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Adipose-derived cardiomyogenic cells: *in vitro* expansion and functional improvement in a mouse model of myocardial infarction

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Aims Cells derived from the stroma vascular fraction (SVF) of mouse adipose tissue can spontaneously give rise to rare, functional, cardiac-like cells *in vitro*. This study aimed to improve the production of adipose-derived cardiomyogenic cells (AD-CMG), to characterize them and to assess their cardiac fate and functional outcomes after their administration in a mouse model of acute myocardial infarction.
Methods and results The culture process optimized to improve *in vitro* cardiac specification consisted of a primary culture of murine SVF cells in semi-solid methylcellulose medium, a selection of AD-CMG cell clusters, and a secondary culture and expansion in BHK21 medium. AD-CMG cells were $CD29^+$, $CD31^-$, $CD34^-$, $CD44^+$, $CD45^-$, $CD81^+$, $CD90^-$, $CD117^-$, and $Flk\text{-}1^-$ and expressed several cardiac contractile proteins. After 1, 2, and 4 weeks of their injection in mice having acute myocardial infarction, a strong presence of green fluorescent protein-positive cells was identified by immunohistochemistry as well as quantitative polymerase chain reaction. Echocardiography showed a significant reduction of remodelling and stability of left ventricle ejection fraction in the AD-CMG cell-treated group vs. controls. Vascular density analysis revealed that AD-CMG administration was also associated with stimulation of angiogenesis in peri-infarct areas.
Conclusion Cardiomyogenic cells can be selected and expanded in large amounts from mouse adipose tissue. They can survive and differentiate in an acute myocardial infarction model, avoiding remodelling and impairment of cardiac function, and can promote neo-vascularization in the ischaemic heart.

1. Introduction

Post-infarction heart failure is closely associated with a major loss of differentiated cardiomyocytes subsequent to ischaemia, which cannot be overcome by the poor renewal of differentiated cardiomyocytes in the heart.[1] This has led many investigators to develop various strategies of cell-based therapy to improve defective contractile performance and heart function,[2] but an efficient and unlimited source of autologous cells able to produce functional cardiomyocytes in the clinical setting is still lacking. Adipose tissue could provide such a suitable source of stem cells, as this adult tissue is abundant and easy to sample with no ethical limitation. Recent work from independent groups states that adipose tissue host cells are able to display various differentiation potentials *in vitro* and *in vivo*.[3–5] Such cells can be isolated from the stroma vascular fraction (SVF) obtained after adipose tissue digestion and mature adipocyte elimination. Thus, SVF cells represent a heterogeneous cell population that has been used after being freshly prepared in some studies or after culture in others. In the latter case, the cells obtained after culture represent a particular cell sub-population restricted to the adherent cell fraction of the SVF, termed ADSCs (adipose-derived stromal cells).[6] Our group previously demonstrated the *in vitro* spontaneous cardiac differentiation of freshly isolated adipose tissue cells from mouse SVF and not from ADSCs.[7] The differentiation rate was relatively low under such basal conditions but was obtained without the addition of the hypomethylant agent 5-azacytidine that is required for bone marrow mesenchymal stem cells (MSCs),[8] or the addition of epidermal growth factor and basic fibroblast growth factor (bFGF) as reported for skeletal muscle Spoc cells.[9]

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The first purpose of this study was to scale-up *in vitro* expansion of adipose derived-cardiomyogenic cells (AD-CMG) and to further characterize these cells. The second objective was to assess their potential to survive, engraft, differentiate into cardiomyocytes, and improve cardiac function after their implantation in a mouse model of acute myocardial ischaemia.

2. Methods

Additional information is provided in a Supplementary material online, *Methods section* with detailed protocols including description of reagents and antibody companies.

2.1 Animals

Eight-week-old C57Bl/6 N mice (Harlan, France) and green fluorescent protein (GFP) mice (kindly provided by Dr Okabe)[10] were housed in conventional animal quarters (SPS barrier facility) under a controlled environment (12 h light/dark cycle at 21°C) with free access to water and a standard chow diet. All procedures were performed in accordance with the European Community guidelines for the care and use of laboratory animals (EEC/No. 07430).

2.2 Cell isolation and culture

After a careful dissection to eliminate any contaminating muscle tissues, cells were isolated from adipose tissue according to the method of Bjorntorp *et al.*[11] with minor modifications.

Freshly prepared SVF cells were seeded (10 000 cells/cm^2) in DMEM-F12 containing 10% new-born calf serum. Six hours after plating, all non-adherent cells were removed by washing and adherent cells were then maintained in culture for 6 days until phenotyping or *in vivo* administration. Sub-confluent cells were harvested by trypsinization 5 min at 37°C and represent ADSCs.

In other experiments, freshly prepared SVF cells were plated (5000 cells/cm^2) and maintained for 2 weeks in methylcellulose (Methocult M3534), where morphology of developing cardiogenic cell clusters was followed as previously reported.[7,12] These clusters were composed of adherent elongated cells and rounded non-adherent cells. Early contracting clusters of cells were then dissected under an inverted phase-contrast microscope and were plated (1500 cells/cm^2) into 30-mm culture dishes coated with 0.1% gelatin and cultured in BHK21 medium containing 10% foetal bovine serum, supplemented with β-mercaptoethanol 10^{-4}M, glutamine 2 mM, pyruvate 1 mM, non-essential amino acid 0.1 mM, and a solution of amphotericin 0.25 μg/mL, penicillin G 100 U/mL, and streptomycin 100 μg/mL. Cell expansion was performed by harvesting cells in suspension in the culture medium every 3 days, centrifugation (600 *g*, 5 min), and plating into new culture dishes coated with 0.1% gelatin and culture in BHK21 medium. For the purpose of this study, only cells from passages 1–4 over a period of 1 month for each passage were used.

2.3 Fluorescence analysis cell sorter

Cells were stained in a phosphate saline buffer containing 0.2% foetal calf serum and incubated with directly conjugated anti-mouse monoclonal antibodies (mAb) at BD Biosciences (Heidelberg, Germany) for 30 min at 4°C. After washings, cells were analysed on a fluorescence analysis cell sorter (FACS Calibur, Becton Dickinson, Mountain View, CA, USA). Data acquisition and analysis were then performed (Cell Quest software, Becton Dickinson). For intracellular staining, paraformaldehyde 4% fixation (10 min, 4°C) was followed by permeabilization with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.5% saponin, 20 min at room temperature. After the labelling with anti-troponin T, a PerCP anti-mouse was used.

2.4 Immunochemistry

Adherent cells, grown in chamber slides, were washed with PBS and fixed overnight at 4°C in 3.7% paraformaldehyde/PBS buffer. After blocking for 1 h in 1% BSA PBS buffer, cells were incubated for 1 h in 0.3% Triton X-100/PBS buffer with primary antibody: mouse antibodies against MLC-2v (1:200), sarcomeric α-actinin (1:500), α-smooth muscle actin (α-SMA; 1:1000), β-MHC, α-MHC, titin, troponin T (1:100), and rabbit antibody against connexin 43 (1:50). After washing, a secondary antibody, Alexa 546, Alexa 350 (1:500), or Alexa Fluor 594 (1:500) conjugated anti-mouse or anti-rabbit IgG (1:300) was applied for an additional 60 min at room temperature. Negative controls were performed with purified mouse IgG or rabbit IgG and yielded no staining.

