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Early homing of adult mesenchymal stem cells in normal and infarcted isolated beating hearts

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Abstract

Little is known on the early homing features of transplanted mesenchymal stem cells (MSCs). We used the isolated rat heart model to study the homing of MSCs injected in the ventricular wall of a beating heart. In this model all types of cells and matrix elements with their interactions are represented, while external interferences by endothelial/neutrophil interaction and neurohormonal factors are excluded. We studied the morphology and marker expression of MSCs implanted in normal hearts and in the border-zone of infarcted myocardium. Early morphological adaptation of MSC homing differs between normal and infarcted hearts over the first 6 hrs after transplantation. In normal hearts, MSCs migrate very early through the interstitial milieu and begin to show morphological changes. Yet, in infarcted hearts MSCs remain in the site of injection forming clusters of round-shaped cells in the border-zone of the infarcted area. Both in normal and infarcted hearts, immunohistochemistry and confocal imaging showed that, besides the proliferative marker proliferating cell nuclear agent (PCNA), some transplanted cells early express myoblastic maker GATA-4, and some of them show a VWF immunopositivity. Moreover, a few hours after injection connexin-43 is well evident between cardiomyocytes and injected cells. This study indicates for the first time that the isolated beating heart is a good model to study early features of MSC homing without external interferences. The results show (*i*) that MSCs start to change marker expression few hours after injection into a beating heart and (*ii*) that infarcted myocardium influences transplanted MSC morphology and mobility within the heart.

Keywords: isolated beating heart • stem cell homing • myocardial injuries • GFP

Introduction

The last few years have seen a surge of interest for the possibility to use stem cell (SC) transplants to repair damaged hearts [1–5]. A wide range of stem/progenitor cell types have been used for cardiac cell therapy, including myoblasts and bone marrow SCs [3, 6]. However, very little is known about the early features of the homing of SC in the receiving heart. In a recent review, Dimmeler *et al.* [6] states that 'the mechanisms for progenitor cells' homing to sites of tissue injury are only understood rudimentarily'. In particular, nothing is known about early (first few hours) cell survival and distribution in

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the receiving beating heart. Thus, many issues remain to be clarified on the process following mesenchymal stem cells (MSCs) implantation into the myocardium [7].

We decided to use the isolated beating heart preparation as a model to throw some light on the early stages of MSC migration, survival and homing in normal and infarcted myocardium.

At present, different methods have been used for homing/integration studies. The common methods are based on experiments *in vitro* in which SCs are co-cultured with different cell types, and *in vivo* where SCs are injected intravascularly or directly into the studied organ [2, 3, 6, 8–11]. However, both techniques (*in vitro* and *in vivo*) have disadvantages. For instance, *in vitro* co-culture cannot include all types of cells and matrix elements that interact in the heart. Moreover, usually myocardiocytes do not contract when in culture or co-culture. We recently reported that MSCs show a limited plasticity when co-cultured with non-beating adult cardiomyocytes [12]. Other results with adult primary-cardiomyocytes and MSCs were controversial and some studies obtained better results if the cardiac cells co-cultured with MSCs were immature [8, 13–15].

In addition to the fact that *in vivo* the early cellular mechanisms involved in the interaction between MSCs and recepient's myocardium have not yet been described it should be considered that the *in vivo* scenario does not allow an adequate comparison between non-ischaemic and post-ischaemic hearts, since in the latter external 'disturbing' events such as endothelial/neutrophil interaction and neurohormonal responses can interfere with differentiation, homing and integration of MSCs [16]. For instance, in myocardial infarction adrenergic discharge and angiotensin release are greatly increased. Therefore, it seems necessary to have an experimental 'clean' condition, in which all types of cells and matrix elements with their interactions are represented, while external 'disturbing' events are excluded or, if necessary, included under a well-defined experimental control.

Since the isolated beating heart perfused with an oxygenated buffer solution instead of blood can provide sufficient 'clean' conditions of investigation, we used this experimental model to study the expression of differentiation/proliferation markers and the morphological aspects of the homing of bone marrow MSCs. We obtained these cells from green fluorescent protein (GFP) stably transfected adult rats. The cells from these animals can be easily recognized in the receiving organ because of their well evident green autofluorescence. Using immunohistochemistry, light and confocal microscopy we studied the early changes induced by host myocardial environment on implanted MSC either in normal or infarcted hearts.

We found that migration and integration occurs earlier in a non-ischaemic heart, whereas MSCs form clusters around the infarcted areas of post-ischaemic hearts. Nevertheless, in both conditions (post-ischaemic and non-ischaemic hearts), few hours after implantation MSCs express new markers of differentiation, which co-exist with the markers already present in cultured cells.

Materials and methods

Isolation of stem cells and cell cultures

To allow grafted cells to be identified in the recipient tissue, donor cells were obtained from transgenic rats expressing the enhanced-GFP under the control of the cytomegalovirus enhancer and the chicken β-actin promoter derived from an expression vector, pCAGGS [17, 18].

MSCs were harvested from femur bone marrow of GFP stably transfected adult rats (body-weight 450–550 g). The rats were housed in plastic cages and were allowed to access water and a standard rodent diet *ad libitum*. The animals received care in accordance with the Italian law (DL-116, Jan. 27, 1992), which complies with the Guide for the Care and Use of Laboratory Animals by the US National Research Council. In brief, they were sacrificed by decapitation after anaesthesia with urethane (1 g/kg i.p.). MSC-GFP were extracted by inserting a 21-gauge needle into the shaft of the bone and flushing it with 30 ml of complete α-modified Eagle's medium (α-MEM) containing 20% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were filtered through a 70-μm nylon filter (Falcon; Franklin Lakes, NJ, USA). The cells from one rat were plated into one 75 cm^2 flask. They were grown in complete α-MEM containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2.

The medium was then replaced with fresh medium and the adherent cells were grown to 90% confluence to obtain samples here defined as passage zero (P0) cells. The P0 MSCs were washed with phosphate-buffered saline (PBS) and detached by incubation with 0.25% trypsin and 0.1%

ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO, USA) for 5–10 min. at 37°C. Complete medium was added to inactivate the trypsin. The cells were centrifuged at 900 × *g* for 5 min., resuspended in 1–10 ml of complete medium, counted manually in duplicate using a Bürker's chamber, and plated as P1 on 58 cm^2 plates (Falcon). Complete medium was replaced every 3–4 days over the 18- to 24-day period of culture (P1–P6) [19, 20].

To verify that the cell population we used for transplantation was composed of MSCs, we showed that the cells were CD90 positive and CD34 negative [21]. Moreover, a differentiation experiment was carried out by adding to culture medium 1 μM Dextamethasone, 1.7 μM insulin, 0.5 mM isobutyl-methylxanthine for 5 days. Under these experimental conditions, it was shown that MSCs differentiated into adipocytes [21].

Isolated hearts

Male Wistar rats ($n = 22$; body-weight 450–550 g) were heparinized (2.500 U. i.m.) and anaesthetized with urethane (1 g/kg i.p.) 10 min later. Then, the hearts were rapidly excised and attached to a perfusion apparatus and retrogradely perfused with oxygenated Krebs-Henseleit buffer containing (in mM) 127 NaCl, 17.7 $NaHCO_3$, 5.1 KCl, 1.5 $CaCl_2$, 1.26 $MgCl_2$ and 11 D-glucose, supplemented with 5 μg/ml lidocaine and gassed with 95% O_2 and 5% CO_2. To detect ventricular and coronary perfusion pressure, the hearts were instrumented as previously described [22–24].

A constant flow was adjusted with a proper pump (Watson-Marlow 313, Falmouth, Cornwall, UK) to obtain a typical coronary perfusion pressure of 80–85 mm Hg during the initial part of stabilization. Thereafter, the same flow level (9 ± 1 ml/min/g) was maintained throughout the experiment. Also temperature of perfusate and hearts was strictly controlled and kept constant (37°C) throughout the experiments. Under these experimental conditions, rat hearts beat spontaneously and regularly for about 6–7 hrs after isolation. After then, heart function begins to be progressively impaired and, for this reason, 6-hrs is the ultimate end-point of the isolated heart model.

