^{POS} CStC were also α-$Sarc^{POS}$, whereas only 6% of BMStC acquired α-Sarc positivity (*Figure 5*). In agreement with the results obtained tracking cells with QDot labelling, in the scarred infarct, the number of $HC^{POS}/GFP^{POS}/\alpha\text{-}Sarc^{POS}$ cells derived from CStC was higher than the number of $HC^{POS}/GFP^{POS}/\alpha\text{-}Sarc^{POS}$ cells derived from BMStC (*Figure 5*). Of note, most BMStC did not exhibit defined sarcomere striations (see Supplementary material online, *Figure S11*). In contrast, CStC-derived cardiomyocytes present in the border area showed clear sarcomere striations (*Figure 5*; see Supplementary material online, *Figure S11*) and reached volumes up to 20 000 μm^3 (*Figure 5*). Noteworthy, about 15% of $HC^{POS}/GFP^{POS}/\alpha\text{-}Sarc^{POS}$ CStC-derived cardiomyocytes had a volume higher than 10 000 μm^3, whereas 100% of $HC^{POS}/GFP^{POS}/\alpha\text{-}Sarc^{POS}$ BMStC-derived cardiomyocytes had a volume lower than 10 000 μm^3 (*Figure 5*). Taken together, these results suggest that CStC were more efficient than BMStC in their ability to generate cardiomyocytes with an adult-like phenotype.

3.6 Cell fusion

To assess fusion at the nuclear level, DNA content was measured in human-derived $GFP^{POS}/\alpha\text{-}Sarc^{POS}$ cardiomyocytes. Nuclei of resident cardiomyocytes, negative and positive for the phosphorylated form of histone H3 ($H3\text{-}S10^{P}$), which is specifically associated with

