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Cardiomyogenic differentiation-independent improvement of cardiac function by human cardiomyocyte progenitor cell injection in ischaemic mouse hearts

Melina C. den Haan [a, #], Robert W. Grauss [a, #], Anke M. Smits [b], Elizabeth M. Winter [c], John van Tuyn [a, b], Daniël A. Pijnappels [a], Paul Steendijk [a], Adriana C. Gittenberger-De Groot [c], Arnoud van der Laarse [a], Willem E. Fibbe [d], Antoine A. F. de Vries [a, b], Martin J. Schalij [a], Pieter A. Doevendans [e], Marie-José Goumans [b], Douwe E. Atsma [a, *]

[a] *Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands*
[c] *Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, The Netherlands*
[d] *Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands*
[e] *Department of Cardiology, University Medical Center Utrecht, Utrecht, The Netherlands*

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Abstract

We previously showed that human cardiomyocyte progenitor cells (hCMPCs) injected after myocardial infarction (MI) had differentiated into cardiomyocytes *in vivo* 3 months after MI. Here, we investigated the short-term (2 weeks) effects of hCMPCs on the infarcted mouse myocardium. MI was induced in immunocompromised (NOD/scid) mice, immediately followed by intramyocardial injection of hCMPCs labelled with enhanced green fluorescent protein (hCMPC group) or vehicle only (control group). Sham-operated mice served as reference. Cardiac performance was measured 2 and 14 days after MI by magnetic resonance imaging at 9.4 T. Left ventricular (LV) pressure–volume measurements were performed at day 15 followed by extensive immunohistological analysis. Animals injected with hCMPCs demonstrated a higher LV ejection fraction, lower LV end-systolic volume and smaller relaxation time constant than control animals 14 days after MI. hCMPCs engrafted in the infarcted myocardium, did not differentiate into cardiomyocytes, but increased vascular density and proliferation rate in the infarcted and border zone area of the hCMPC group. Injected hCMPCs engraft into murine infarcted myocardium where they improve LV systolic function and attenuate the ventricular remodelling process 2 weeks after MI. Since no cardiac differentiation of hCMPCs was evident after 2 weeks, the observed beneficial effects were most likely mediated by paracrine factors, targeting amongst others vascular homeostasis. These results demonstrate that hCMPCs can be applied to repair infarcted myocardium without the need to undergo differentiation into cardiomyocytes.

Keywords: cardiomyocyte progenitor cell • myocardial infarction • paracrine factors • angiogenesis • remodelling

Introduction

In recent years, it has been demonstrated that cell therapy can improve left ventricular (LV) function in animal models for myocardial infarction (MI) and in patients with acute MI. Several cell types have been studied to date, with bone marrow (BM)-derived mononuclear cells including haematopoietic stem cells (HSCs) [1] and mesenchymal stem cells (MSCs) [2] as the most extensively investigated cell populations. However, also embryonic stem cells [3], skeletal myoblasts [4] and endothelial progenitor cells [5] have shown cardiac regeneration potential in animal MI models.

Although most of the clinical studies applying BM-derived cells revealed no significant side effects, stem cell-induced improvements in cardiac function remained modest [6], comparable to currently used treatment modalities for post-MI patients [7]. The improvement of LV function appears to result from neovascularization

[#]Both authors contributed equally to this study.
*Correspondence to: D. E. ATSMA, M.D., Ph.D.,
Department of Cardiology, Leiden University Medical Center,
P.O. Box 9600, 2300 RC Leiden, The Netherlands.
Tel.: +31 71 526 2020
Fax: +31 71 526 6809
E-mail: D.E.Atsma@lumc.nl

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[8, 9], reduction of apoptosis [10] and improvement of scar compliance [11], probably through paracrine effects. Cardiomyogenic differentiation of engrafted BM-derived stem cells is uncertain [12], and at best seems to be a very rare event [13].

The recent identification of populations of cardiac stem or progenitor cells (CPCs) that reside in the heart itself, has generated new opportunities for cell-based therapy, since these cells are capable of cardiac regeneration [14]. We recently isolated human cardiomyocyte progenitor cells (hCMPCs) from foetal hearts [15]. These cells express the stem cell markers stem cell antigen-1 (Sca-1)-like protein and islet-1 (Isl-1), and the early cardiac transcription factors GATA-4 and Nkx2.5. hCMPCs are able to differentiate into spontaneously beating cells, when stimulated with the DNA methyltransferase inhibitor 5-azacytidine. Further maturation is achieved by adding transforming growth factor-β (TGF-β) to the culture medium. Differentiated hCMPCs are able to form highly conductive gap junctions and to generate ventricular cardiomyocyte-like action potentials [15]. Previously, we showed that hCMPCs transplanted into infarcted mouse myocardium differentiated into both cardiomyocytes and vascular cells and that hCMPC-treated animals displayed less deterioration of cardiac function in the long term (3 months) compared to control mice [16].

To learn more about the mechanism and the time course of these beneficial effects, we now analysed the effects of hCMPC injection into the infarcted mouse heart after a short term (2 weeks).

To this end, we investigated the *in vivo* behaviour of undifferentiated hCMPCs in an immunocompromised mouse model 2 weeks after acute MI and assessed (1) the engraftment and differentiation state of the intramyocardially injected hCMPCs and (2) the effects of intramyocardial hCMPC injection on LV function by small animal magnetic resonance imaging (MRI) and pressure–volume (PV) analysis.

Materials and methods

See online data supplement for more details.

Animals

All experiments were approved by the Committee on Animal Welfare of the Leiden University Medical Center, Leiden, the Netherlands. To avoid rejection of injected human cells, experiments were performed in 8- to 10-week-old male non-obese diabetic/severe combined immunodeficient (NOD/scid) mice (Charles River Laboratories, Maastricht, the Netherlands). The animals were housed in filtertop cages and were given standard diet and water with antibiotics and antimycotics *ad libitum.* The experiments conformed to the principles of Laboratory Animal Care formulated by *the Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23, revised 1996).

Isolation and expansion of hCMPCs

For human foetal tissue collection, individual permission using standard informed consent procedures and prior approval of the Medical Ethics Committee of the University Medical Center Utrecht, Utrecht, the Netherlands, were obtained. hCMPCs were isolated by magnetic cell sorting (MACS; Miltenyi Biotec, Sunnyvale, CA) using Sca-1–conjugated beads, as described previously [15]. To facilitate their identification *in vivo*, hCMPCs were transduced with a human adenovirus vector encoding the enhanced green fluorescent protein (eGFP) as previously described [17].

Myocardial infarction model and cell implantation

Myocardial infarction was induced as described previously [18]. Briefly, animals were anaesthetized and intubated. After left thoracotomy, the left anterior descending (LAD) coronary artery was ligated using a 7-0-prolene suture (Johnson and Johnson, New Brunswick, NJ, USA). Twenty minutes after MI, animals received 20 μl culture medium (M199; Invitrogen, Bleiswijk, The Netherlands) containing 2×10^5 hCMPCs (MI + hCMPC group) or 20 μl culture medium containing no cells (MI + vehicle group) by intramyocardial injections at five sites in the infarcted area. Sham-operated animals were used to determine baseline characteristics (Sham group). In the MI + hCMPC group 25 animals were included, 11 animals reached the end-point. In the MI + vehicle 26 animals were included, 12 animals reached the end-point. In the sham group 16 animals were included, 10 animals reached the end-point. Only measurements of the animals that reached the 15-day end-point were taken into account in the analysis of the performed experiments.