In group 1 (normal hearts, $n = 11$), after a period of stabilization (20 min.), MSC-GFP were injected in the anterior part of the free wall of the left ventricle in the area perfused by the left descending coronary artery (LDCA).

In group 2 (infarcted hearts, $n = 11$), after a period of stabilization (20 min.), the LDCA was occluded for 30 min. followed by 30 min. of reperfusion before the MSC-GFP were injected. The occlusion of the LDCA was obtained with a ligature around the artery. The ligature was then loosened for full re-flow.

Hearts of both groups were removed from the apparatus for histological fixation (see below) at four different times after MSC injection (5 min., 2 hrs, 4 hrs, 6 hrs after injection).

All the buffer compounds were obtained from Sigma (St Louis, MO, USA). Heparin was obtained from Roche (Milan, Italy).

MSCs transplantation

The MSCs at P6 were washed with PBS and detached by incubation with 0.25% trypsin and 0.1% EDTA (Sigma, St Louis, MO, USA) for 5–10 minutes at 37°C. Complete medium was added to inactivate trypsin. The cells were centrifuged at 900 × *g* for 5 min. and counted using a Bürker's chamber. Then 1×10^6 cells were resuspended in 300 μl of PBS. In the heart of group 1, these cells were then injected in the wall of the left ventricle with a 27.5-gauge needle. In the hearts of group 2, the injection was performed in the wall of the left ventricle close to the border zone of the previously ischaemic area. In this model the ischaemic area is easily recognizable as it turn pale-coloured in a few seconds after coronary occlusion. Ischaemia was confirmed by a drop in left ventricular developed pressure.

Light and confocal imaging

Tissues were fixed with 4% paraformaldehyde for 2 hrs and then washed in a solution of 0.2% glycin in 0.1 M phosphate buffer (pH 7.2) for 30 min. Then the tissues were embedded in increasing solutions of sucrose (7.5% for 1 hr, 15% for 1 hr, 30% over-night) in 0.1 M phosphate buffer and then in a solution 1:1 of PBS/sucrose 30% and OCT for 30 min. Finally, the tissues were embedded in 100% optimal cutting temperature (OCT) and stored at –80°C.

The whole heart was cut in 10 μm thick slices transversally to the axis and starting from the apex. All sections were analysed by confocal laser microscopy (see later on for technical details) to detect the presence of GFP-positive transplanted cells. On the sections where GFP-positive cells were found, either double immunofluorescence or haematoxylin and eosin staining were carried out as described below. Although the objective of this analysis was to describe qualitatively the changes occurring to transplanted MSCs, in two hearts (one normal and one infarcted, at 4 hrs after injection) quantitative estimation of the volume of cell migration was also carried out by the Cavalieri method [25]. In one section out of each 50 sections, the area of migration was measured on reconstructed pictures of the whole heart profile (Fig. 3). Then, the mean area was finally multiplied by the number of sections obtaining thus an estimation of the entire myocardial volume through which GFP-positive cells migrated.

For double immunofluorescence, the sections were rinsed in PBS, blocked with normal goat serum (NGS, 1%) for 1 hr and then incubated overnight with primary antibodies against CD90 (Thy-1.1 mouse, monoclonal, 1:50, BD Pharmingen), Connexin-43 (rabbit, polyclonal, 1:400, Sigma, St. Louis, MO, USA), GATA-4 (rabbit, polyclonal, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA), PCNA (mouse, monoclonal, 1:1000, Sigma, St. Louis, MO, USA), α-actinin (mouse, monoclonal, 1:800, Sigma, St. Louis, MO, USA), troponin T (mouse, monoclonal, 1:50, Lab Vision Corporation, Westinghouse, CA, USA), Von Willebrand Factor (VWF) (rabbit, policlonal, 1:200, Sigma, St. Louis, MO, USA).

After washing in PBS, sections were incubated with TRITC antimouse IgG (Dako, Milano, Italy) or CY3 anti-rabbit IgG (Jackson Immuno Research, Cambridgeshire, UK) for 1 hr at room temperature. Sections were finally washed again in PBS and mounted with a fluorescent mounting medium (Dako, Milano, Italy).

The slides were observed with a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany), which incorporates two lasers (Argon and HeNe) and is equipped with an inverted Axiovert 100M microscope. Confocal fluorescence images were taken using a 20× Plan-NEOFLUAR objective with a numerical aperture (NA) of 0.50 and a 40× Plan-NEOFLUAR objective with a NA of 0.75. An electronic zoom with a magnification ranging from 1× to 8× was employed to obtain the magnifications indicated in each figures. To visualize GFP fluorescence, we used excitation from 488-nm Argon laser line and emission passing through a band-pass (BP) 505–530 filter passing wavelengths 505 nm to 530 nm to the detector. Images created with the BP 505–530 filter were digitally coloured green. To visualize TRITC and CY3 red fluorescence we used excitation from 543-nm HeNe laser line and emission passing through a high-pass filter (LP 560) which passes wavelengths superior to 560 nm to the detector. Images created with the BP 505–530 filter were digitally coloured green. Images created with the LP 560 filter were digitally coloured red.

After confocal observation and digital image acquisition, the slides with evidence of GFP-positive cells were stained with haematoxylin and eosin, observed and photographed with a DM4000B microscope equipped with a DFC320 digital camera and a IM50 image manager system (Leica Microsystems, Wetzlar, Germany). Finally, the fluorescent and light microscope images were superimposed to allow an accurate morphological description of GFP-positive cells.

In addition, to verify that the observed fluorescence was not due to the tissue autofluorescence of the heart, some slides were immunostained with an anti-GFP antibody (rabbit, polyclonal, 1:100, Abcam, Cambridge, UK) and then incubated with an anti-rabbit biotinylated secondary antibody (Dako, Milan, Italy). Sections were then processed with peroxidase-conjugated Vectastain ABC kit (Vector, Burlingame, CA, USA), revealed with diaminobenzidine (Sigma, St Louis, MO, USA), and observed and photographed with the DM4000B light microscope.

Assessment of antibody specificity

For specificity assessment, all antibodies were checked by secondary antibody labelling with omission of the primary antibody, a procedure that led to no immunopositivity. In addition, we have also carried out a 'morphology-based specificity test' [26], consisting of the same immunolabelling protocol to stain sections from other tissues or cells in which antigens are known to be present with a clear localization easily detectable from a morphological viewpoint. Normal heart was used for specificity assessment of connexin-43, VWF, α-actinin and troponin T and normal small intestine for a-PCNA because of the presence of the easily detectable pool of fast dividing crypt cells. Cultures of MSCs were used for specificity assessment of a-CD90 and cultured myoblasts (H9C2 cell line, ECACC, UK) for a-GATA-4. All these tests supported the specificity of each reagent by demonstrating that it specifically labels only the tissue elements where the immunogen is known to be present (data not shown).

Results

Figure 1 illustrates the appearance of suspended GFP-positive MSCs before (Fig. 1A and B) and 5 min. after transplantation (Figs 1C–E) in a normal heart. The strong green autofluorescence makes transplanted MSCs easily recognizable inside the injection part of myocardium where they are concentrated (Fig. 1C). Moreover, in the same slide haematoxylin and eosin staining reveals the clear morphological difference between MSCs (*e.g.* basophil cytoplasm) and the surrounding cardiomyocytes (*e.g.* eosinophil cytoplasm) (Fig. 1D). Finally, transplanted MSCs were also easily recognizable after anti-GFP immunostaining (Fig. 1E).

Figures 2, 3 and 4 illustrate the observations made on hearts removed 2, 4 and 6 hrs after MSC transplantation.