For tissue samples, the heart was quickly removed after mice anaesthesia at Days 7, 14, and 28 after cell administration, cut longitudinally along main axis, and fixed overnight at 4°C in 3.7% paraformaldehyde. The tissue was paraffin-embedded and cut into 6 μm sections. Protocols for immunostaining were described in the Supplementary material online, *Methods section*. Fluorescence imaging was performed with a confocal microscope (Zeiss, LSM 510) or with a fluorescent wide field microscope (Leica DMR). AEC visible imaging was visualized under a Leica DMRB microscope.

For lectin quantification, Isolectin B_4 (1:500) and Strept AB Complex/Peroxidase (Dako, Spain) were used. After performing the staining, pictures were taken in the peri-infarct region, on a Nikon Eclipse E800 microscope equipped with epifluorescence optics and digital images were analysed using imaging software (Jay Image). The total number of stained vessels was counted and then divided by the area of the photograph in order to obtain vessel density.

For smooth muscle quantification, the total area of the tissue was divided by the total area occupied by stained vessels in the picture. The result is expressed as the percentage of α-SMA-positive areas.

2.5 Real-time quantitative PCR

For GFP cell-tracking, DNA from heart tissue samples was isolated with QIAmp® DNA Mini Kit (Qiagen, France). Mixing increasing amounts of DNA from GFP donor mice with DNA from C57Bl/6 N recipient mice made the standard curve for quantifying GFP and 36B4 genes in the heart of recipient mice.

Total RNA from AD-CMG in BHK21 culture and RNA from mouse heart were isolated and reverse-transcribed before quantitative PCR was performed. The nucleotide sequences of the PCR primers and the protocols used were indicated in the Supplementary material online, *Methods section*.

2.6 Mouse model of acute myocardial infarction

C57Bl/6 N mice between 8- and 12 weeks old (22–28 g) were used. After intraperitoneal injection of Ketamine (75 mg/kg) and Xylasine (7.5 mg/kg), they underwent tracheal intubation for ventilation with a rodent ventilator (Hugo Sachs elektronik, March-Hugstetten, Germany). Gaseous anaesthesia was maintained with 2% Isoflurane. After a left thoracotomy through the fifth intercostal space, pericardium was opened and left descending artery was ligatured with an 8/0 polypropylene suture (Ethicon, Johnson & Johnson, Brussels). In the acute model, immediately after ligation, a total volume of 10 μL containing 1×10^5 cells was injected in three points of the ischaemic area (pale area downstream from the ligature). For echocardiographic functional studies, we slightly modified the model of acute myocardial infarction. We used a sub-acute model to provide data before (baseline) and 3 days after infarction. We performed injection of the cells or medium (for the control group) 3 days after the ligation of the coronary artery, through the same intercostal approach. The day of injection was the first day of echocardiographic follow-up (D0).

2.7 Functional assessment by echocardiography

Blind echocardiographic functional assessments were performed for both groups, before infarction for baseline, immediately before injection (D0, 3 days after infarction), 1 (D7), and 4 (D28) weeks after injection. Echocardiographic evaluations were performed using a commercially available VIVID 7 (General Electric) echocardiographic system equipped with a 14-MHz linear-array transducer (Agilent, Andover, MA, USA).

Left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured from the LV M-mode tracing with a sweep speed of 200 mm/s at the papillary muscle level. Other cardiac parameters were also measured in the two-dimensional view both in long- and short-axis. Ejection fraction (EF) was measured by single-plane area length and calculated according to the formula: EF $(\%) = [(0.85\ \mathrm{LVAD}^2/\mathrm{LVLD}) - (0.85\ \mathrm{LVAS}^2/\mathrm{LVLS})]/(0.85\ \mathrm{LVAD}^2/\mathrm{LVLD}) \times 100$ (LVAD, left ventricular area in end-diastole; LVAS, left ventricular area in end-systole; LVLD, left ventricular long-axis length in end-diastole; LVLS, left ventricular long-axis length in end-systole).[13] Each value was the average of three measurements.

2.8 Statistical analyses

All data are expressed as mean $\pm$ SEM. Student's *t*-test was used to analyse statistical significance. *P*-values corresponded to a two-tailed paired *t*-test for each group follow-up analysis, and a two-tailed unpaired *t*-test for the group comparison. All $P < 0.05$ were considered statistically significant using Prism software (GraphPad software, San Diego, CA, USA).

3. Results

3.1 A three-step process to highly improve culture of AD-CMG

As already described,[7] the spontaneous emergence of beating clusters of cardiac-like cells among various other phenotypes was observed when adipose tissue stromal cells (SVF) were plated in methylcellulose The weak frequency (around one cluster for 1400 plated SVF cells) and the small amount of cells subsequently recovered by such a procedure were inappropriate for cell transplantation. In order to strongly improve the yield of cardiomyogenic cells in culture, we designed an optimized protocol that consisted of the following three-step procedure (*Figure 1A*): (i) a primary culture of SVF cells in methylcellulose until the emergence of AD-CMG cell clusters; (ii) a dissection step under an inverted microscope to select and pick up these clusters in methylcellulose; (iii) a final culture of the selected cells in BHK21 liquid medium. Two weeks later, about 60% of the cells were troponin T^+ and 40% positive for MLC2v, a marker for more mature ventricular cardiomyocytes. Two distinct morphologies of AD-CMG could be identified in BHK2 culture: rounded cells that were non-adherent and elongated contractile cells that were adherent onto the plastic dish (*Figure 1A*). Non-adherent rounded cells could be easily harvested, without using trypsin, by collecting the culture medium and centrifugation. From this observation, and to avoid the use of trypsin, we performed a large amount of cell expansion by collecting and plating cells present in suspension in the culture medium. Each plating is considered a passage. Using this three-step culture protocol, 200×10^6 adherent AD-CMG cells and 15×10^6 non-adherent AD-CMG cells could be obtained from 1 g of fat tissue (corresponding to 20×10^6 freshly prepared SVF cells) within 4 weeks without using trypsin and spontaneously contracting.

Adherent and non-adherent populations of AD-CMG obtained from the described procedure were $47.1 \pm 7\%$ ($n = 6$) and $54 \pm 3.8\%$ ($n = 12$) troponin T^+, respectively (*Figure 1A*). In comparison, troponin T^+ cells were not detected in freshly prepared SVF cells and represented only 2% in cells collected from methylcellulose. Thus, the amount of troponin T^+ cells is significantly increased when AD-CMG are cultured in BHK21.

The primary culture of SVF cells in methylcellulose remained necessary to identify the cardiomyogenic cells and then select them. The secondary culture in liquid medium BHK21 allowed large AD-CMG expansion. A direct plating of SVF cells in BHK21 supported the emergence of AD-CMG; however, the culture was very heterogeneous and poor as only 10% of the cells were troponin T^+.