Cardiac MRI

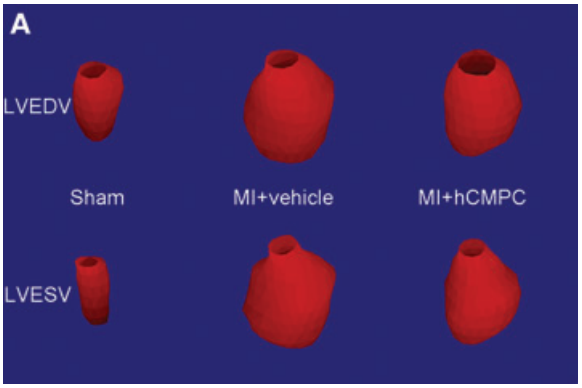
Left ventricular volumes and functions were serially assessed at day 2 and 14 after surgery by high-field (9.4 T) MRI as described previously [19]. All data were analysed by manual tracing of endocardial and epicardial borders with the MR Analytical Software System (MASS) for Mice (MEDIS, Leiden, the Netherlands). End-diastolic and end-systolic phases were identified automatically, after which LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV) and LV ejection fraction (LVEF) were computed.

Pressure–volume loop analysis to assess LV function

At day 15 a 1.4-F pressure-conductance catheter (SPR-719; Millar Instruments, Houston, TX) was introduced *via* the right carotid artery, positioned in the left ventricle and connected to a Sigma-SA signal processor (CD Leycom, Zoetermeer, the Netherlands) for online display and recording of LV pressure and volume signals. Parallel conductance and LV pressure–volume signals were measured as described previously [20–22]. All data were acquired using Conduct-NT software (CD Leycom) at a sample rate of 2000 Hz and analysed off-line with custom-made software.

Histological examination

At day 15 after MI, the mice were killed, weighed and their hearts and lungs were excised. Lung weight was measured immediately after excision and following freeze-drying for 24 hrs. The wet weight/dry weight ratio was used as a measure of pulmonary congestion. The hearts were fixed by immersion in buffered 4% paraformaldehyde and embedded in paraffin. Serial transverse sections of 5 μm were cut for (immuno)histological analyses.



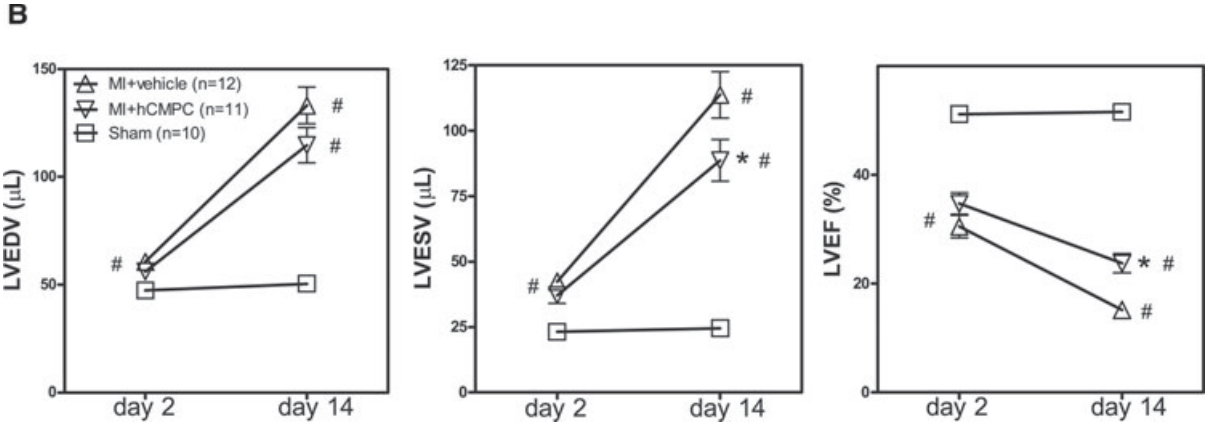


Fig. 1 Assessment of LV function at day 14 by MRI. Representative 3D reconstructions of LV end-diastolic volume (EDV) and LV end-systolic volume (ESV) for sham-operated animals (Sham), animals receiving vehicle only (MI + vehicle) and animals receiving hCMPCs (MI + hCMPC) (**A**). Graphs summarizing the results of the anatomical and functional analysis of the left ventricle by MRI for all three experimental groups: EDV, ESV and ejection fraction (EF) (**B**). $*P < 0.05$ *versus* MI mice that received vehicle only. $\#P < 0.05$ *versus* sham-operated animals.

Assessment of hCMPC engraftment and differentiation

Human cardiomyocyte progenitor cell engraftment was assessed by immunostaining using an anti-GFP antibody. Double immunostainings were performed to investigate differentiation of eGFP-labelled hCMPCs. Serial sections were immunostained using antibodies against human CD31 (also known as platelet endothelial cell adhesion molecule-1 (PECAM-1)), α-smooth muscle actin (ASMA), α-sarcomeric actin (αSA), cardiac troponin I (cTnI), cardiac troponin T (cTnT) and atrial natriuretic factor (ANF). Primary antibodies were visualized with appropriate secondary biotinylated IgG and Qdot-655-streptavidin conjugates. GFP-specific labelling was visualized using Alexa Fluor 488–conjugated IgGs.

Morphometric analysis

To determine the angiogenic effects of hCMPC transplantation, vascular density was assessed by quantifying the number of murine CD31-positive vessel per mm^2. The effect of hCMPC transplantation on cell proliferation and reparative nuclear DNA synthesis in donor and recipient cells was evaluated by nuclear staining with an anti-proliferating cell nuclear antigen (PCNA) antibody. Double immunostainings were performed to identify PCNA-positive cell types. Serial sections were immunostained using antibodies against CD31, ASMA and cTnI. The effect of hCMPCs transplantation on scar composition was assessed by staining for collagen type III. Left ventricular collagen type III density was expressed as the ratio of the percentage of collagen type III–positive tissue in the left ventricle to that in the right ventricle of the same section. To analyse the extent of the total collagen deposition after hCMPC transplantation, sections were stained with picro-sirius red. Total collagen deposition was determined by the area stained tissue within the left ventricle as a percentage of the whole left ventricle.

The effect of hCMPCs engraftment on LV wall thickness was quantified at two separate border zone areas, at the midpoint of the infarct region and averaged for all three measurements.

Statistical analysis

Numerical values were expressed as mean ± standard deviation (SD). Comparisons of parameters between the Sham, MI + vehicle and MI + hCMPC groups were performed using one-way analysis of variance, with Bonferroni correction. *P* values less than 0.05 were considered statistically significant.

Results

Human cardiomyocyte progenitor cells preserve systolic heart function

Cardiac volumes and ejection fractions were evaluated 2 and 14 days after MI by small animal MRI. Representative images of systolic and diastolic 3D reconstructions of MRI short axis views for all three experimental groups at day 14 are shown in Figure 1A. Already 2 days after MI, both the MI + vehicle group and the MI + hCMPC group had undergone cardiac remodelling

Table 1 Pressure–volume loops-derived left ventricular function indices for sham-operated, MI + vehicle and MI + hCMPC mice