The pattern of migration inside the host tissue (Figs 2 and 3) and the acquisition of a new morphological phenotype (Fig. 4) revealed the different influence of normal and infarcted hearts on MSCs homing.

In regards to migration, our observations showed that in normal hearts (Figs 2A–C and Fig. 3A) MSCs

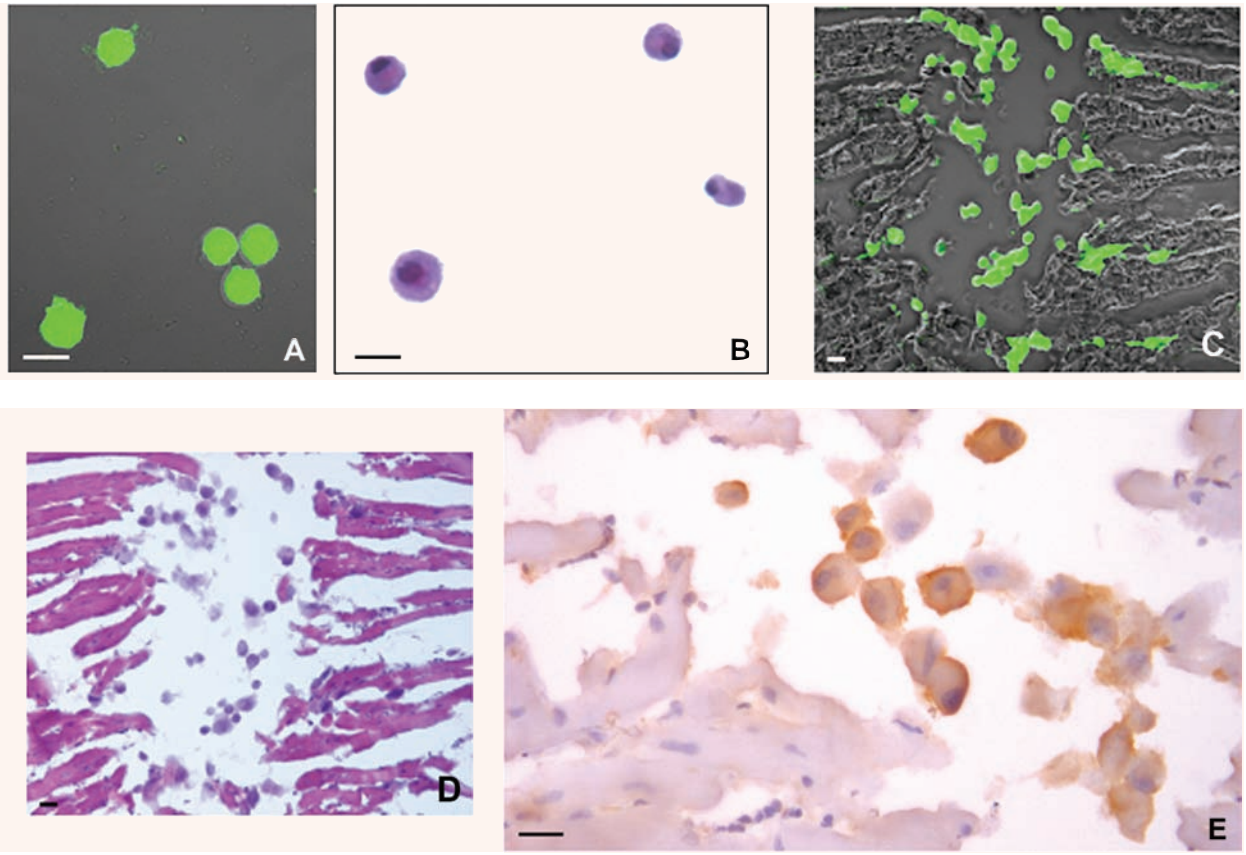

Fig. 1 Images of suspended green fluorescent protein (GFP)-positive mesenchymal stem cells (MSCs) before (**A, B**) and 5 min. after transplantation (**C–E**) in a normal beating heart. (**A, C**) Direct images of fluorescence merged with contrast; (**B, D**) Haematoxylin/eosin staining; (**E**) Peroxidase of a-GFP antibody contrastained with haematoxylin. After haematoxylin/eosin staining MSCs appear with a basophil cytoplasm while the surrounding cardiomyocytes have an eosinophil cytoplasm. Scale bars: 20 μm.

migrated very early (2 hrs) in the myocardium (Fig. 2A) spreading throughout a large myocardial volume ($1.22 \pm 0.16 \times 10^{12}$ μm^3) within 4–6 hrs (Fig. 2B and C, Fig. 3A). In contrast, in infarcted hearts most MSCs remained concentrated in clusters located near the site of injection (Figs 2D–F, Fig. 3B) occupying a myocardial volume 1000 times smaller than in normal hearts ($1.01 \pm 0.26 \times 10^9$ μm^3) (Fig. 3B).

The acquisition of a new morphological phenotype by the transplanted cells in non-ischaemic heart is shown in Figure 4. Starting 2 hrs after transplantation, some MSCs still maintained the original histological aspect (Figs 4A–D), while others began to acquire new morphological features, in particular a plasma eosinophilia similar to that of the surrounding cardiomyocytes (Fig. 4E, F asterisks). This was not the case of infarcted myocardium (data not shown).

Anti-PCNA immunostaining showed that some of the MSCs continued to proliferate after 6 hrs of transplantation both in normal and in infarcted hearts (Fig. 5).

Sequential direct GFP confocal imaging followed by immunostaining with anti-GFP antibody (Fig. 6) shows that the observed fluorescence was not due to autofluorescence of cardiomyocytes.

Despite the fact that changes in the morphological phenotype demonstrated by histological examination were observed in the normal hearts only (Fig. 4), confocal imaging after a-connexin-43 (Fig. 7) and a-GATA4 (Fig. 8A and B) immunolabelling showed similar signs of differentiation of transplanted MSCs in