'Long-term' culture of AD-CMG (15 passages of non-adherent cells) revealed that cells retained their cyclic 'proliferation/differentiation' potency that produced a constant quantity of adherent and non-adherent cells (*Figure 1B*). However, phenotype and protein-expression analysis showed that AD-CMG cell characteristics were modified with increasing passages. In non-adherent cells, cardiac troponin T expression was decreasing with time (*Figure 1C*). In adherent confluent AD-CMG cells, we also observed that the expression of the cardiac proteins MLC-2v and troponin was decreasing with passages (*Figure 1D*), even though cells still expressed cardiac mRNA of MLC-2v (*Figure 1E*), MEF2c, Islet1 (data not shown). This suggested that the cardiac phenotype was not efficiently maintained with time in such culture conditions. As the myotube morphology was conserved in adherent AD-CMG during passages, they were tested for skeletal markers. It appeared that in late passages the majority of the cells did express the myoblast transcription factors myogenin (*Figure 1D*). Surprisingly, immunocytochemistry studies revealed that it is possible to find in BHK21 culture, some cells co-expressing cardiac proteins (MLC2v, cardiac TnT) together with skeletal muscle protein myogenin (Supplementary material online, *Figure S1*). In early passages (1–4) of BHK21 cultures, AD-CMG were mainly composed of cells that expressed cardiac markers (over 60%) with rarer numbers of them co-expressing the skeletal protein myogenin (data not shown). Taking this result into consideration, subsequent experiments were strictly performed with AD-CMG cells from passages 1 to 4.

3.2 Comparison of the two AD-CMG populations obtained *in vitro*

As detailed in *Figure 2A*, both types of cell shared the following pattern of expression: $CD29^+$, $CD44^+$, $CD81^+$, $CD31^-$, $CD45^-$, $CD90^-$, $CD133^-$, c-kit^-, Flk-1^-, and were partially positive for Sca-1. The main differences between the two morphologies were that only adherent cells were positive for CD38 (42%), CD73 (74%), CD105 (39%), and CD34 (10–20%). As only 47.1% of the adherent cell population expressed troponin T, we analysed the phenotype of the troponin T-negative adherent sub-population in comparison to ADSCs classically referred to as adipose tissue mesenchymal cells. This troponin T-negative AD-CMG

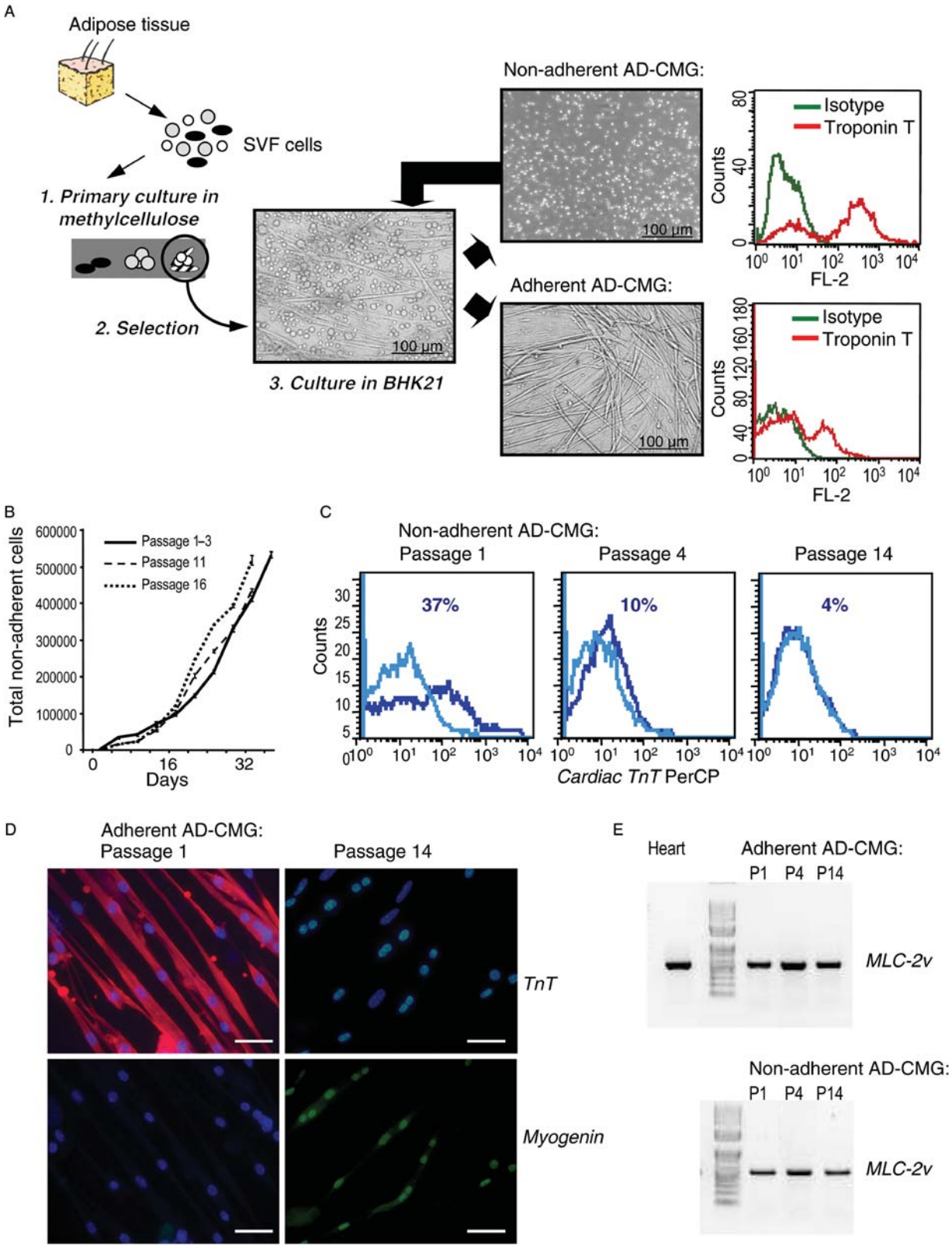

Figure 1 Isolation and *in vitro* expansion of AD-CMG according to the three-step culture procedure. (*A*) Starting with 1 g of adipose tissue 20×10^6 SVF cells could be obtained and plated in methylcellulose (5000 cells/cm^2) and represented (step 1). Emerging contracting clones could be selected (step 2) and expanded in liquid medium culture (step 3). Each plating of 200 000 non-adherent cells lead to the formation of a confluent adherent cell layer within a week (1×10^6 cells) which in turn produced 100 000 non-adherent cells every 2 days for 4 weeks. AD-CMG cells culture in BHK21 developed into two distinct cell morphologies; an adherent and elongated cell population and a non-adherent and rounded cell population able to renew and to give rise in turn to both populations *in vitro*. Both populations expressed troponin T$^+$ as indicated by FACS histogram (representative of one experiment). (*B*) Number of non-adherent AD-CMG cells produced in early and late culture passages. (*C*) FACS analysis of troponin T-positive cell population in the non-adherent AD-CMG cells collected at different passages. (*D*) Immunolabelling of confluent adherent AD-CMG cells for troponin T and Myogenin. Nuclei were labelled in blue with DAPI. (*E*) RT–PCR analysis of MLC-2v expression in adherent and non-adherent AD-CMG cells.