	Sham	MI + vehicle	MI + hCMPC	*P* value: sham *versus* vehicle	*P* value: vehicle *versus* hCMPC
HR (bpm)	431 ± 53	465 ± 52	440 ± 52	0.163	0.261
CO (ml/min.)	9.9 ± 4.6	4.7 ± 1.6	6.1 ± 2.7	0.002	0.140
LVESV (μl)	36 ± 14	81 ± 23	55 ± 18	<0.001	0.011
LVEDV (μl)	57 ± 21	90 ± 25	69 ± 22	0.004	0.048
LVESP (mmHg)	88 ± 18	72 ± 13	67 ± 10	0.033	0.379
LVEDP (mmHg)	7 ± 3	17 ± 7	14 ±10	0.001	0.538
Tau (msec.)	14 ± 4	21 ± 5	16 ± 4	0.008	0.040
dP/dt_{max} (mmHg/sec.)	8380 ± 1684	3946 ± 1432	4105 ± 1595	<0.001	0.808
$-dP/dt_{min}$ (mmHg/sec.)	5898 ± 1245	3085 ± 949	3282 ± 951	<0.001	0.633
SW (mmHg/μl)	1967 ± 826	481 ± 297	643 ± 302	<0.001	0.218
LVEF (%)	40 ± 10	12 ± 4	20 ± 6	<0.001	0.002
ESPVR					
Slope: E_{es} (mmHg/μl)	2.81 ± 2.16	1.86 ± 0.39	2.28 ± 1.18	0.211	0.320
Intercept: ESV_{int} (μl)	31 ± 11	82 ± 22	59 ± 20	<0.001	0.042
PRSW					
Slope: M_W (mmHg/μl)	56 ± 22	31 ± 16	46 ± 8	0.020	0.043
Intercept: EDV_{int} (μl)	39 ± 9	109 ± 25	80 ± 22	<0.001	0.028
dP/dt_{max}-EDV					
Slope: S_{dP} (mmHg/μl)	167 ± 83	119 ± 48	112 ± 47	0.169	0.790
Intercept: EDV_{int} (μl)	38 ± 15	105 ± 34	90 ± 32	<0.001	0.370
EDPVR					
Slope: E_{ed} (mmHg/μl)	0.58 ± 0.62	1.41 ± 0.88	0.83 ± 0.52	0.034	0.127
Intercept: EDV_{int} (μl)	75 ± 26	92 ± 21	77 ± 27	0.136	0.220

HR: heart rate; CO: cardiac output; LVESV: left ventricular end-systolic volume; LVEDV: left ventricular end-diastolic volume; LVESP: left ventricular end-systolic pressure; LVEDP: left ventricular end-diastolic pressure; Tau: relaxation time constant; dP/dt_{max}: maximum rate of pressure increase; dP/dt_{min}: maximum rate of pressure decrease; SW: stroke work; LVEF: left ventricular ejection fraction; ESPVR: end-systolic pressure–volume relationship; E_{es}: end-systolic elastance; ESV_{int}: end-systolic volume intercept; PRSW: preload recruitable stroke work relation (SW *versus* EDV); EDV_{int}: end-diastolic volume intercept; EDPVR: end-diastolic pressure–volume relationship; E_{ed}: end-diastolic stiffness.

as shown by an increase in LVEDV (61 ± 2 μl and 56 ± 3 μl, respectively) and LVESV (42 ± 2 μl and 37 ± 3 μl, respectively), as well as a considerable decrease in LVEF (30 ± 2% and 35 ± 2%, respectively), compared to Sham (LVEDV: 48 ± 2 μl, LVESV: 24 ± 1 μl and LVEF: 51 ± 1%) (Fig. 1B). Two weeks after MI, the MI + vehicle group showed substantial ongoing LV remodelling and a further decrease in LVEF. However, this decrease in LVEF was significantly less in the MI + hCMPC group than in the MI + vehicle group (24 ± 2% *versus* 15 ± 1%; $P < 0.05$). Likewise, the increase in LVESV in the MI + hCMPC group was significantly less than that in the MI + vehicle group (89 ± 8 μl *versus* 114 ± 9 μl; $P < 0.05$) (Fig. 1B).

Human cardiomyocyte progenitor cells improve mechanical performance

Pressure–volume loop data confirmed the extensive LV remodelling after MI. This effect was attenuated by injection of hCMPCs, as demonstrated by significant differences in LVESV, LVEDV, LVEF and relaxation time constant (Tau) between animals that received cells and those that were injected with vehicle only (Table 1). In addition, the intercept of the end-systolic PV relationship (ESPVR) as well as the slope and intercept of the preload recruitable stroke work relation (PRSW) significantly improved in the MI + hCMPC group compared to the MI + vehicle group,

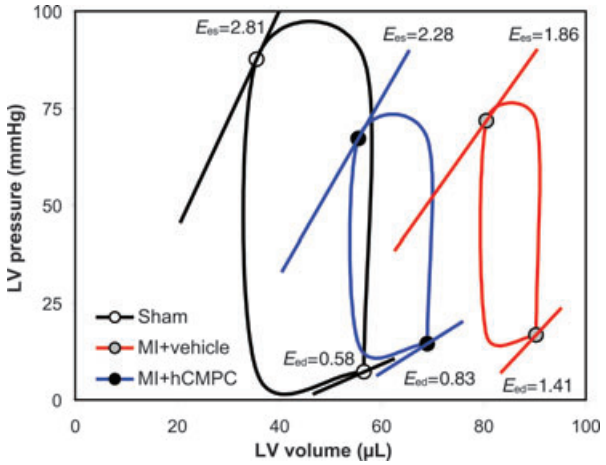


Fig. 2 Pressure–volume loops in the sham, MI + vehicle and MI + hCMPC groups of mice at day 15 after MI (based on mean LV end-diastolic and LV end-systolic pressures and volumes). The oblique lines represent the end-systolic (E_{es}) and end-diastolic (E_{ed}) pressure–volume relations.

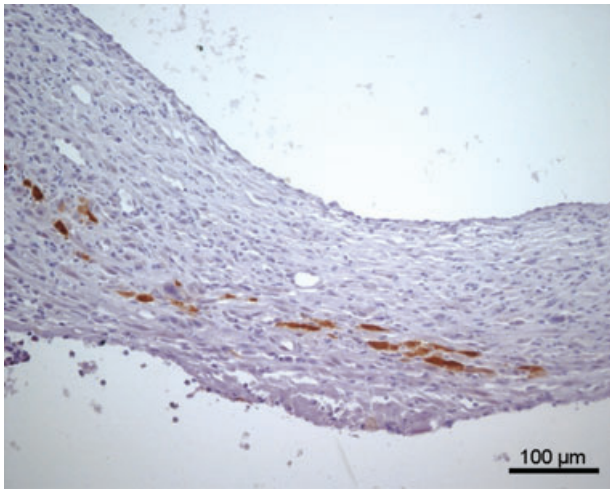


Fig. 3 Immunohistological staining of engrafted GFP-labelled cells (brown) 15 days after MI and intramyocardial hCMPC transplantation.

indicating improvement of LV systolic function by injected hCMPCs (Table 1).

Summarized schematic PV loops (based on mean LVESV, mean LV end-systolic pressure (LVESP), mean LVEDV and mean LV end-diastolic pressures (LVEDP), mean LV end-systolic PV relationships (slope: E_{es}) and mean LV end-diastolic PV relationships (slope: E_{ed}) are presented in Figure 2.

Human cardiomyocyte progenitor cells engrafted in the infarcted murine myocardium do not undergo cardiomyogenic differentiation within the first 15 days after transplantation

Human cardiomyocyte progenitor cells were labelled with GFP to assess engraftment. Fifteen days after cell transplantation, injected hCMPCs were predominantly observed in the infarcted anterolateral wall and border zone of the infarcted area. An engraftment rate of 3.6 ± 1.0% of the total number of injected cells was identified in the hearts of animals treated with hCMPCs (Fig. 3). Human cardiomyocyte progenitor cells were absent in the non-infarcted posterior and septal walls. Serial sections were examined at day 15 to identify GFP-positive cells co-expressing differentiation markers (Fig. 4). None of the injected hCMPCs stained positive for the human endothelial cell-specific marker CD31. The cardiomyocyte-specific markers cTnI, cTnT and ANF were present in the heart. However, none of the injected hCMPCs expressed these markers. The injected cells did express ASMA and αSA, but the staining pattern of the latter protein was diffuse, and without cross-striations at this moment in time (Fig. 4).