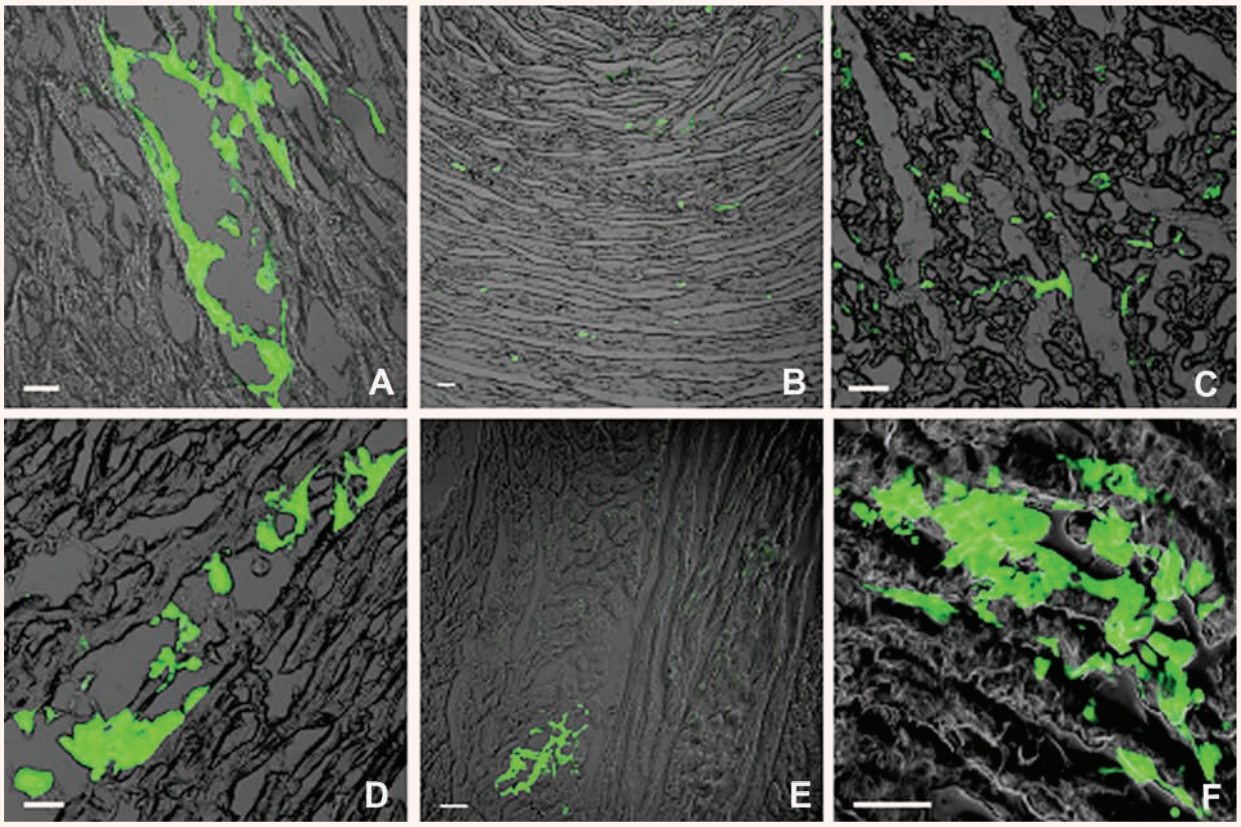

Fig. 2 Confocal imaging in normal (**A–C**) and ischaemic (**D–F**) hearts withdrawn 2 (**A,D**), 4 (**B,E**) and 6 (**C,F**) hours after GFP-positive MSCs transplantation. In normal hearts, MSCs migrate very early in the myocardium while in infarcted hearts most MSCs remain concentrated in clusters. Scale bars: **A, B, E**: 50 μm; **C, D, F**: 20 μm.

both normal and ischaemic heart. In myocardium of both normal (Fig. 7A–C). and infarcted hearts (Fig. 7D and E), connexin-43, a protein that is mainly localized in the intercalated disks (Fig. 7 arrows), was present between resident cardiomyocytes and transplanted MSCs only 2 hrs after transplantation. It is noteworthy that, though the location was different, even in P6 cultured MSCs we detected the presence of a-connexin-43 (Fig. 7F–H) as also reported by Gallo *et al.* [12] in P3-P10 MSCs.

In some MSCs, transplanted in both normal (Fig. 8A) and infarcted (Fig. 8B) hearts, nuclear a-GATA-4 immunolabelling confirms the presence of differentiation towards cardiomyocyte phenotype. It is noteworthy that a-GATA-4 immunolabelling does not label cultured MSCs (data not shown).

Transplanted MSCs are negative for a-α-actinin and a-troponin T, two important myocardial markers (Fig. 8C and D) in both normal and infarcted hearts. By contrast, a-VWF immunostaining was detected in some injected MSCs only in normal (Fig. 9A and B) and not in infarcted hearts (Fig. 9C and D).

Results of a-CD90 immunostaining in normal (Fig. 10A) and infarcted (Fig. 10B) hearts showed that most of the MSCs still maintain CD90-immunopositivity. Figure 10C shows a long chain of cells well integrated in the surrounding myocardium of a normal heart. These cells, which are still CD90 positive (Fig. 10E and F), are recognized as transplanted MSCs for the strong green autofluorescence (Fig. 10D and F).

Discussion

Since the initial phase after injection is critical for cell survival [27], adequate knowledge of the early homing of MSCs in the infarcted myocardium may improve the

outcome of SCs therapy which so far has been known to produce controversial results [1, 4, 5, 28].

Our results show that isolated heart model can be a simple and effective method suitable to study early SC survival and distribution in a beating heart, both in the presence and absence of myocardial infarction.

We show that implanted MSCs start to change marker expression a few hours after they are transplanted in a beating heart. Moreover we show that infarcted myocardium influences implanted MSC morphology and mobility within the heart.

Arrested (non-beating) isolated hearts have been used by Anversa's group to asses intramilieu migration of *in vivo* injected SCs using two-photon confocal microscopy [29]. In the present investigation, explanted normal and infarcted isolated beating hearts were used for the first time to study the early aspects of homing of MSCs injected in the left ventricular wall.

GFP-stable transfected MSCs were used as easy to track when surrounded by other types of cells. In fact, the a-GFP antibody confirmed that the fluorescence was due to the actual presence of the protein into MSCs and not to autofluorescence of cardiomyocytes [30].

In this model, endothelial/neutrophil interaction and neurohormonal influences can be either excluded or, if necessary, included under proper control if required by the experimental protocol. In our protocol the model was used in that it offered the possibility to study and compare the homing in normal and infarcted beating hearts without any extracardiac interference.

It is surprising that in all normal hearts, MSCs migrate through the interstitial milieu very rapidly and, in some cases, acquire a new morphological phenotype similar to the surrounding cardiomyocytes already a few hours after transplantation. In fact very early after the injection in a beating heart, some MSCs (*i*) acquire an eosinophil aspect, (*ii*) align with actual cardiomyocytes in contact with them in the sites where cardiomyocytes expose connexin-43 and (*iii*) express the differentiation marker GATA-4 at nuclear level, which was not detectable in cultured MSCs, while markers of mature cardiomyocytes (α-actinin, troponin T) were still not expressed. While other cells acquire a phenotype of endothelial (a-VWF immunopositivity). Yet, in all the infarcted hearts MSCs, which maintain their typical round shape, do not migrate but concentrate in the ischaemic border zone.

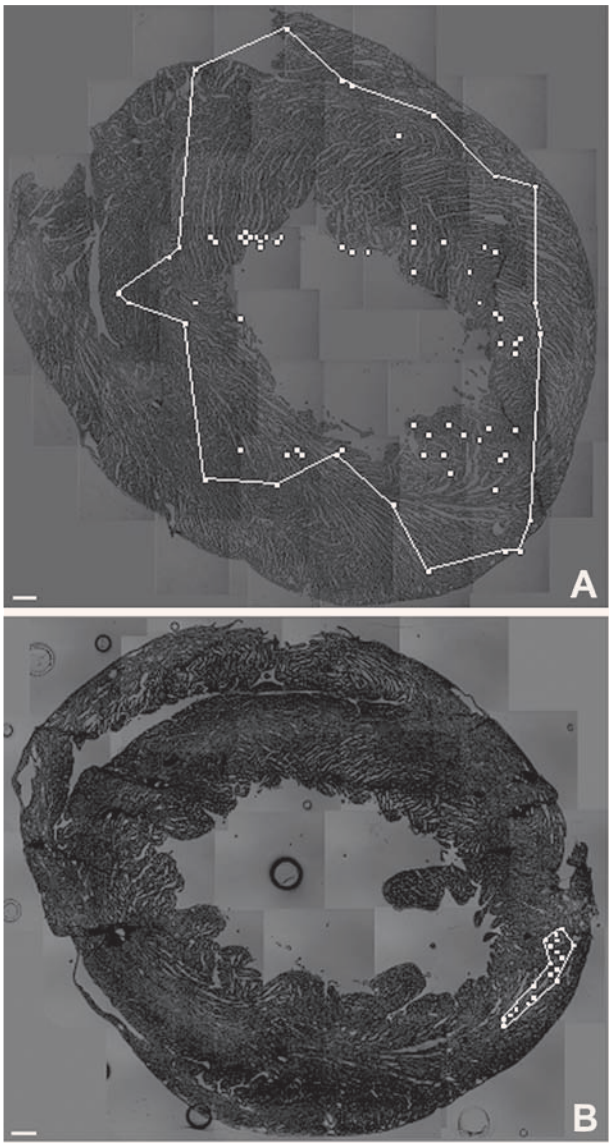

Fig. 3 Confocal image reconstruction of the whole heart profile for migration analysis. (**A**) Reconstruction of normal heart 4 hrs after MSCs injection. (**B**) Reconstruction of infarcted heart 4 hrs after MSCs injection. White points: presence of green MSCs. White lines: area of migration. Scale bars: 500 μm.