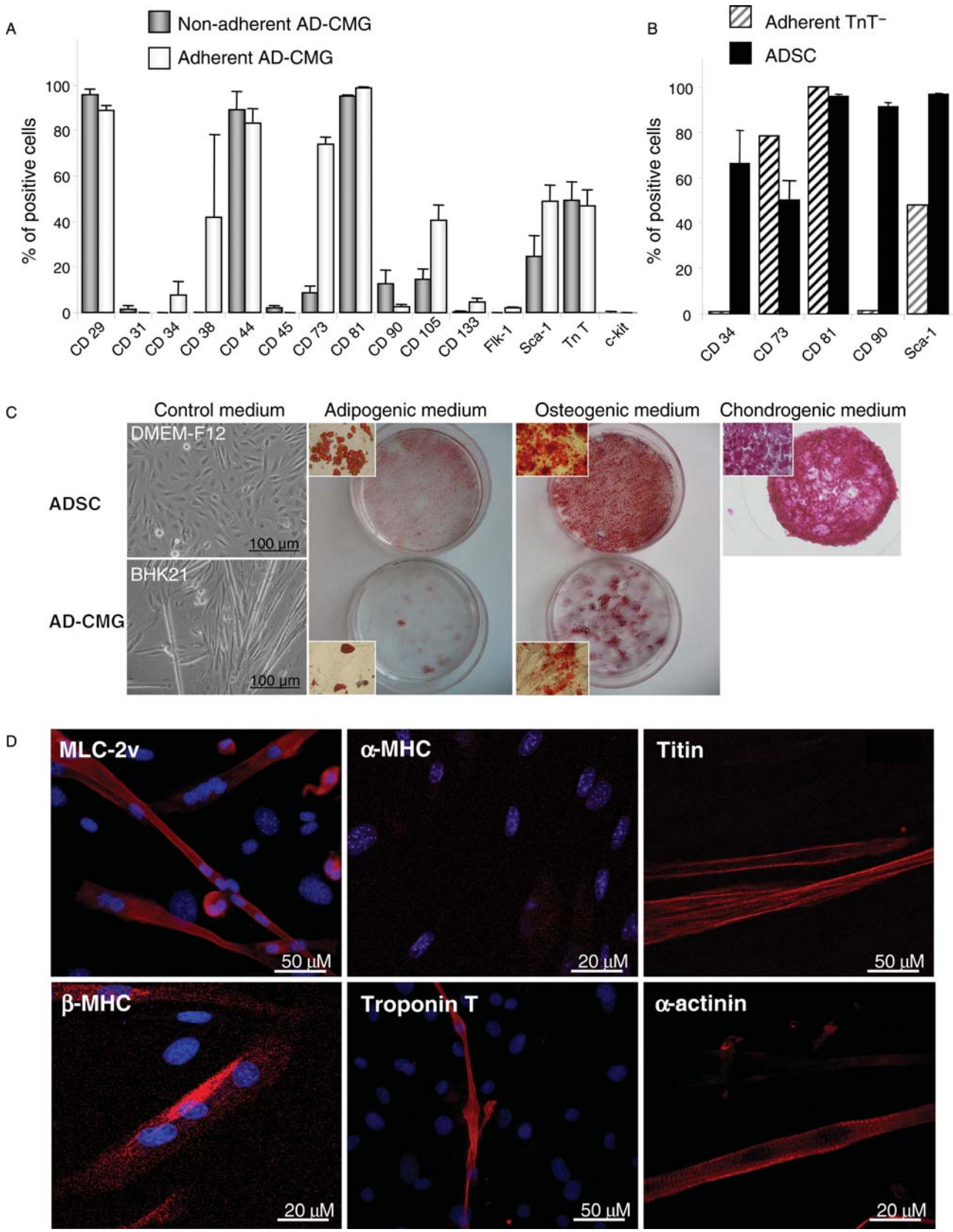

Figure 2 Characterization of AD-CMG cells in culture. (*A*) Phenotype of adherent and non-adherent AD-CMG cell populations obtained in BHK21 after the three-step culture process. (*B*) Phenotypes of the troponin T-negative sub-population present in adherent AD-CMG compared with ADSCs. (*C*) Comparative differentiation potentials of AD-CMG and ADSCs. (*D*) *In vitro* AD-CMG protein expression. Adherent AD-CMG cells cultured for 15 days in BHK21 were specifically stained with anti-MLC-2v, titin, sarcomeric α-actinin, β-MHC, and troponin T antibodies. No specific labelling was obtained for α-MHC at this point. Nuclei were labelled in blue with DAPI.

population can be distinguished from ADSCs based on CD34, CD90, and Sca-1 expression (*Figure 2B*). In order to complete AD-CMG comparison to ADSCs, both cell types were cultured in standardized adipogenic, osteoblastic, and chondrogenic media. As previously reported, ADSCs efficiently differentiated towards adipocyte, osteogenic, and chondrogenic phenotypes. Some AD-CMG cells were still able to differentiate into adipocytes and osteoblasts, but to a lesser extent. AD-CMG cells did not survive in chondrogenic culture conditions (*Figure 2C*).

Protein expression relative to *in vitro* cardiac differentiation was analysed by immunocytochemistry on adherent fibres (*Figure 2D*) showing the presence of cardiac proteins such as MLC-2v, titin, sarcomeric α-actinin, cardiac troponin T, and β-MHC, but no expression of α-MHC.

3.3 *In vivo* differentiation

To further investigate and analyse their cardiomyogenic potential, AD-CMG cells were transplanted in a mouse model of acute myocardial infarction. According to *in vitro* results, only AD-CMG cells collected from the first to the fourth passage after the three-step culture procedure were used. Immediately after infarction, 45 mice were transplanted by direct injection into ventricular wall of 1×10^5 cells from four different preparations of GFP-expressing cells: non-adherent AD-CMG cells collected in suspension in culture medium ($n = 9$), adherent AD-CMG cells obtained after trypsinization of the cell layer ($n = 7$), the mix of both cell types (1:1 ratio, $n = 23$), and ADSCs as a control for non-cardiac-committed cells ($n = 6$). Surprisingly few (from non-adherent AD-CMG) or no (from adherent AD-CMG) GFP cells could be identified 7 or 15 days post-injection when administered separately (see Supplementary material online, *Table S1*). When ADSCs were injected, rare GFP cells were identified in injected hearts (five out of six) but always displayed a fibroblast-like shape and were negative for cardiac markers such as MLC-2v and troponin T (data not shown). In contrast, after 7 days, GFP-expressing cells were found in all hearts of animals injected with the mix (11 of 11). A specific GFP labelling was evidenced within the infarct zone (*Figure 3A*). Moreover, some GFP-positive cells were located in the boarding limit, suggesting the emergence of a regenerating zone surrounding the ischaemic area (*Figure 3B*). These cells featured a cardiac morphology and were in the same spatial orientation as the neighbouring native myocardium (*Figure 3A*). They were very abundant, forming a real graft of myocardium-like tissue. These GFP-positive cells presented striations and were also expressing cardiac markers such as MLC-2v (*Figure 4A*) and troponin T (*Figure 4B*). Staining with antibodies against MyoD or myogenin was never detected, as well as any evidence of adipose or osteoblast differentiation (Oil Red-O and Alizarin Red-specific staining, respectively). After 15 days and 1 month, cells were still present in most of the grafted hearts (five of seven and four of five, respectively) and presented the same features (data not shown). To precisely quantify the post-injection chimerism, we set up real-time quantitative PCR analysis based on the presence of the genomic GFP sequence. When mice were sacrificed immediately after cell injection, the quantification revealed that only 7% of the injected cells where really engrafted. Seven days after the injection in the acute model of myocardial infarction, $5.3 \pm 1.9\%$ of the total injected GFP^+ AD-CMG cells were detected. Taken together, these data demonstrated that the injection of 100 000 GFP^+ cells lead to the engraftment of 7000 cells and 7 days later 75% of GFP^+ engrafted cells were still present. Considering this GFP-positive population that survived 7 days after administration, 88% of the cells were co-expressing MLC-2v and 51% the cardiac troponin T as estimated after immunolabelling.