Human cardiomyocyte progenitor cells alter vascular density

Left ventricular functional improvements after hCMPC transplantation in the ischaemic heart may occur through paracrine effects. Therefore, we studied the effects of hCMPCs on neovascularization, cellular proliferation and scar composition in the anterolateral wall (infarct zone) and lateral wall (border zone) of the heart (Figs 5–8). The presence of MI promoted neovascularization in the border zone, but not in the infarcted area (Fig. 5A–D). In hCMPC-transplanted mice, the number of vessels was significantly higher in both the infarct and border zone (Fig. 5A) than in mice treated with vehicle (Fig. 5B), which demonstrates a positive effect of hCMPCs on neovascularization (Fig. 5D). None of the hCMPCs expressed hCD31, indicating all vessels were derived from host tissue (Fig. 4).

Human cardiomyocyte progenitor cells are capable of self-renewal and increase cellular proliferation of host tissue

Proliferating cell nuclear antigen was up-regulated in both the infarct and border zone of hCMPC-treated mice (Fig. 6A) when compared to mice treated with vehicle only (Fig. 6B), demonstrating a stimulatory effect of transplanted hCMPCs on cell proliferation (Fig. 6D). This increase in cell proliferation involved both hCMPCs and host tissue (data not shown). The presence of MI altered proliferation rate only in the border zone (Fig. 6A–D). Proliferating cell nuclear antigen expression was present predominantly in cells that were also positive for the smooth muscle cell marker ASMA, with a significant up-regulation of PCNA in animals

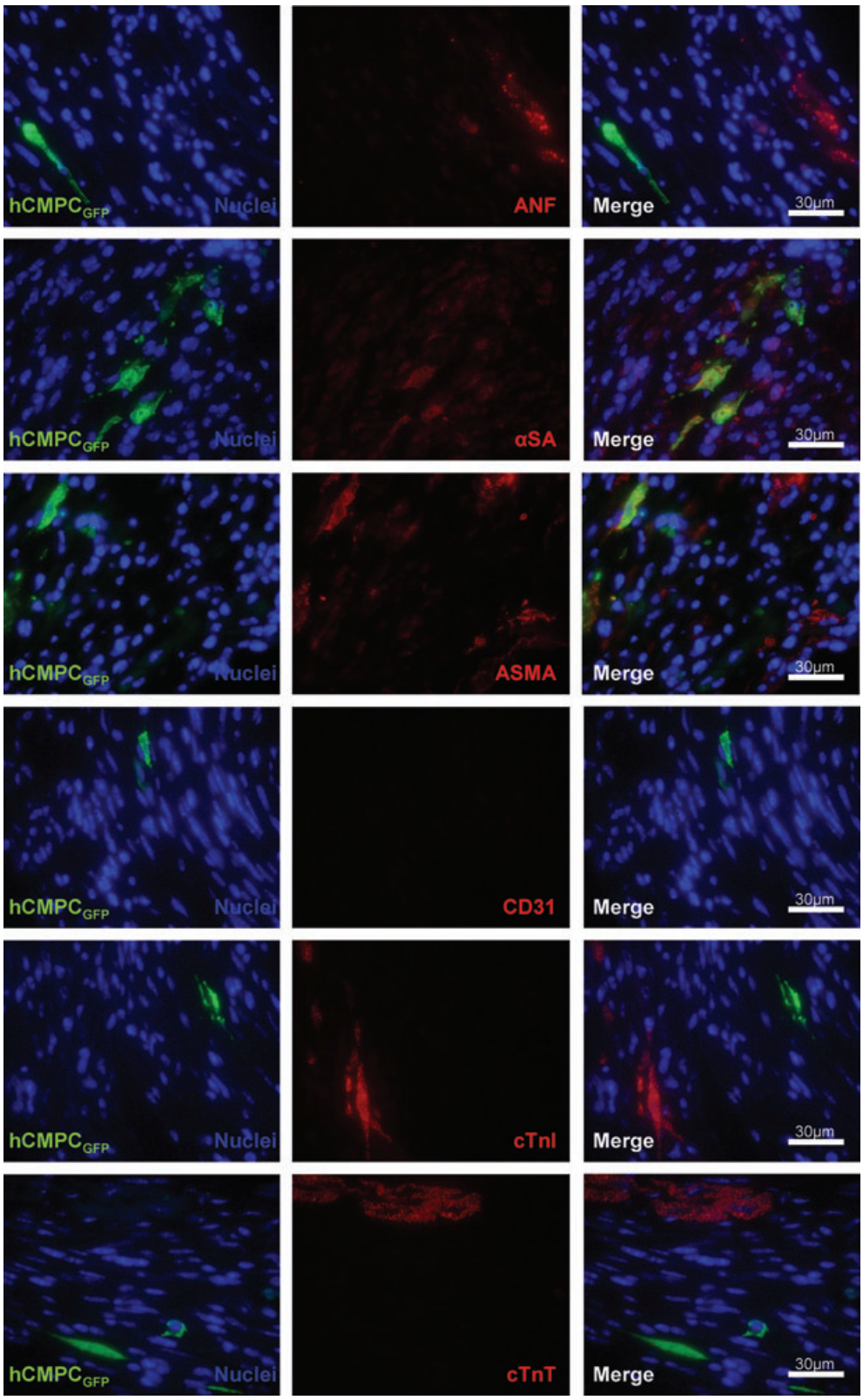


Fig. 4 Double immunofluorescent stainings for GFP and cardiac markers to assess the differentiation capacity of hCMPCs. The left column shows engrafted hCMPCs in green (Alexa 488) and nuclei in blue (Hoechst 33342), the middle column shows staining for the indicated cardiac marker (Qdot 655) and the right column is a merge of both images. hCMPCs show a diffuse staining for α-smooth muscle actin (ASMA) and α-sarcomeric (α-SA) actin 15 days after injection. Control immunostainings of human tissue sections for the human endothelial cell-specific marker CD31 were performed in parallel (data not shown).