The very rapid differentiation process which is activated in normal hearts is surprising and can be tentatively explained in the light of the presence of differentiating factors normally secreted by adult myocardiocytes. In support to this hypothesis, an *in vitro* study has recently shown that co-culture of

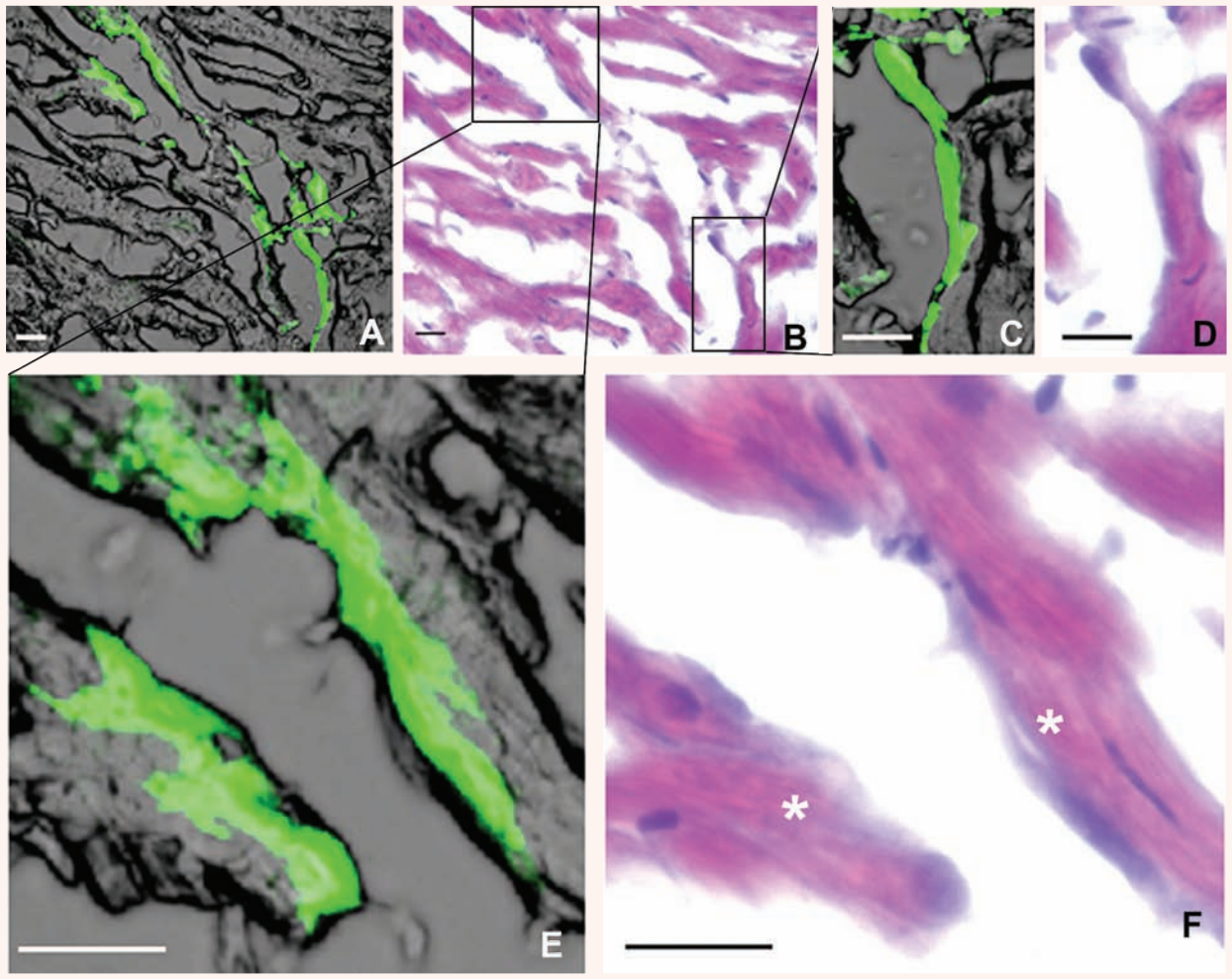

Fig. 4 Confocal and haematoxylin/eosin imaging of suspended GFP-positive MSCs injected in normal isolated hearts harvested after 2 hrs. Panels **C, D** are magnifications of the rectangular box in **B**; panels **E, F** are magnifications of the squared box in **B**. The MSC in **D** shows the basophil cytoplasm typical of this cell population. Asterisks in **F** point to MSCs that acquired an eosinophil myocardiocyte-like cytoplasm. Scale bars: 20 μm.

myocardiocytes with MSCs induces an early differentiation of the latter cell type [12]. By contrast, after ischaemia there might be a prevalence of soluble factors promoting cell survival rather then differentiation which could explain the different MSCs behaviour in infarcted hearts.

These findings are consistent with those of other authors, who have shown that chemo-attractant factors are released by the infarcted area [31–35] and that in an ischaemic zone, SCs migrate very slowly through the interstitial milieu of isolated arrested hearts [1].

Notably, we showed that connexin-43 was already present in cultured MSCs, and that CD90 immunolabelling persists also in MSCs that have already undergone phenotype changes in normal hearts suggesting that, in early homing times, there might be a co-existence of markers of different differentiation stages. This occurrence should then be taken into consideration when interpreting results of cell transplantation experiments.

As said, the morphological changes (elongation, eosinophilia) and integration within surrounding myocardium occur earlier in normal than in

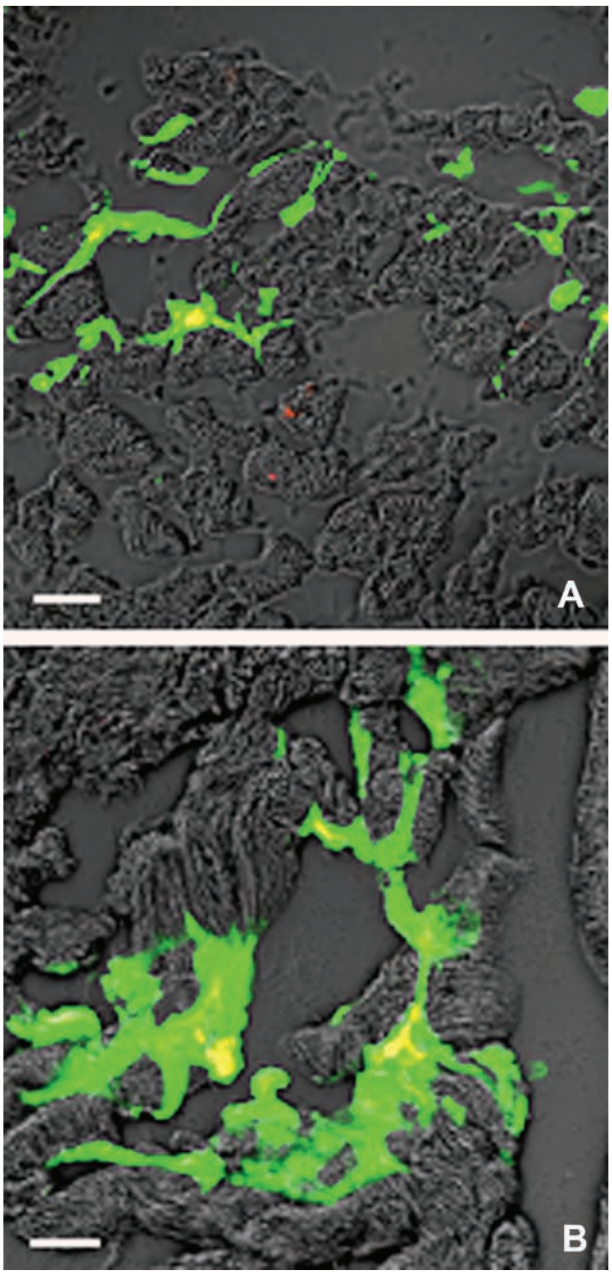

Fig. 5 Confocal imaging of direct fluorescence GFP-positive MSCs (green) and a-PCNA immunolabelling (red). Where the red labelling is superimposed to the green, the system displays it yellow. MSCs continue to proliferate 6 hrs after injection both in normal hearts (**A**) and in infarcted hearts (**B**). Scale bars A: 50 μm; B: 20 μm.