3.4 Functional study

The extent of GFP^+ cells with cardiac features after the injection of the mix in acute heart infarction prompted us to determine whether AD-CMG cells could protect or restore LV function in such an ischaemic heart. To investigate this point, we set-up a protocol to follow the longitudinal evolution of heart function. An initial echocardiography was performed before coronary artery ligation (baseline). Three days after surgery, we performed a new echocardiography in order to randomize infarcted mice according to their cardiac function loss before the cell transplantation. Then, the cell-treated group received 1×10^5 AD-CMG cells ($n = 9$), whereas the control group received the same volume of acellular medium ($n = 8$). Systolic function (based on LVEF) and estimated remodelling (based on diameters and volumes) were assessed by echocardiography after 7 (D7) and 28 days (D28).

No significant differences could be observed for left ventricular end-diastolic volume (LVEDV), left ventricular end-systolic volume (LVESV), and LVEF, between the two groups before surgery (baseline). Three days after coronary artery ligation and immediately before injections (D0), all these parameters were similarly impaired in both groups ($P > 0.146$) indicating that sizes of myocardial infarction were comparable between the two groups (*Figure 5A*, D0 between both groups). After injection, LVEF of control mice significantly decreased, whereas LVEF of AD-CMG-treated group remained stable. At 28 days after injection, LVEF was significantly higher in the cell-treated group than in the control group ($P = 0.0201$; *Figure 5A*).

Regarding remodelling, a significant dilatation of the LV of control mice was noted after 28 days, as indicated by significant increase of LVEDV as well as LVESV. In contrast, infarct hearts treated with AD-CMG experienced a non-significant dilatation. Moreover, the comparison of LVEDV and LVESV after 28 days showed a significant lower dilatation in the cell-treated group compared with control group ($P = 0.046$ and 0.039, respectively; *Figure 5A*). In these animals, the presence of cells co-expressing GFP and MLC-2v was checked and identified in five of the nine hearts treated at D28 (*Figure 5B*).

The functional contribution of transplanted cells was questionable with regard to the limited amount of GFP cells quantified by Q-PCR. To determine if additional mechanisms could be involved in the improvement of cardiac function, we analysed the angiogenic effect of transplanted cells in the injured heart tissue. Vascular density was determined 15 days after transplantation by quantification of the lectin-positive and α-SMA-positive vessels in the scar area. The number of capillaries per square-millimetre as well as the number of α-SMA-positive vessels was significantly increased ($P < 0.05$), suggesting that neovascularization

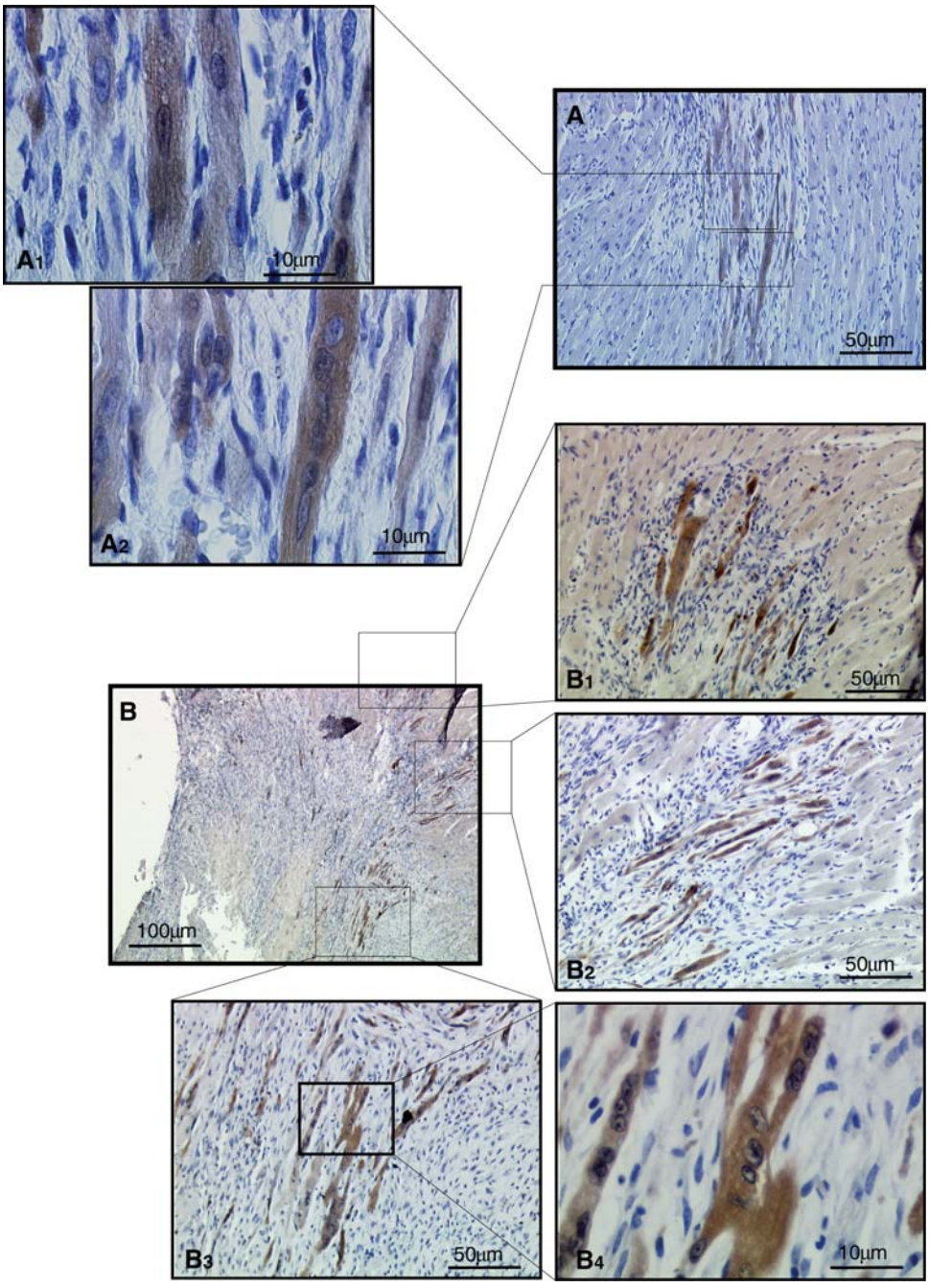

Figure 3 Immunohistochemistry of GFP-expressing cells 7 days after transplantation in a mouse model of acute myocardial infarction. GFP is labelled by a specific antibody revealed in brown. (*A*) GFP-expressing AD-CMG were identified within the infracted area. (*B*) GFP-expressing cells were present in multiple integration sites in boarding areas. GFP-expressing cells adopt the same spatial organization than the surrounding cardiac myocytes.