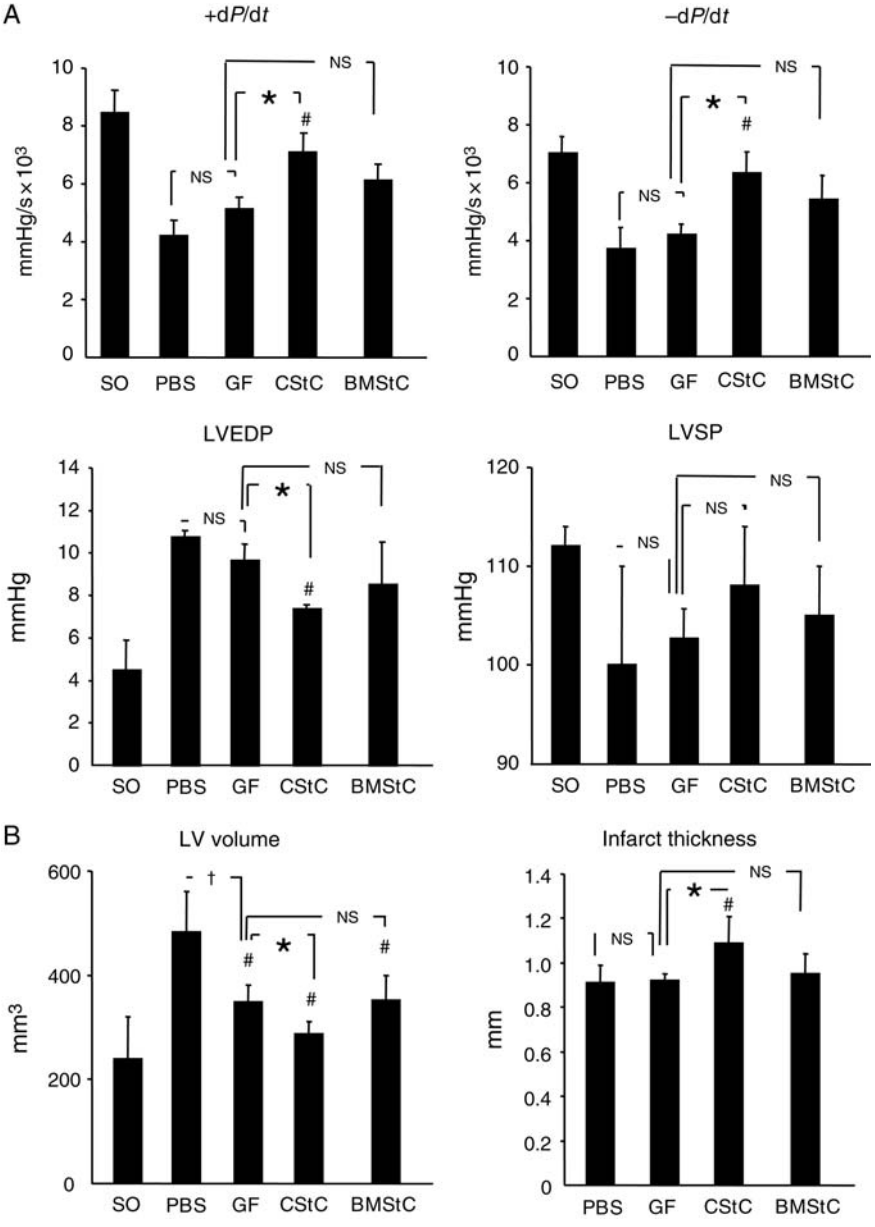

Figure 4 Effect of cell transplantation on LV function and remodelling. (*A*) Haemodynamic evaluation of LV function was performed 3 weeks after injection of CStC or BMStC supplemented with GF. Control groups were treated with PBS alone and with PBS + GF (GF). SO, sham-operated rats ($n = 6$); PBS, infarcted hearts treated with saline ($n = 6$); GF, infarcted hearts treated with PBS supplemented with GF ($n = 6$); CStC, infarcted hearts treated with CStC supplemented with GF ($n = 7$); BMStC, infarcted hearts treated with BMStC supplemented with GF ($n = 6$). CStC injection improved +d*P*/d*t* and reduced LVEDP vs. GF alone; in contrast, BMStC had no significant effect on these haemodynamic parameters. Neither CStC nor BMStC had a significant effect on LVSP compared with GF alone. (*B*) GF, BMStC, and CStC were all efficient in reducing ventricular dilation compared with saline injection, although the effect of CStC was more pronounced than that of BMStC and GF. Importantly, the infarcted wall was thicker in CStC- than in BMStC- and GF-treated hearts. $^{\#}P < 0.05$ vs. GF; $^{\dagger *}P < 0.05$.

chromosome condensation and dynamics during mitosis,[23] were used as controls for 2n or 4n DNA content, respectively. Double DNA content, correspondent to 4n chromosomes, was present only in mitotic H3-S10^{P}-positive cells. In addition, the volume of normal adult rat cardiomyocyte[24] ranges from 30 000 to 80 000 μm^3; therefore, fusion between human stromal cells and resident cardiomyocytes appears unlikely, as the stromal cell-derived cardiomyocytes shown here never reached volumes higher than 20 000 μm^3. Fusion between cytoplasm and polynucleation was also excluded as all HC^{POS} cardiomyocytes analysed in the present investigation were mononucleated (*Figure 5*).

3.7 CStC and BMStC contribution to new vessel formation

Both CStC and BMStC treatment resulted in a higher number of arterioles within the infarcted region (*Figure 6*) compared with PBS and GF injection; further, the effect of CStC on the total