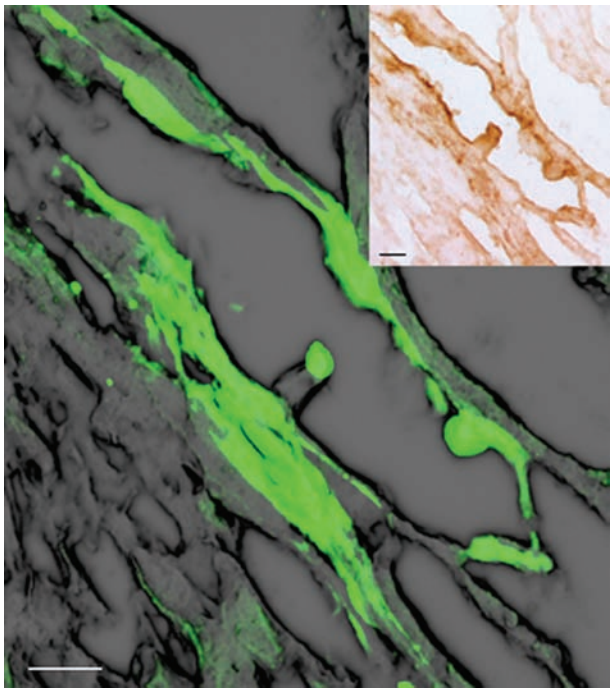

Fig. 6 Confocal imaging of direct fluorescence of GFP-positive MSCs injected in normal isolated hearts 2 hrs after injection. The upper right box shows peroxidase immunostaining with a-GFP antibody of the same slide demonstrating that the observed fluorescence was not due to autofluorescence of cardiomyocytes. Scale bars: 20 μm.

ischaemic hearts (Figs 4 and 10). Yet, MSCs injected in both normal and post-ischaemic hearts showed accelerated expression of makers of differentiation (Figs 8 and 9), when compared with what observed in co-culture of MSCs and adult cardiomyocytes [12]. The presence of minor morphological changes in infarcted hearts might be attributed to the limited contact with healthy tissue depending on the limited migration from the ischaemic border zone [29] and the consequent difficulty of integration between mesenchymal and host cells in the damaged area.

The above findings and the fact that in infarcted hearts MSCs concentrate in the border zone of ischaemia, where they continue to proliferate as shown by anti-PCNA immunolabelling (a marker of the S phase), supports the idea that MSCs may regenerate infarcted myocardium [2] and may integrate with normal myocardium. Therefore, the delay in morphological changes may not be detrimental because it allows proliferation of MSCs where repairing is required. In fact, a too early and advanced differentiation may reduce the proliferation power of the implanted cells.

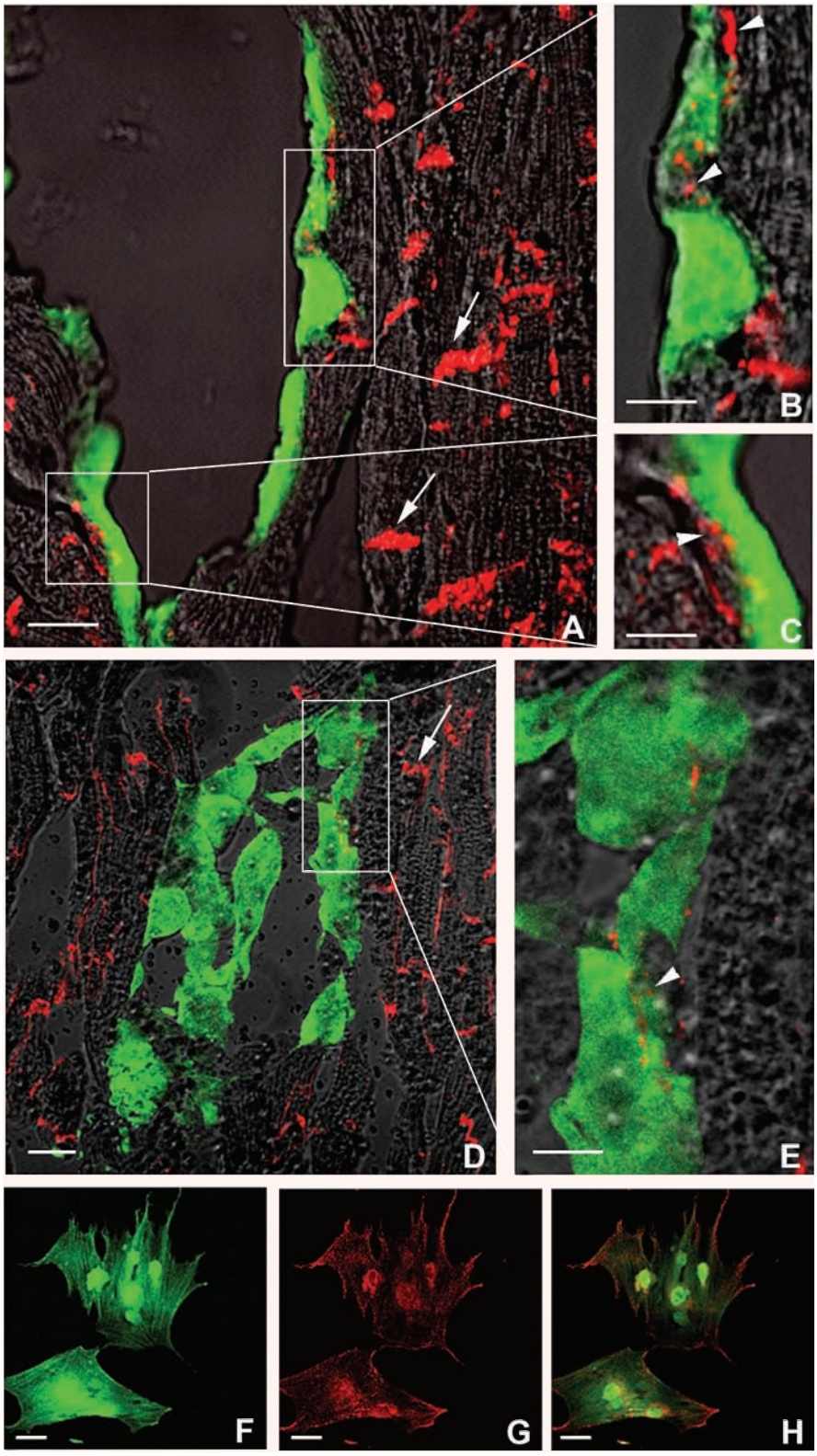

Fig. 7 Confocal imaging of direct fluorescence GFP-positive MSCs (green) and immunostaining with anti-connexin-43 antibody (red) in normal (**A–C**) and infarcted (**D, E**) hearts. Panel **B, C** are magnifications of rectangular and square box in panel **A**. Panel **E** is magnification of square box in panel **D**. Arrows point to the typical connexin-43 localization at the intercalated disc. Arrowheads point to connexin-43 localization between cardiomyocytes and injected MSCs. **F**: GFP autofluorescence of MSCs in culture; **G**: MSCs in culture stained with connexin-43 (red); **H**: merging of green autofluorescence and red staining with connexin-43. Scale bars **A, D, F–H**: 20 μm; **B, C, E**: 10 μm.