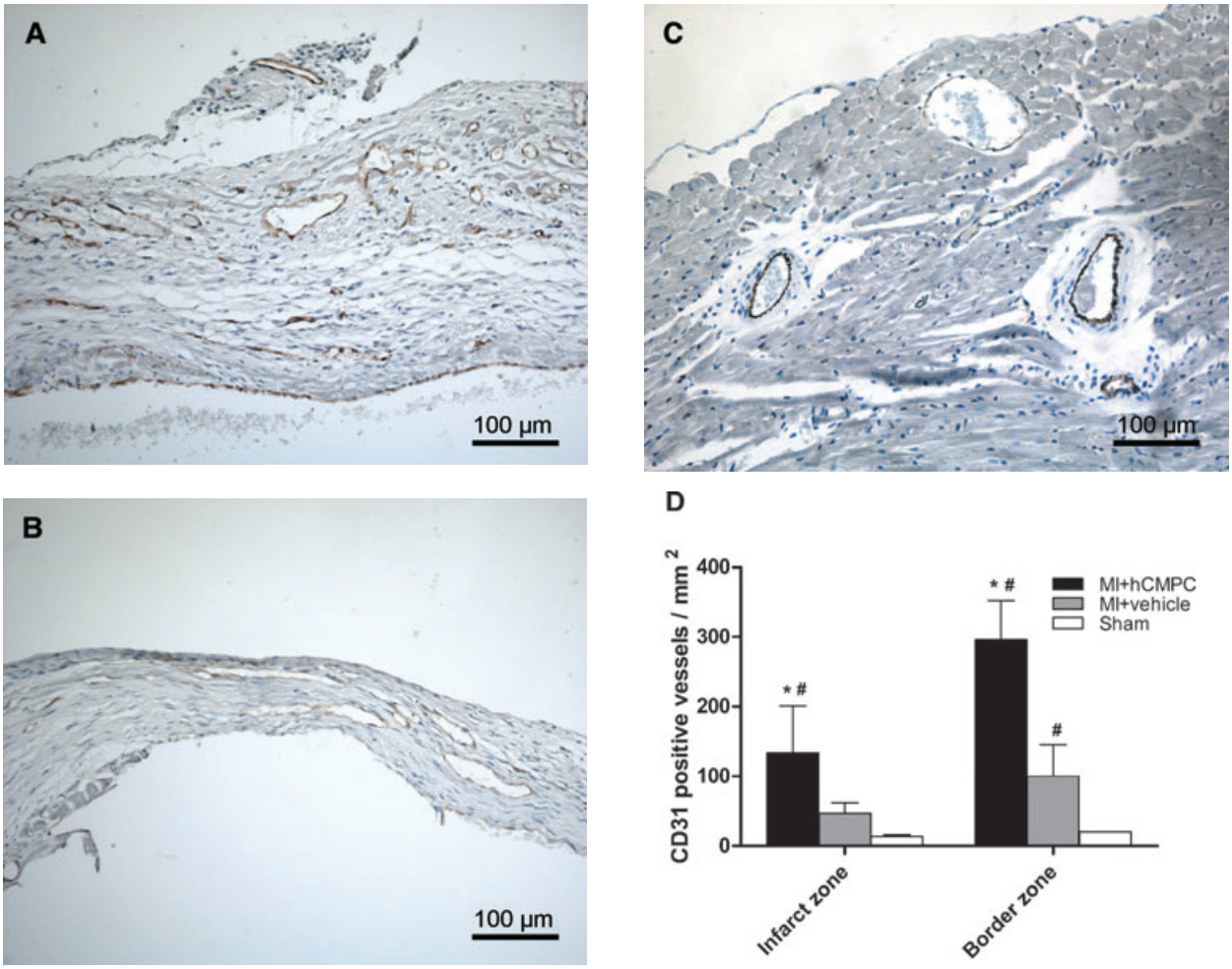


Fig. 5 Photomicrographs of representative sections of the LV wall 15 days after MI showing CD31-positive murine (*i.e.* host) vessels (brown). The vascularity in hearts that received hCMPCs (**A**), was higher than in hearts of vehicle-treated (**B**) and sham-operated mice (**C**). Quantification of CD31 staining (D). $*P < 0.05$ *versus* MI mice that received vehicle only. $\#P < 0.05$ *versus* sham-operated animals.

with a myocardial infarction. CD31-positive nuclei showed a comparable PCNA expression between all groups. Cardiac troponin I–positive nuclei had the lowest expression of PCNA, when compared to ASMA and CD31 staining, but were also comparable between all groups (Fig. 6E).

Human cardiomyocyte progenitor cells only suppress collagen type III density but do not decrease total collagen deposition after MI

Myocardial infarction was associated with increased total collagen density in the left ventricle, which treatment with hCMPCs could not prevent (Fig. 7A–D). Injection of hCMPCs only resulted in a significantly lower collagen type III deposition in the border zone and infarct region (Fig. 8A), when compared to mice treated with vehicle only (Fig. 8B–D) [23]. Quantification of LV wall thickness showed a significant thinning of the infarcted wall, when compared to sham-operated animals. A non-significant trend towards an attenuated process of wall thinning was observed in animals treated with hCMPCs, when compared to mice treated with vehicle ($P = 0.056$) (Fig. 7E).

Assessment of pulmonary congestion

Pulmonary water accumulation (wet weight–dry weight ratio) at day 15 was assessed for all experimental groups. In the MI + vehicle group there was a significant increase in lung fluid compared to the Sham group (0.19 ± 0.02 g *versus* 0.14 ± 0.01 g; $P < 0.05$). In contrast, in the MI + hCMPC group pulmonary water accumulation was not significantly different from that in the Sham group (0.15 ± 0.01 g *versus* 0.14 ± 0.01 g; $P =$ ns).