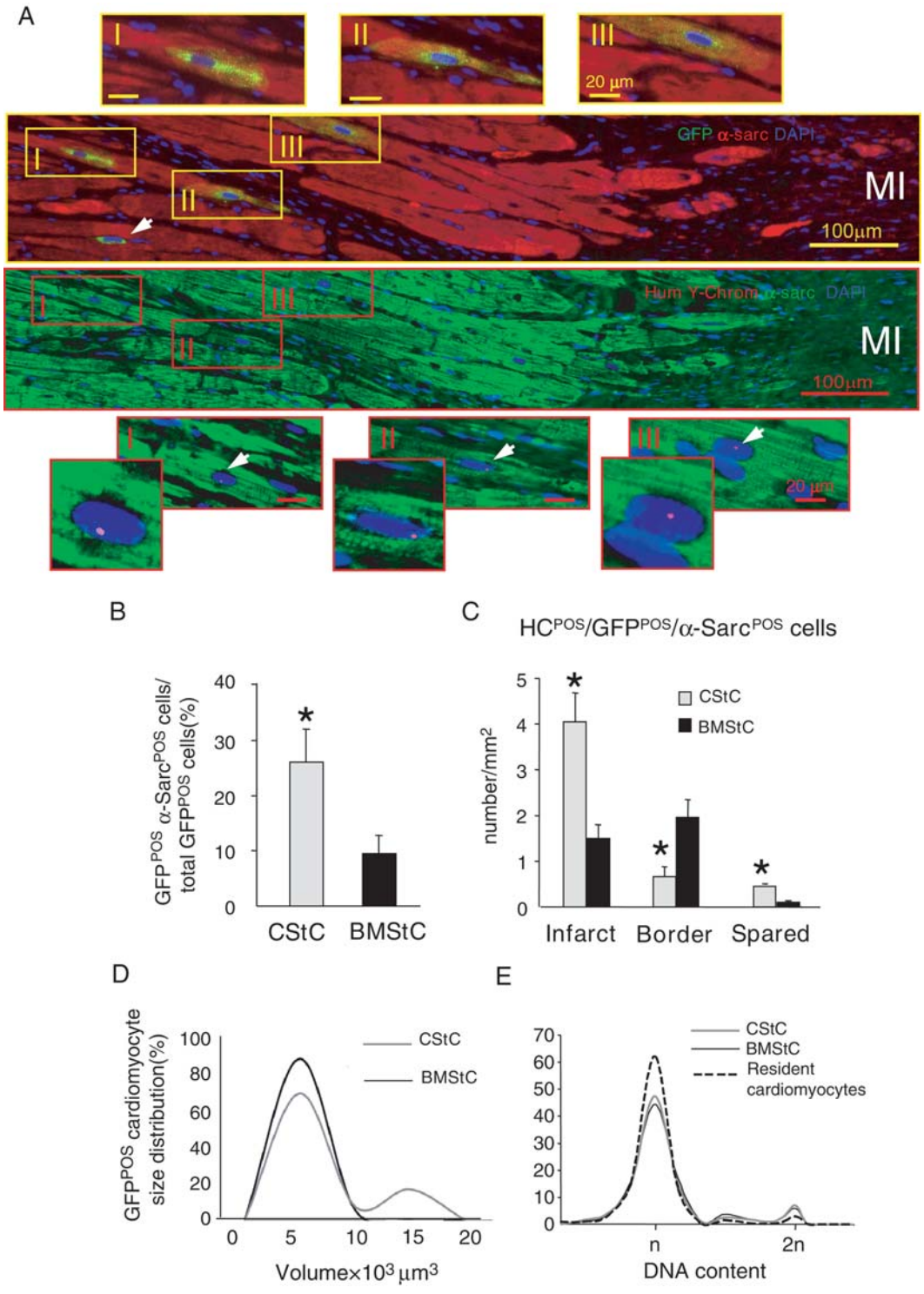

Figure 5 Human CStC and BMStC differentiation *in vivo*. (*A*) The upper central panel shows GFP^{POS} (green)/α-$Sarc^{POS}$ (red) cardiomyocytes with defined sarcomere striations derived from CStC and located in the infarct (MI) border zone. The white arrow points to a GFP^{POS}/α-$Sarc^{NEG}$ cell. The lower central panel corresponds to the serial section confirming, by FISH for human chromosome Y (HC, red), the human origin of α-$Sarc^{POS}$ (FITC)-labelled cells. The areas in the rectangles (I, II, and III) are shown at higher magnification. (*B*) The percentage of GFP^{POS}/α-$Sarc^{POS}$ cardiomyocytes over the total counted GFP^{POS} cells. $^*P < 0.05$ vs. BMStC. (*C*) The density of HC^{POS}/GFP^{POS}/α-$Sarc^{POS}$ cardiomyocytes. $^*P < 0.05$ vs. BMStC. (*D*) CStC- and BMStC-derived cardiomyocytes size distribution. Notably, a significant fraction of CStC-derived cardiomyocytes reached volumes $>15\,000\ \mu m^3$. (*E*) DNA content distribution of CStC-, BMStC-derived, and rat resident cardiomyocytes.