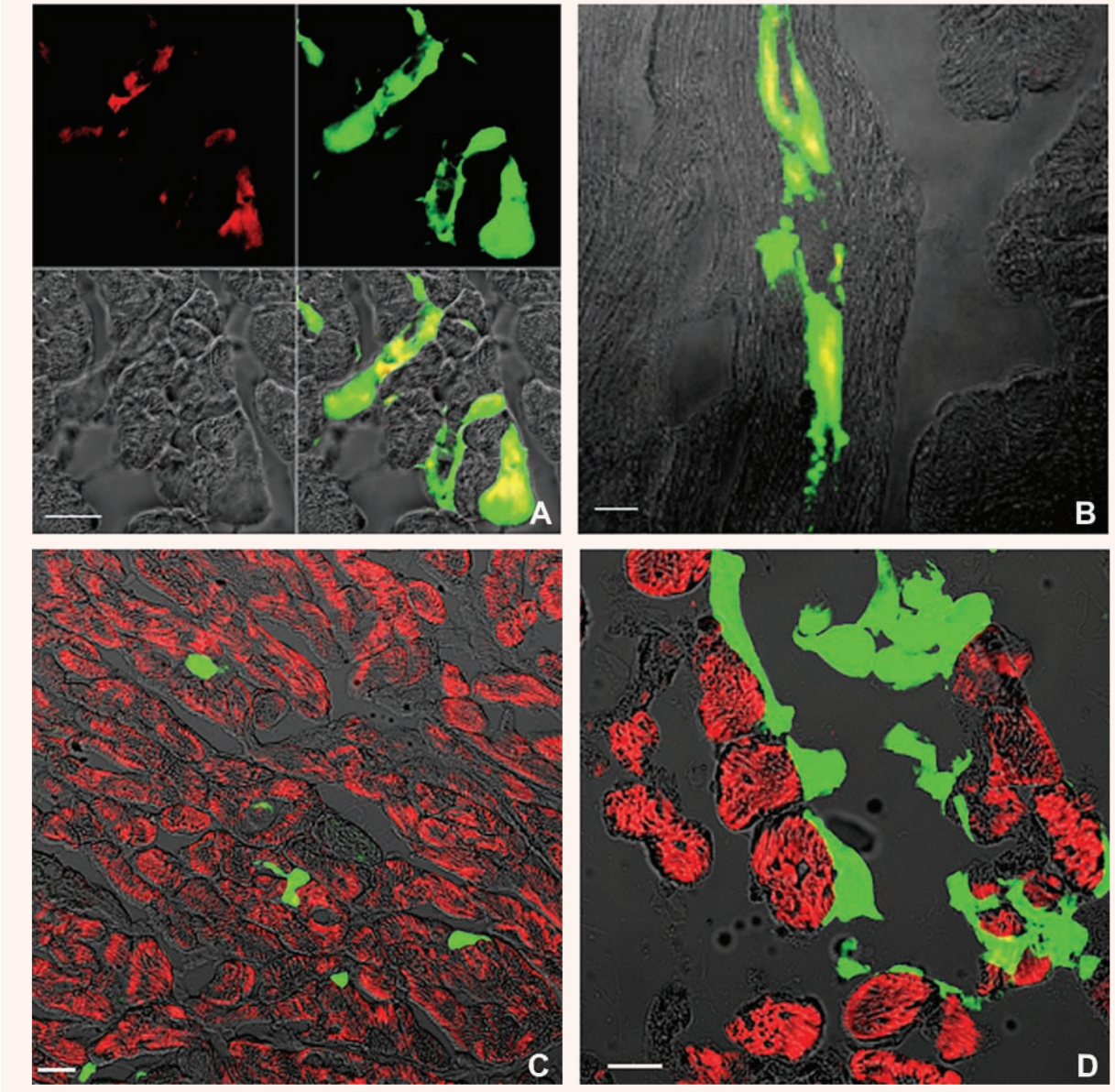

Fig. 8 Confocal imaging of direct fluorescence GFP-positive MSCs (green) and nuclear immunostaining with anti-GATA-4 antibody (red) in normal (**A**) and infarcted (**B**) hearts. (**C, D**) Immunostaining with α-actinin antibody (red in **C**) or troponin T (red in **D**) in normal hearts 4 hrs after injection. Where the red labelling is superimposed to the green, the system displays it yellow. Scale bars: 20 μm.

The faster differentiation in the normal than in infarcted heart is also supported by the observation that, only in normal hearts MSCs were found to be positive to VWF, indicating that their differentiation has begun also towards endotheliocites.

Beside the cell-to-cell contact, the faster phenotype changes could be due to the co-existence in non-ischaemic isolated beating heart of various factors (*e.g.* multiple cell-to-cell interaction, mechanical and electrical stimulation) which, *in vitro*, have been suggested to

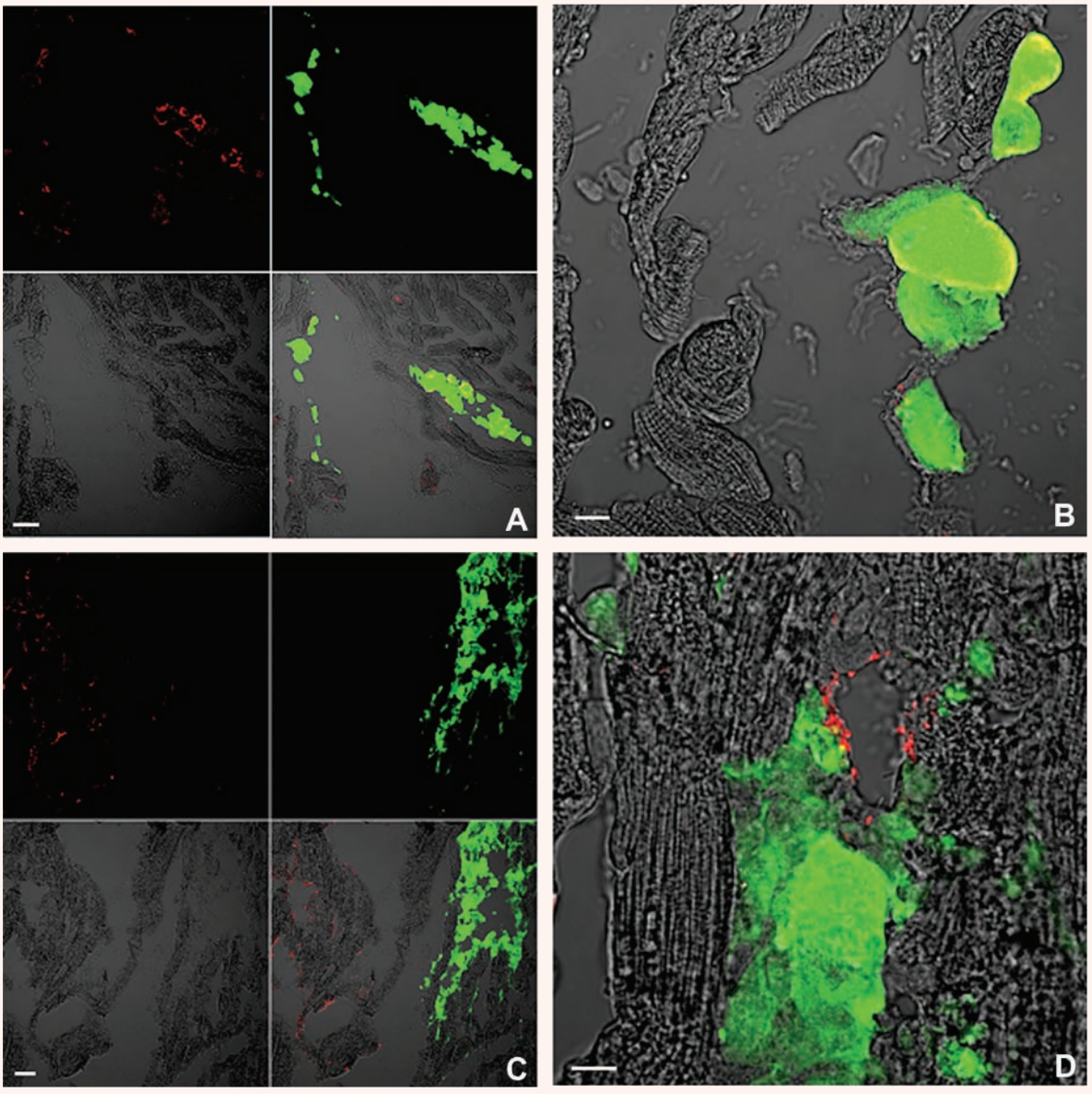

Fig. 9 Confocal imaging of direct fluorescence GFP-positive MSCs (green) and immunostaining with Von Willebrand Factor (VWF) antibody (red). (**A, B**) Normal heart 4 hrs after MSCs injection; (**C, D**) Infarcted heart 4 hrs after MSCs injection. Scale bars **A, C**: 50 μm; **B, D**: 10 μm.