was improved in the cell-treated group compared with the control group (*Figure 6A*). The size of the capillaries ranged from 5 to 10 μm, whereas αSMA-positive vessels were characterized by a thicker wall composed of layers of smooth muscle (positively stained) and a size well over that of the capillaries (>15 μm). This result was sustained by the fact that administered AD-CMG cells were able to express the anti-apoptotic factor HGF and the pro-angiogenic factor VEGF in culture (*Figure 6B*). This result indicated that transplanted AD-CMG cells may improve cardiac function through their differentiation potential and stimulation of neovascularization process, possibly involving the paracrine effect.

4. Discussion

In this study, we designed an efficient culture procedure to obtain large amounts of AD-CMG. These cells lead to substantial survival in an acute ischaemic context and to functional benefit.

We previously reported the *in vitro* spontaneous differentiation of adipose-derived cells toward functional cardiomyocytes.[7] We overcome the low frequency by defining the best procedure to obtain large quantities of pre-committed cells. This consists in a primary culture in clonogenic methylcellulose conditions until the apparition of clusters with contractile lengthened cells. These cells can

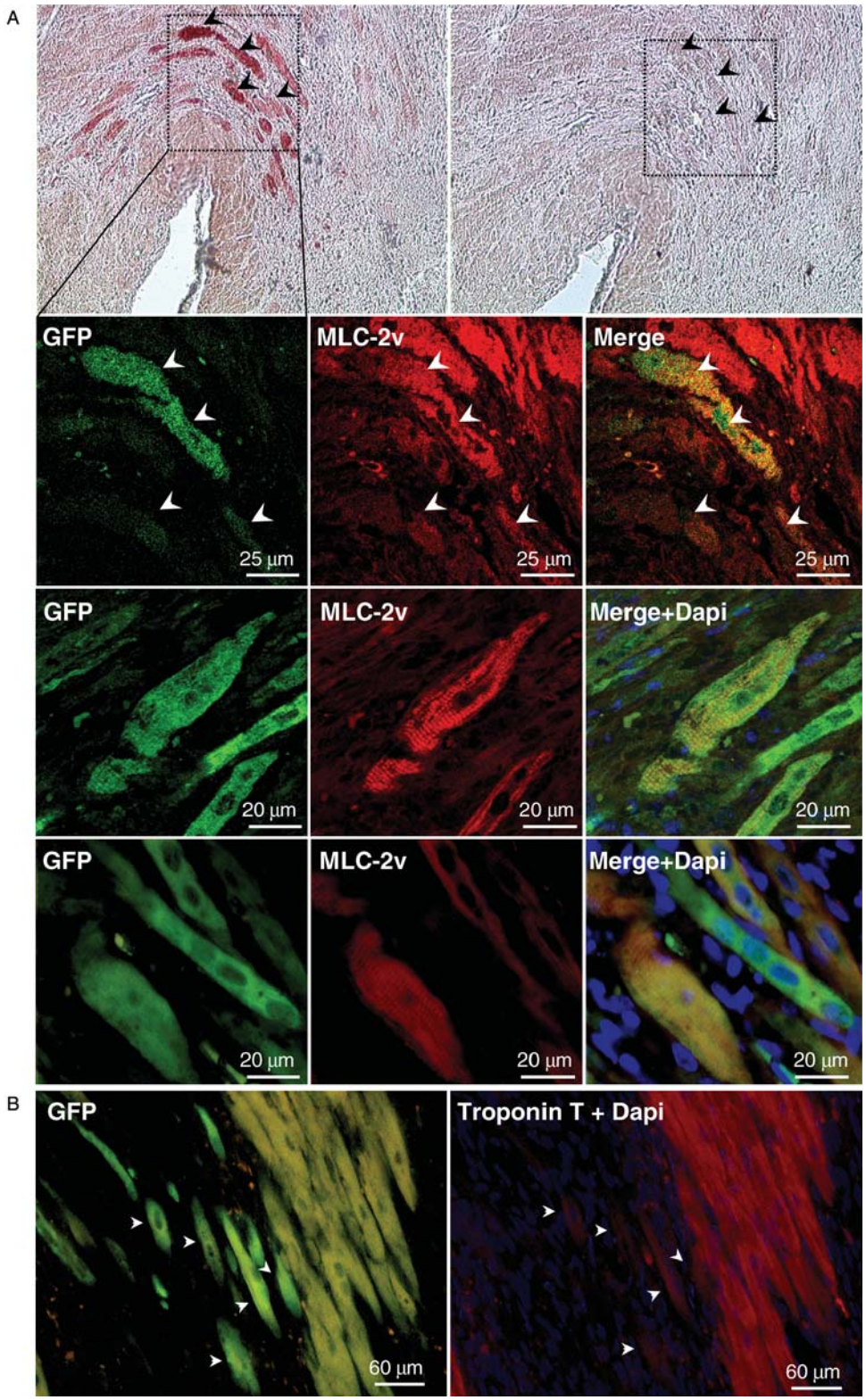

Figure 4 *In vivo* cardiac differentiation of GFP-expressing AD-CMG cells 7 days after transplantation in acute myocardial infarction. Adherent and non-adherent AD-CMG cells (ratio 1:1) prepared from the three-step culture procedure were injected. (*A*—Top) Immunohistochemistry on heart tissue for the presence of GFP revealed in red (left) vs. the isotypic control (right). (Middle) Immunofluorescence confocal microscopy using specific anti-MLC-2v (red) and anti-GFP (green) antibodies. The merged picture evidenced the presence of native cardiac myocytes (only red) and cells co-expressing the GFP and MLC-2v proteins (yellow). (*B*) Immunofluorescence wide-field microscopy using anti-GFP antibody (left) showing that GFP cells were present in infarcted areas (green, see arrows) and were distinct from native myocardium (autofluorescence in yellow). Right panel showed that cells specifically labelled with the anti-GFP antibody were also stained with the anti-troponin T antibody (red, see arrows). Native myocardium is also stained with the anti-troponin T antibody (red). Nuclei were labelled in blue with DAPI.