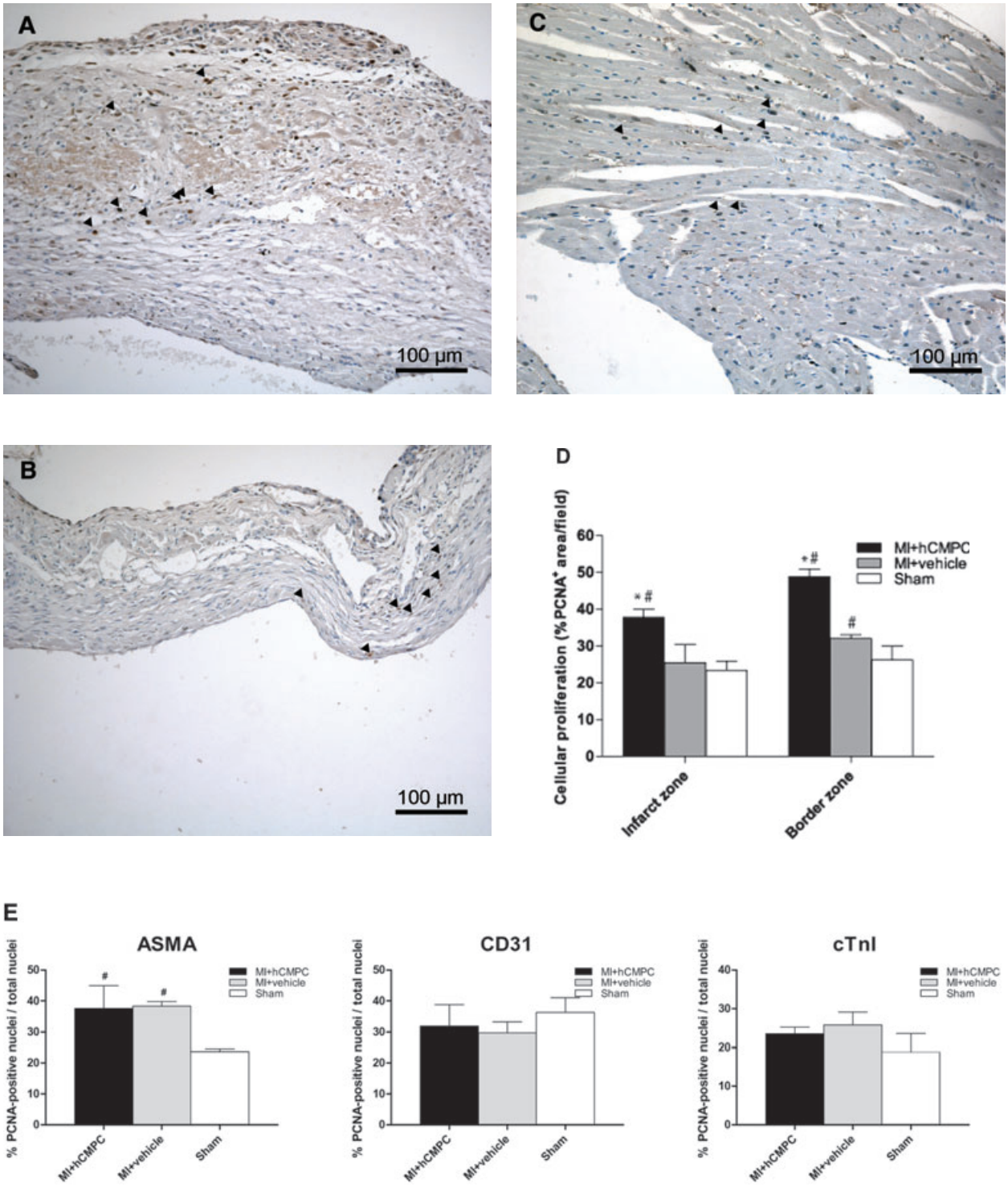
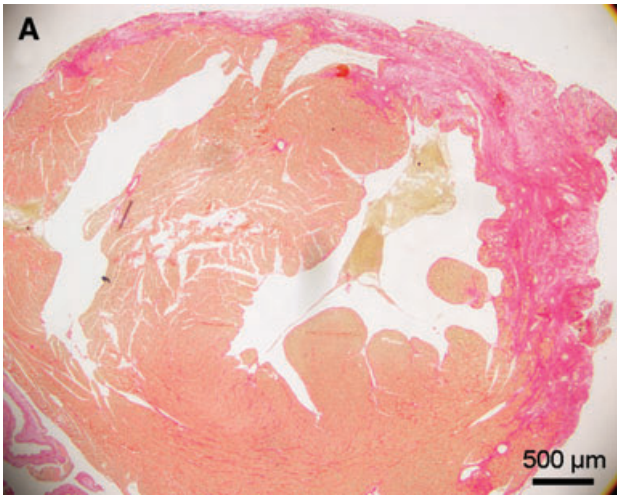
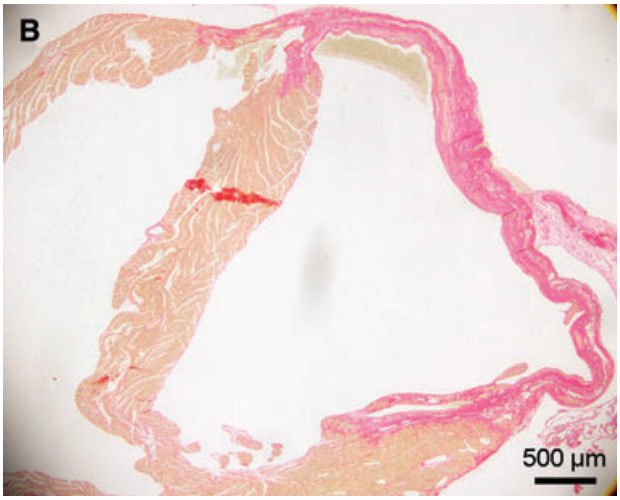
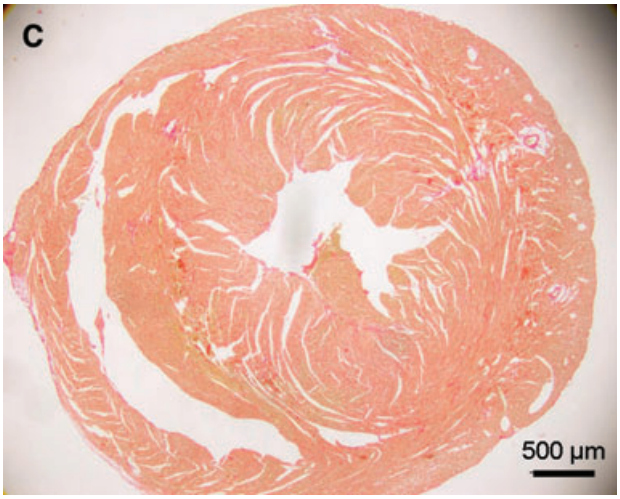
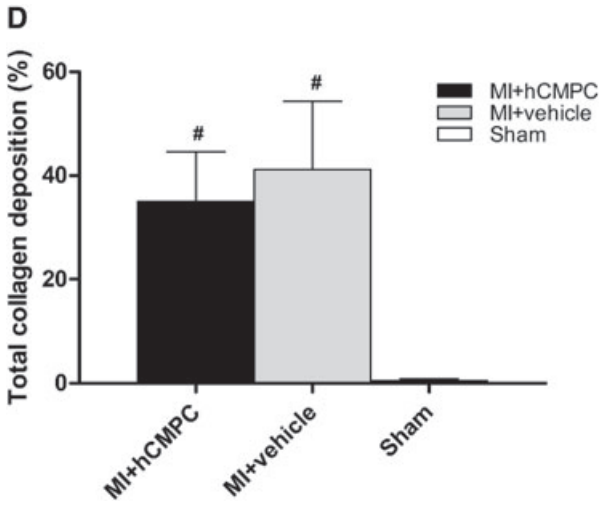


Fig. 6 Photomicrographs of representative sections of the LV wall 15 days after MI followed by immunostaining for the cellular proliferation marker PCNA (brown, marked with triangle). The number of PCNA-positive nuclei is augmented in hearts treated with hCMPCs (**A**), when compared to those of animals that received vehicle only (**B**) or were sham-operated (**C**). Quantification of PCNA staining (**D**). Quantification of double immunostainings for PCNA and ASMA, CD31 and cTnI (**E**).* $P < 0.05$ *versus* MI mice that received vehicle only. # $P < 0.05$ *versus* sham-operated animals.









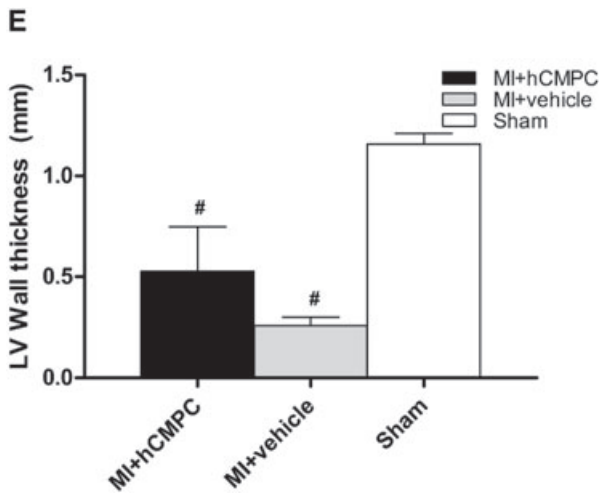


Fig. 7 Photomicrographs of representative sections of the heart 15 days after MI showing total collagen (red) by Sirius red staining. MI increased the total collagen deposition in the left ventricle in both animals treated with hCMPCs (**A**) and animals treated with vehicle only (**B**), when compared to sham-operated mice (**C**). Quantification of Sirius red staining (**D**). Quantification of LV wall thickness 15 days after MI shows a non-significant trend towards an attenuated wall thinning in animals treated with hCMPCs, when compared to animals treated with vehicle only ($P = 0.056$) (**E**). $\#P < 0.05$ *versus* sham-operated animals.