be singularly able to induce differentiation, even in the absence of ischaemic-related stimuli [4, 28]. Moreover, it must be considered that in the isolated beating heart coronary flow is 10–12-fold higher than in the in situ preparation [22–24]. This higher flow causes the intercellular milieu to change much more rapidly so that the SCs found after 4–6 hrs in the isolated heart have been exposed to a number of milieu variations that in *in situ* conditions may occur after days from the event [36]. Thus, it may be argued that in a few hours an isolated heart model can give information that *in vivo* and *in vitro* may require a longer period of time,

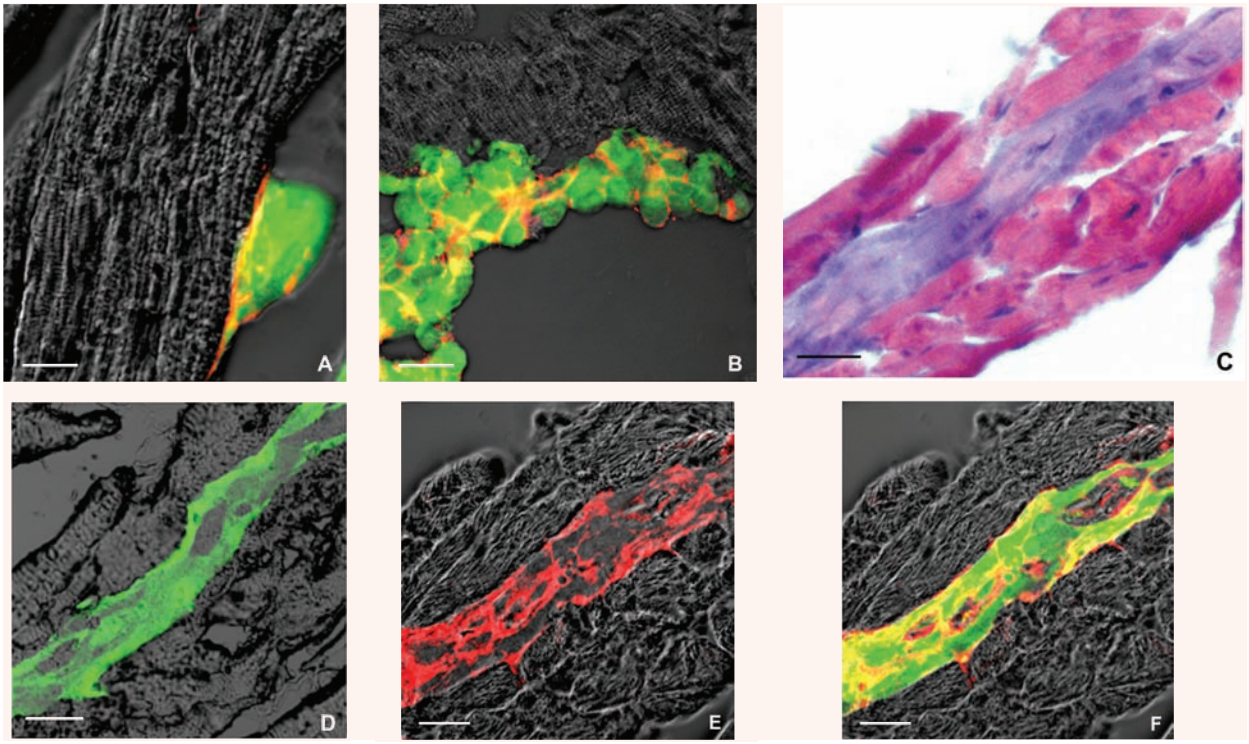

Fig. 10 Confocal imaging of direct fluorescence GFP-positive MSCs (green) and sarcolemmal immunostaining with anti-CD90 antibody (red) in normal (**A**) and infarcted (**B**) hearts. Panels **C–F** show pictures of serial sections (from a normal heart withdrawn 6 hrs after transplantation) which after confocal imaging of GFP autoflourescence (green) were stained with haematoxylin and eosin (**C**) and a-CD90 immunolabelling (**E, F,** red). Where the red labelling is superimposed to the green, the system displays it yellow (**F**). Scale bars **A**: 10 μm; **B–F**: 20 μm.

because rapid changes in the milieu, in the absence of extra-cardiac interference, are likely to induce faster response of MSCs. The prompt initiation of differentiation in normal hearts clearly indicates that this rapid response is possible.

Methodological considerations

Despite the rapidity of events, we recognize that our isolated beating heart model is limited by the fact that it is inadequate for studying the fate of transplanted cells over a long period of observation. The reason is that, after 6–7 hrs of isolation, heart function begins to be impaired. For the same reason, the model is not adequate for studying survival of transplanted cells. Our rough estimates of the total number of GFP^+ cells detectable in isolated hearts up to six hours after transplantation suggest that more of 50% of the injected amount of cells survive in these experimental conditions. However, longer observation times are needed to follow up survival rates and, to this end, *in vivo* studies are required.

The absence of blood could be another limitation. Yet with buffer perfused hearts, it is possible to prevent the presence of potentially confounding external factors, such as inflammatory and redox events which follow endothelial/neutrophil interaction. If required, the model can also be used to study the role of each external factor by administering them to the isolated heart individually or in association. Thus, for instance, the heart can be perfused with whole or partial blood elements.

Moreover, important determinants of myocardial response to ischaemia (organ temperature, heart rate, ventricular volume, coronary perfusion and collateral flow) are kept under the control of the researcher, whereas *in vivo* they are extremely variable and can influence the results of cell implantation.

Although it was beyond the aims of the present study, ideally the clean conditions of the isolated heart models might also permit to carry out quantitative

stereological assessment [37] of MSCs behaviour after transplantation. However, it has been pointed out that stereological assessment is particularly difficult when dealing with fluorescent labelling [38], because the data obtained with this procedure may vary significantly depending on various factors that may generate unpredictable variability in the intensity of fluorescence at the time of quantitative analysis (*e.g.* fluorescence bleaching can be related to many different variables and remains difficult to keep under control in fluorescence microscopy). Our experience has shown that this limitation is particularly evident in the *in vivo* heart in which GFP positive MSCs were injected 4 weeks before the *post-mortem* examination because of the autofluorescence of the myocardial tissue (unpublished observations).

Conclusions

The isolated beating heart model provides a simple and efficient method to study early SC homing in a cardiac environment, in which the various intrinsic and extrinsic factors may be strictly controlled. This model can be used to answer emerging questions about the early migration, proliferation and differentiation of SCs injected into the heart muscle. We have shown that in early homing times, in the MSCs there is a co-existence of markers (PCNA, CD90, connexin-43, GATA-4, VWF) and histological signs of different degree of integration with the surrounding myocardium. We have also observed that MSCs differently integrate, migrate and change shape, whether they are injected in normal or infarcted heart. Yet data and hypothesis presented here are just few examples of scenarios in which this method can be used. Further hypotheses fertile for experimental interventions may arise in which the isolated beating heart experimental model may be suitable.

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