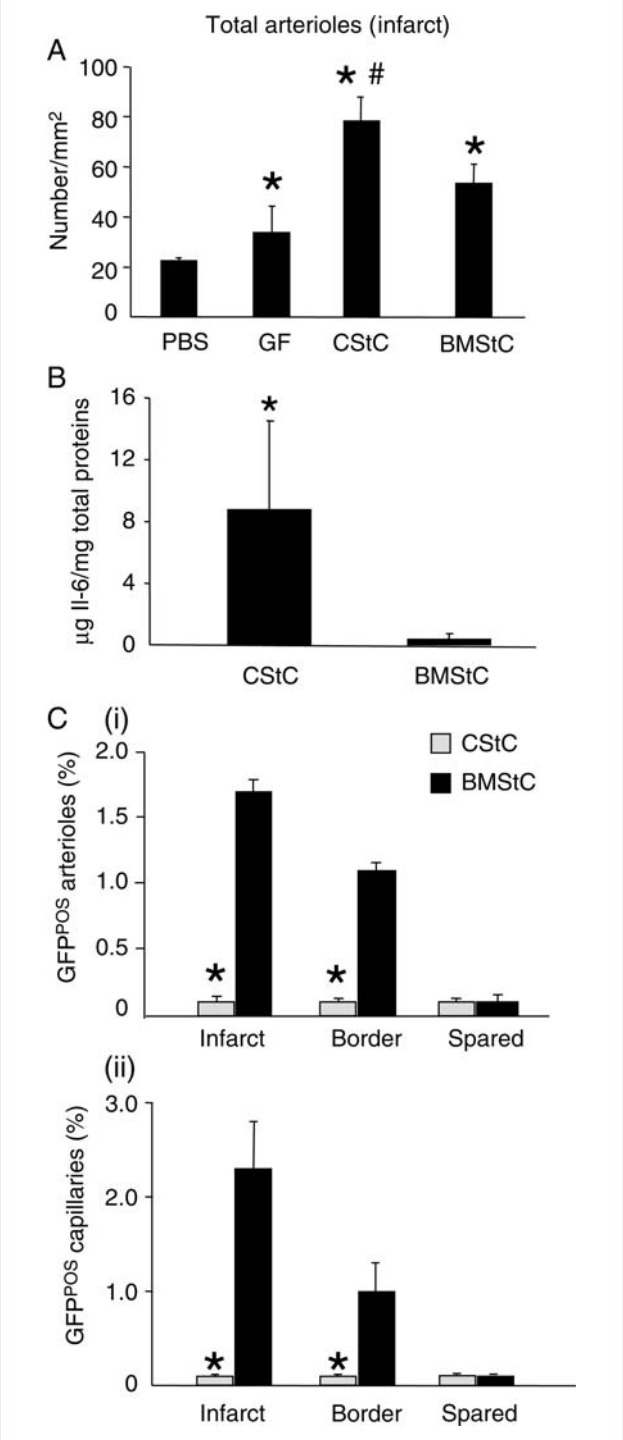

Figure 6 CStC and BMStC promote arteriole development *in vivo*. (*A*) Arteriole density within the infarcted myocardium (*$P < 0.01$ vs. BMStC; #$P < 0.01$ vs. PBS and GF). (*B*) Enzyme-linked immunosorbent assay indicating that IL-6 secretion was increased in CStC supernatants compared with BMStC; $n = 4$; *$P < 0.05$. (*C*) (i) The percentage of $GFP^{POS}/\alpha\text{-}SMA^{POS}$ arterioles derived from the differentiation of GFP^{POS} CStC and GFP^{POS} BMStC ($n = 6$; *$P < 0.01$ vs. BMStC). (ii) The percentage of GFP^{POS}/vWF^{POS} capillaries derived from the differentiation of GFP^{POS} CStC and GFP^{POS} BMStC ($n = 6$; *$P < 0.01$ vs. BMStC). The percentage of GFP^{POS} arterioles and capillaries is referred to the total number of counted arterioles and capillaries, respectively.

number of arterioles was more marked than BMStC's. This is in agreement with the higher production of pro-angiogenic factors like IL-6 and LIF[25,26] observed in CStC compared with BMStC (see Supplementary material online, *Figure S6*, *Table S9*, and Results).

In order to differentiate the paracrine action from the direct stromal cell contribution to blood vessel development, $GFP^{POS}/\alpha\text{-}SMA^{POS}$ arteriole and GFP^{POS}/vWF^{POS} capillary profiles were quantified both in the infarcted region and in the border area (*Figure 6*). In these regions, although higher for BMStC than CStC, the differentiation process was very low for both cell types, representing only 1.7 and 2.2% of total arterioles and capillaries, respectively. In the spared region, the contribution of both types of stromal cells to arteriole and capillary development was minimal and similar between the two cell types (*Figure 6*).

4. Discussion

In the present study, we characterized the unselected population of CStC in comparison with BMStC derived from the same patient and we examine whether CStC were more oriented than bone marrow cells to reconstitute their tissue of origin.