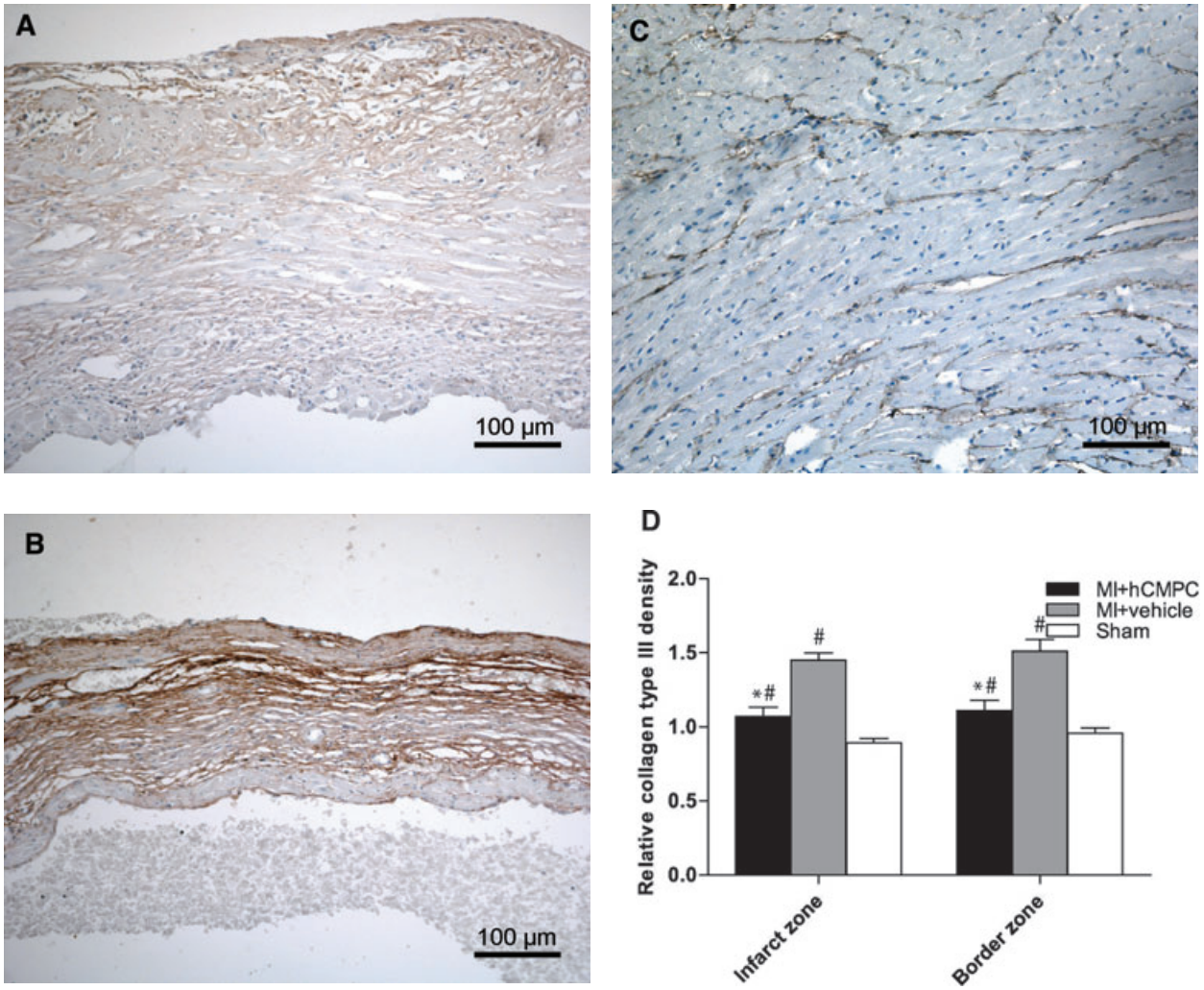


Fig. 8 Photomicrographs of representative sections of the LV wall 15 days after MI followed by immunostaining for the extracellular matrix component collagen type III. Collagen type III was less prominent in animals treated with hCMPCs (**A**), than in those that received vehicle only (**B**) while the hearts of sham-operated mice contained the lowest amount of collagen type III (**C**). Quantification of collagen type III staining (**D**). LV collagen type III intensity is expressed as ratio of the percentage of collagen type III–positive tissue in the left ventricle to that in the right ventricle of the same section. $*P < 0.05$ *versus* MI mice that received vehicle only. $\#P < 0.05$ *versus* sham-operated animals.

Discussion

The main findings of the present study are that 2 weeks after hCMPC injection in the infarcted heart of an immunocompromised mouse model we observe (*i*) significant preservation of LV systolic function, (*ii*) hCMPCs engraftment in the ischaemic area, without cardiomyogenic differentiation and (*iii*) attenuation of the adverse ventricular remodelling process, probably by paracrine factors. The present study therefore demonstrates the ability of hCMPCs to alleviate the deleterious effects of MI prior to their cardiomyogenic differentiation [16].

As Beltrami *et al.* proposed that cardiomyocytes may re-enter the cell cycle and undergo mitotic division, there has been discussion about the regenerative capacities of the heart [24]. Recent studies have confirmed this finding [25, 26], but it remains controversial whether the number of newly formed cardiomyocytes is actually sufficient to contribute to the injured myocardium [27]. Hsieh *et al.* suggested that progenitor cells might play a role in this process as they provided evidence that these cells may contribute to the process of cardiomyocyte renewal after injury of the heart [28]. However, the contribution of progenitor cells appears to be limited during normal ageing, as main cardiac regeneration then occurs through pre-existing cardiomyocytes [28]. In contrast to the small amount of regeneration that occurs in mammalian hearts, zebrafish are able of cardiac regeneration [29]. Recent studies showed an indisputable role for

pre-existing cardiomyocytes as cardiac renewal source, while the contribution of progenitor cells was minimal at best [30, 31]. Zuo *et al.* recently provided evidence for another important role for stem or progenitor cells in a rat myocardial infarction model. Injected MSCs released paracrine factors which acted on the native cardiomyocytes and resulted in less vulnerability to apoptosis [32]. This observed phenomenon of stem cell-mediated cardioprotection, has been reported earlier [33, 34]. Beltrami *et al.* demonstrated that the heart contains a pool of CPCs displaying endogenous regenerative potential [35]. So far CPC populations residing in postnatal hearts have been reported in rats [35], mice [36] dogs [34] and human beings [37]. These populations were identified by expression of several marker proteins, including c-kit [34, 36, 37], Isl-1 [38] and Sca-1 [39]. Few studies have described CPC isolation from human tissue. Messina *et al.* isolated clusters of fibroblast-like cells, termed cardiospheres, from human heart biopsies that expressed the endothelial markers KDR and CD31, as well as the stem cell markers CD34, Sca-1 and c-kit [37]. In co-culture with adult rat cardiomyocytes these cells differentiated into cardiomyocyte-like cells that displayed spontaneous beating [37]. Furthermore, intramyocardial injection of these cells in NOD/scid mice following induction of acute MI resulted in expression of endothelial, smooth muscle and cardiomyocyte markers with preservation of cardiac function [37, 40]. The observed improvement of cardiac function appears to be cell-type specific, as recent studies show that fibroblast are not able to improve cardiac function, in contrast to cardiac progenitor cells [40, 41].

Earlier and in the current study we were able to isolate hCMPCs from the human foetal heart. These cells are able to grow in culture for at least 25 passages [42]. We have previously reported that when hCMPCs are stimulated with 5-azacytidine and TGF-β, spontaneously beating cardiomyocytes can be identified within the culture after approximately 3 weeks [15]. Both undifferentiated hCMPCs and beating hCMPC-derived cardiomyocytes were intramyocardially transplanted in an animal model with long-term follow-up. Both cell types preserved cardiac function and underwent phenotypic changes *in vivo*, including expression of sarcomeric proteins. Twelve weeks after injection, intramyocardial human grafts expressed troponin I and myosin light chain 2a [16].

Having established that intramyocardially injected undifferentiated hCMPCs spontaneously differentiated into cardiomyocytes *in vivo* after 12 weeks and prevented deterioration of cardiac function, we now studied the short-term effects of the intramyocardial administration of undifferentiated hCMPCs in a mouse myocardial infarction model.