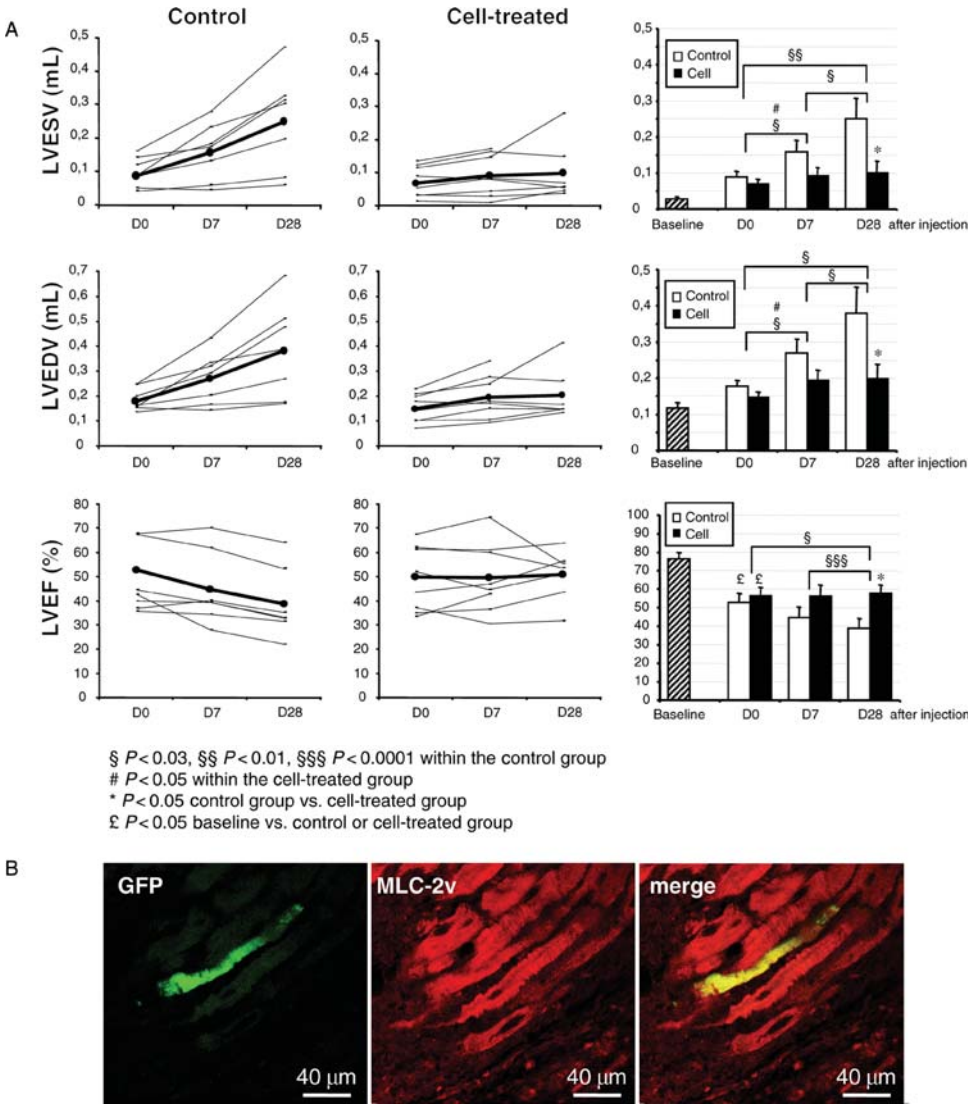

Figure 5 Functional analysis by echocardiography. (*A*) Evolution in end-systolic volumes (LVESV, top), end-diastolic volumes (LVEDV, middle), and left ventricular ejection fraction (LVEF, bottom) at Days 0, 7, and 28 after cells or medium injection. The thick traces represent average values. In the control group, LVESV and LVEDV significantly increased during the follow-up, whereas they were stable for the cell-treated group. Twenty-eight days after injection, LVEF values were significantly better in the cell-treated group compared with the control group. Histograms represent the comparison of the functional values variations between D0, D7, and D28. (*B*) Immunohistochemistry showing co-expression of GFP and MLC-2v in a GFP-expressing AD-CMG cell identified 28 days after injection.

be harvested and subsequently cultured in liquid BHK21 medium. Culture expansion is then achieved by plating every 2 days cells released in suspension without any treatment by proteolytic enzyme. We then distinguish a poorly adherent rounded cell population from an adherent elongated cell population.

Cardiac-like cells obtained in methylcellulose clusters were strictly expressing various cardiac proteins, with the exception of α-MHC,[12] probably because of limited differentiation level *in vitro*. In this study we found that such a feature is maintained at least during the first passage when cells are transferred in BHK21 culture conditions. With increasing number of passages, cardiac protein expression was no longer detected by immunostaining even though MLC-2v and MEF-2c genes were still expressed (RT–PCR data not shown). In parallel, AD-CMG cells co-expressing cardiac and skeletal proteins were detected. In parallel, the skeletal phenotype was maintained at the protein and mRNA levels, suggesting that this medium could induce or select myogenic differentiation process from AD-CMG cells. Regardless of the answer, the myoblastic phenotype of AD-CMG was never detected after *in vivo* transplantation.

Among the different antigens, AD-CMG cells are $CD29^+$, $CD44^+$, $CD81^+$ but $CD31^-$, $CD34^-$, $CD45^-$, $CD90^-$, $CD117^-$ (c-kit), and $Flk\text{-}1^-$. This phenotype does not correspond to any phenotype already described. Particularly, they are almost negative for CD34 as well as for CD90, which are specific features of ADSCs in primary culture. CD34 expression tends to decrease with successive passages, but ADSCs remain $CD90^+$ like bone marrow MSCs.[4] This strongly suggests that AD-CMG cells do not belong to the classic stromal cell lineage. This is consistent with the differentiation potentials displayed by both adipose-derived cell types. Indeed, the adipogenic, osteogenic, and chondrogenic potential of ADSCs is strongly impaired in AD-CMG cells. A recent work reports that CD29 is an efficient marker to detect and select cardiogenic progenitor cells from brown adipose tissue.[12] This marker is not decisive