CStC and BMStC are remarkably similar to the expression of mesenchymal-associated antigens (i.e. CD105 and CD44) and revealed positivity for both pericytes (i.e. CD146) and fibroblast markers (i.e. vimentin and human fibroblast surface antigen). This finding does not surprise considering that human bone marrow mesenchymal cells have recently been reported to show a broad structural and functional overlap with dermal fibroblasts.[27] Importantly, skin fibroblasts also differentiate into osteocytes and adipocytes[28] and show immunoregulatory properties.[27] On the other hand, marrow mesenchymal cells *in vivo* exhibit fibrogenic potential.[29] Therefore, based on phenotypical criteria, it is difficult to clearly discriminate between fibroblasts and mesenchymal cells[30] of cardiac and bone marrow origin. For these reasons, we defined these cells as 'stromal cells'.

Despite the important similarities described above, significant differences between CStC and BMStC emerged at the biological and molecular level: CStC were, in fact, less competent than BMStC in producing adipocytes and osteocytes but more efficient in the expression of cardiovascular lineage markers. In addition, CStC and BMStC significantly diverged at the transcriptional level. CStC expressed early (GATA4) and late (MYLC-2a) cardiac mRNA and were identified by a specific miR signature unmodified by differentiation stimuli, suggesting that the expression of these miR is only minimally affected by the extracellular environment. A growing body of evidence indicates that many miR play important roles during development,[16] highlighting their causal role in establishing cell fate. Consequently, not only miR represent biomarkers useful to discriminate between stromal cells of cardiac or bone marrow origin, but they may also be involved in establishing their tissue-specific plasticity and therapeutic properties.

Notably, some of the miR up-regulated in CStC (miR-126 and -146a) are highly expressed in proliferating cardiomyocyte precursors isolated from the foetal human heart (CMPC[18]), suggesting potential analogies between CMPC and CStC. miR-126 is also known for its role in angiogenesis and angiogenic response.[17] Its higher expression in CStC compared with BMStC may therefore account for their increased *in vitro* ability to acquire the expression of endothelial

markers, although this effect *in vivo* may be mitigated by locally provided environmental signals.

The results we obtained transplanting CStC/BMStC in the border zone of rat chronic MI are in line with prior works, describing that the injection of skin fibroblasts,[31] bone marrow cells,[2] and ventricular cells[32] ameliorates the performance of infarcted hearts. Accordingly, in the present work, we demonstrated that CStC not only improve cardiac function, but participates to tissue regeneration more efficiently than their bone marrow counterpart. Importantly, CStC persisted longer within the cardiac tissue and migrated within the scar, promoted angiogenesis, and differentiated into adult-like cardiomyocytes more efficiently than BMStC. In this regard, the *in vitro* ability of CStC to invade collagen layers and secrete enzymes able to modulate extracellular matrix turnover (i.e. MMP-1) may provide the molecular basis for the higher efficiency of CStC to invade the post-MI tissue stabilizing or improving cardiac function and/or lessen cardiac dysfunction.

In vivo, the evidence in favour of CStC/BMStC differentiation into endothelial/smooth muscle cell lineages was minimal, suggesting that paracrine mechanisms had a higher importance than direct differentiation in the formation of new vessels. This possibility was supported by the secretome analysis, since both IL-6 and LIF were increased in CStC-conditioned media. Others found that both IL-6 and LIF are induced during the infarct healing phase and known to modulate angiogenesis protecting cardiomyocytes from apoptosis.[33] The observation that CStC production of IL-6 and LIF was higher than that of BMStC is in agreement with their superior ability to promote angiogenesis within the infarcted region.

Interestingly, only in CStC-derived cardiomyocytes, a clear evidence of sarcomere formation and volumetric dimensions compatible with those of adult cardiomyocytes could be observed. In this view, it is noteworthy that CStC acquired adult dimensions and sarcomere-specific striations only in the border zone, suggesting that a cell-to-cell contact with viable myocardium may be required for efficient differentiation into mature cardiomyocytes.

The observation that the cardiomyogenic response induced *in vitro* from CStC/BMStC is a very rare event is in agreement with recent reports[34] and at least in part attributable to the fact that our limited knowledge about the mechanisms by which cardiomyogenic differentiation occurs makes it difficult to define standardized *in vitro* efficient differentiation protocols. These findings are only apparently in contrast with the relative high efficiency of CStC in generating cardiomyocytes *in vivo*, as the environment of the healing infarct is likely more prone to sustain tissue-specific differentiation than unspecific epigenetic agents.[35]

In conclusion, cardiac-derived cells appear as a population of potentially relevant clinical interest, being able to acquire the cardiomyocyte phenotype and to promote angiogenesis via paracrine mechanisms more efficiently than their bone marrow counterpart, thus indicating the importance of tissue specificity when planning cell therapy strategies.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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