Left ventricular function of the heart was assessed by MRI and PV measurements [20]. Human cardiomyocyte progenitor cell transplantation significantly preserved the systolic function of the LV, as the increase in LVESV and the deterioration of LVEF after MI were attenuated in the MI + hCMPC group in comparison to the MI + vehicle group. Pressure–volume relationships showed a significantly lower LVEDV in the MI + hCMPC group than in the MI + vehicle group. Injection of hCMPCs also resulted in a significant decrease in the relaxation time constant, indicating a faster isovolumic relaxation (*i.e.* improved diastolic function). These findings support the concept that transplantation of hCMPCs causes attenuation of the pathological remodelling process that normally occurs after MI. The preservation of cardiac function that was observed in the present short-term study was previously found to be sustained at 4 and 12 weeks after MI [16].

A significant accumulation of lung fluid, indicative of pulmonary congestion, occurred in the MI + vehicle group only, suggesting that symptoms of overt LV failure remained absent in the MI + hCMPC group. Although we hypothesized that hCMPCs may differentiate into functional cardiomyocytes *in vivo* after 2 weeks, we only detected a diffuse staining pattern of α-SA and expression of ASMA in injected hCMPCs. As no fully developed cardiomyocytes with sarcomeric cross-striation were observed, an active contractile contribution of the hCMPCs is unlikely. Instead, the observed beneficial effect of hCMPCs transplantation on cardiac function and structure are considered to result from paracrine pathways.

Nagaya *et al.* have demonstrated that intramyocardial transplantation of rat MSCs improved cardiac function in a rat model of dilated cardiomyopathy, which was associated with increased capillary density and secretion of angiogenic factors, including vascular endothelial growth factor A (VEGF-A), hepatocyte growth factor and adrenomedullin [8]. In the present study, we also observed increased vascular density in both the infarct and the border area of the hCMPC-injected hearts when compared to mice treated with vehicle only [16]. Chimenti *et al.* injected human cardiosphere-derived cells intramyocardially after MI and observed an increase in vascular density, that was mainly due to paracrine effects as only a small number of vessels was derived from human donor tissue [41].

As in the present study no hCMPCs were observed in the vascular linings, we conclude that injected hCMPCs have stimulated neovascularization in the infarcted heart in a paracrine fashion. This coincides with the previous finding that undifferentiated hCMPCs excrete VEGF-A, a potent stimulator of angiogenesis [43, 44].

Proliferating cell nuclear antigen acts as a processivity factor for DNA polymerase δ by encircling the template DNA and is involved in nuclear DNA synthesis and repair. As the PCNA content of cells changes during the cell cycle, reaching a peak at the G1 to S phase transition, it can be used as a marker for cell proliferation [45]. In the present study PCNA up-regulation most likely reflects an increase in cellular proliferation rather than DNA repair, since the conditions affecting DNA damage do not differ between treatment arms. Proliferating cell nuclear antigen was up-regulated in cells of donor and recipient origin in the infarct and border zone of the heart, indicating increased cellular proliferation in these regions. Double immunostainings revealed that PCNA expression was equally present in endothelial cells and cardiomyocytes in all groups. However, ASMA expressing cells showed a significantly higher PCNA expression in the myocardial infarction group, which is in line with previous studies [46, 47]. After myocardial infarction scars were shown to undergo rapid changes in their content of myofibroblasts, where during the proliferative phase fibroblasts undergo phenotypic changes leading to expression of contractile

proteins such as ASMA [47]. Hatzistergos *et al.* have demonstrated that injection of MSCs in the infarcted heart increased the number of mitotic endogenous cardiomyocytes. This significantly higher level of host cardiomyocyte turnover reached its maximum at 2 weeks and decreased to normal levels by 2 months [48]. Following intracoronary administration of CPCs in a MI/reperfusion model, Tang *et al.* also demonstrated increased proliferation of especially the transplanted cells. Moreover, the injection of CPCs induced division of endogenous CPCs in both infarcted and non-infarcted areas [49].

In the present study, we observed that total collagen deposition in the left ventricle increased substantially after MI. Treatment with hCMPC could not inhibit overall myocardial fibrosis, but attenuated the collagen type III density in the infarcted heart. Collagen type III is a constituent of the extracellular matrix and, together with collagen type I, an important contributor to the pathological heart remodelling after MI and the consequential loss of cardiac function [23]. As both the LVESV and LVEDV were better preserved in mice treated with hCMPCs, this may be partly explained by this observed phenomenon.

Berry *et al.* demonstrated earlier the potential of stem cell therapy in the prevention of cardiac remodelling in a myocardial infarction model. Intramyocardially injected MSCs preserved cardiac function after MI by inhibiting fibrosis and LV dilatation, thereby conserving myocardial thickness 8 weeks after MI [11]. Mesenchymal stem cells did not differentiate into cardiomyocytes, but attenuated the remodelling process conceivably through paracrine effects [11].

Ever since stem cell therapy has emerged as a putative treatment for ischaemic heart disease, low engraftment rates of the delivered cells have remained an issue. A limitation of this study is the low observed engraftment rate of the cells 15 days after injection. Direct intramyocardial injection ensures targeted delivery of the cells into the ventricular wall. However, a significant portion of the injected cells wash away by blood flow or are lost by leakage from the injection site [50–52]. But when comparing the intramyocardial injection method with intracoronary or intravenous delivery methods, intramyocardial injection has a preference in case of higher engraftment [53, 54], reduction of infarct size [55] and repairing injured myocardium [54]. Future studies are necessary to further improve acute cell retention and engraftment, thereby increasing the beneficial effects of injected cells.

An alternative to the cell injection approach is mobilization of stem cells to the site of injury [56]. The importance of the presence of injected cells at the site of injury is clearly demonstrated by a recent study from Huber *et al.* Intraperitoneal administration of parathyroid hormone (PTH) increased the mobilization of BMCs and homing of these cells towards the ischaemic myocardium, when compared to control animals. Altogether this attenuated the cardiac remodelling process and enhanced cardiac function in animals which received PTH [57]. Furthermore, a recent study from Theiss *et al.* provided evidence that mobilization and homing of BMCs can be improved by granulocyte-colony stimulating factor (G-CSF) application and dipeptidylpeptidase IV (DPP-IV) inhibition. Homing cells were able to improve myocardial perfusion and attenuated the process of cardiac remodelling. The combination of G-CSF treatment and DPP-IV inhibition, a dual stem cell based therapy, also increased the pool of resident cardiac stem cells [58], which may contribute to the process of cardiomyocyte renewal after injury of the heart [28], an important mechanism which may also play a role in the current study.

So far many small and large animal studies have been performed, generating excitement and prompting investigators to translation of these results into the clinic. The results from placebo controlled trials show that the use of autologous and allogenic BM-derived cells is safe, and overall stem cell therapy positively affects cardiac function, suggesting that improvement over existing pharmaceutical therapy can be achieved [59]. A more recent meta-analysis by Wen *et al.* already suggests that direct injection of BM-derived cells has beneficial effects on cardiac function over regular therapy alone [60]. Cardiac stem cell therapy with hCMPC transplantation most likely requires autologous stem cells, since the immune status of cardiac progenitor cells has not been thoroughly investigated yet. Whether injection of hCMPCs has extra beneficial effects in comparison to conventional pharmaceutical treatment in patients with ischaemic heart disease is a question that remains unanswered so far.

In conclusion, transplantation of hCMPCs into the infarcted heart limits deterioration of cardiac function and attenuates the cardiac remodelling process already 2 weeks after injection. As the hCMPCs had not differentiated into cardiomyocytes at this time point, paracrine stimulation of neoangiogenesis and cell proliferation are considered key factors in preserving short-term cardiac function. These results demonstrate the potential of hCMPCs to treat the infarcted heart.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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