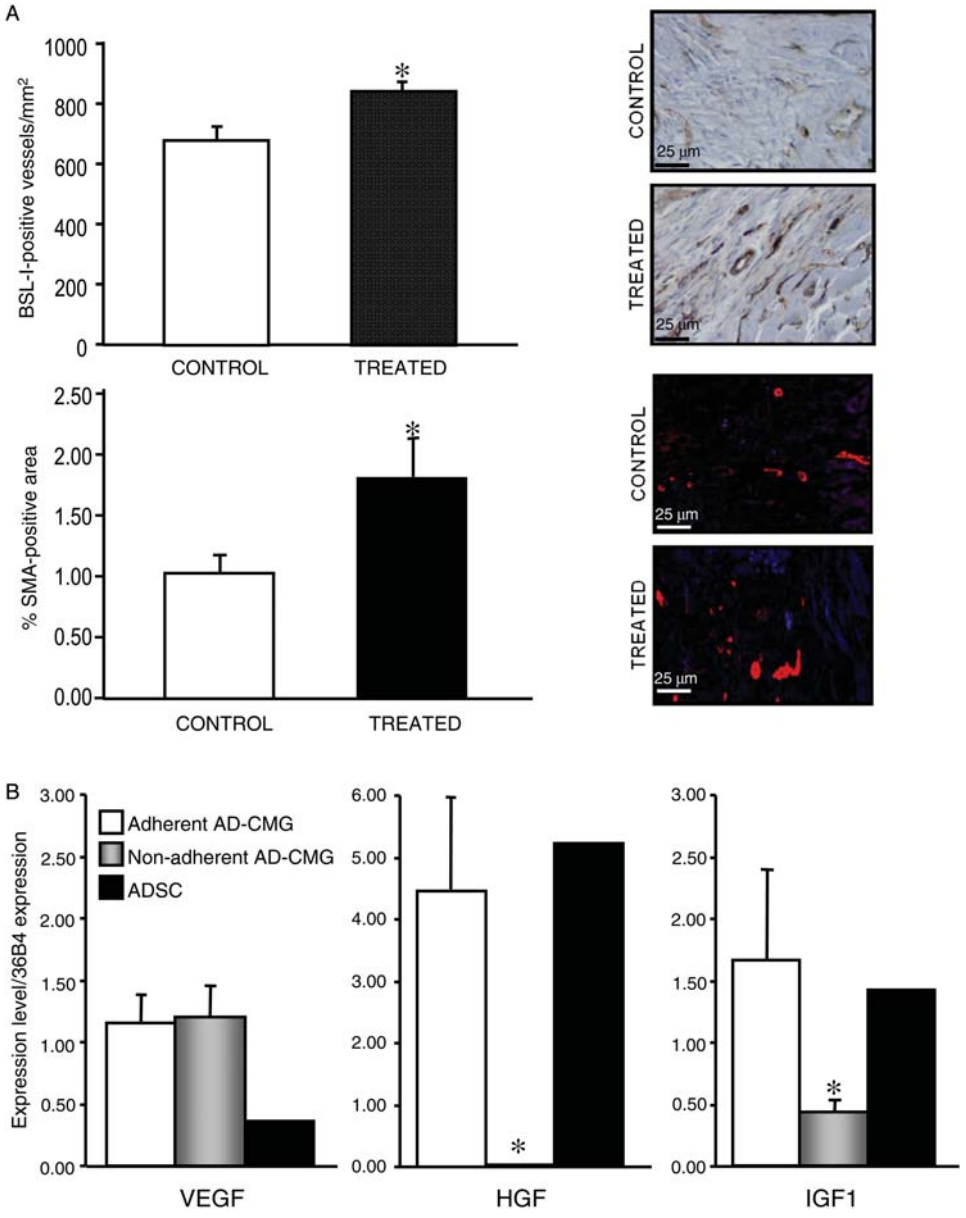

Figure 6 Neovascularization after AD-CMG transplantation. (*A*) Angiogenesis was determined by the number of lectin-positive and α-SMA-positive vessels per square-millimetre in the infarct area in the cell-treated and control group of animals. Images represents lectin-stained sections and α-SMA-positive sections. (*B*) Quantitative RT–PCR to determine the expression level of VEGF, HGF, and IGF1 in adherent and non-adherent AD-CMG in culture.

for cardiac differentiation, since ADSCs in liquid medium also expressed CD29 (95%, data not shown) without initiating any cardiac differentiation *in vitro* and *in vivo*. Adherent and non-adherent populations can be clearly distinguished by CD73 and CD38 expression. As many other adherent cell types express CD73 classically referred to as a mesenchymal marker,[14] the physiological relevance of its expression remains completely open and needs to be investigated. The other differential marker CD38 is not yet described as expressed by cardiogenic cells. It is a multifunctional enzyme that generates two potent $Ca2^{+}$-releasing signal metabolites.[15] Its ligand is CD31 and it can interact with connexin 43. Among various phenotypes, including smooth muscle cells, it is also expressed by haematopoietic precursors. Taken together, the results show that different parameters argue in favour of heterogeneity in AD-CMG cells as morphology, adhesion properties, and antigen features.

In vitro optimal selection, pre-commitment, and expansion are decisive to insure AD-CMG cells survival *in vivo* but are not sufficient. Our results also demonstrate that the recipient niche is critical. The experimental model of ischaemic myocardium with fresh infarction (used in our study and others),[12] is an environment prone for our cells to survive and to reach a cardiac phenotype, whereas a chronic infarction model is not (see Supplementary material online, *Table S2*). As the expression of numerous factors (cytokines, growth factors, extracellular matrix proteins, etc.) changes during the myocardial infarction evolution and can influence the fate of the grafted cells,[16] the crucial signals are difficult to delineate. To the same extent, co-administration of a mixture of the two AD-CMG cell types from the same culture (adherent and non-adherent populations) survived, whereas the same cell populations fail when administrated separately.

Functional outcomes after injections of AD-CMG cells indicate that they can overcome the natural evolution of ischaemic cardiomyopathy, preventing dilatation of the LV. This anti-remodelling effect has often been attributed to a paracrine mechanism of action of various grafted cell types.[2] If this mechanism can partly explain the observed effect, cardiac features and spatial organization of these grafted cells suggest that they could also actively participate in mechanical function. However, only limited engraftment evidences is provided here, as no direct coupling of AD-CMG cells to host tissue could be identified (expression of connexin 43 in GFP cells). This last point would need to be precisely investigated by specific experiments on coupling.[17] Thus, our data suggest that significant cardiomyogenesis of AD-CMG cells occurs after their transplantation and is also probably supported by paracrine effect on host tissue regeneration, as the amount of differentiated transplanted cells could not account for all functional recovery by itself. A non-specific fusion event associated with observed cardiac regeneration cannot be excluded but cannot explain by itself our results because no cardiomyocyte phenotype was observed when a single cell population was engrafted. Moreover, it was excluded in another study using cardiomyogenic cells derived from adipose tissue in which authors concluded that effective repair depended at least on the differentiation of adipose tissue cells into cardiomyocytes.[12] An additional mechanism taking place in this functional recovery is neovascularization. We evidenced that the number of capillaries and larger vessels is increased in cell-treated animals. To date, no direct evidence highlights a vascular differentiation potential *in vivo*, as no AD-CMG co-expressing GFP and lectin or α-SMA could be identified in heart samples analysed. Secretion of pro-angiogenic and anti-apoptotic factors by AD-CMG can be assumed and corroborates significant expression levels of VEGF and HGF in AD-CMG cells in culture. Myoblast markers were never evidenced during our investigations. This can be explained using early passages deprived of myoblast-committed cells. However, it is reasonable to propose that an acute ischaemic situation in the heart is an appropriate situation to direct AD-CMG cell fate into cardiac lineage more than to the myoblast phenotype.

In conclusion, the existence and selection of adipose-derived cardiomyogenic cells allow obtaining from a few adipose tissues large amounts of cells for *in vivo* administration, thanks to an optimized three-step culture procedure. Implanted in an adapted acute ischaemic environment, these cells can survive and acquire cardiac features with beneficial effects preventing remodelling and impairment of LV function. These promising results have to be reproduced in other species. Obviously, further studies are required from these seminal results to open the way to a putative clinical application. Indeed, it will be necessary, on one hand in acute myocardial infarct to finely investigate the comparative benefit of AD-CMG or ADSC transplantation according to the duration of their purification process and the short time available for the cell transplantation and, on the other hand, in chronic myocardial infarct to understand why these cells cannot be efficiently transplanted.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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