

# Long-term benefit of intracardiac delivery of autologous granulocyte–colony-stimulating factor-mobilized blood CD34<sup>+</sup> cells containing cardiac progenitors on regional heart structure and function after myocardial infarct

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## Background aims

Starting from experimental data proposing hematopoietic stem cells as candidates for cardiac repair, we postulated that human peripheral blood (PB) CD34<sup>+</sup> cells mobilized by hematopoietic growth-factor (G-CSF) would contain cell subpopulations capable of regenerating post-ischemic myocardial damages.

## Methods

In a phase I clinical assay enrolling seven patients with acute myocardial infarct, we directly delivered to the injured myocardium autologous PB CD34<sup>+</sup> cells previously mobilized by G-CSF, collected by leukapheresis and purified by immunoselection. In parallel, we looked for the eventual presence of cardiomyocytic and endothelial progenitor cells in leukapheresis products of these patients and controls, using flow cytometry, reverse transcription-quantitative (RTQ)-polymerase chain reaction (PCR), cell cultures and immunofluorescence analyzes.

## Results

The whole clinical process was feasible and safe. All patients were alive at an average follow-up of 49 months (range 24–76 months).

Improvement of heart function parameters became obvious from the third month following cell reinjection. Left ventricular ejection fraction values progressively and dramatically increased with time, associated with PetScan demonstration of myocardial structure regeneration and revascularization and New York Heart Association (NYHA) grade improvement. Furthermore, we identified PB CD34<sup>+</sup> cell subpopulations expressing characteristics of both immature and mature endothelial and cardiomyocyte progenitor cells. In vitro CD34<sup>+</sup> cell cultures on a specific medium induced development of adherent cells featuring morphologies, gene expression and immunocytochemistry characteristics of endothelial and cardiac muscle cells.

## Conclusions

Mobilized CD34<sup>+</sup> cells contain stem cells committed along endothelial and cardiac differentiation pathways, which could play a key role in a proposed two-phase mechanism of myocardial regeneration after direct intracardiac delivery, probably being responsible for the long-term clinical benefit observed.

## Keywords

Acute myocardial infarct, cardiomyocyte, CD34<sup>+</sup>, cell therapy, stem cells.

## Introduction

When Orlic and his group documented the efficacy of murine lineage-negative *c-kit*-positive (Lin<sup>-</sup>*c-kit*<sup>+</sup>) bone marrow (BM) cell transplantation for repairing experimental myocardial infarction [1], they were a trigger for rapid clinical application in patients with acute myocardial infarction (AMI). More than 10 clinical trials using mostly autologous BM mononuclear cells (MNC) have now been completed, showing disparate results in terms of heart function improvement [2–12]. Although they demonstrate both the feasibility and safety of intracoronary or direct intramyocardial cell reinfusion, they do not answer very crucial issues. First, BM MNC represent very heterogeneous cell populations, making it impossible to determine which cell type, if any, would be implied, once reinfused, in cardiac repair, and estimate which doses should be reinfused. Moreover, patients enrolled in most clinical trials have relatively preserved ventricular function with low risk of death or development of congestive heart failure (CHF), creating a challenge for demonstrating significant improvements in cardiac function. The enrolment of patients with poor prognosis and the most favorable risk–benefit ratio thus makes sense [13,14].

Among BM MNC, hematopoietic stem cells (HSC) were first proposed as the best candidates for cardiac repair in animals [1,15]. Since 2003, several experimental works using human HSC, bearing CD34 and CD45 antigens (Ag), in SCID mice have strongly suggested that they can differentiate into cardiomyocytes and endothelial progenitors [16,17].

We hypothesized in 2002 that subpopulations of peripheral blood (PB) CD34<sup>+</sup> cells mobilized by granulocyte–colony-stimulating factor (G-CSF) would have the capacity to regenerate post-AMI lesions in humans. We effectively identified in mobilized CD34<sup>+</sup> cells the presence of small cell subpopulations characterized as endothelial and cardiomyocyte progenitors. At the same time, we started a phase I clinical assay, of which preliminary results have been reported previously [18], to assess the effects of direct intracardiac delivery of immunoselected PB CD34<sup>+</sup> cells in AMI patients with poor short-term prognosis and scheduled for coronary artery bypass graft (CABG) surgery.

## Methods

### *In vitro* studies

#### *Cell sources*

G-CSF-mobilized PB cells were obtained from fresh or thawed leukapheresis products (LKP). LKP were harvested

from 12 cancer patients (five multiple myelomas, three lymphomas, four acute leukemia in complete remission) scheduled to undergo HSC transplantation (controls), and from seven cardiac patients enrolled in the clinical study. Informed consent and ethical committee approval were granted in all cases.

#### *CD34<sup>+</sup> cell isolation*

In all cases but four controls (numbers 6–9), fresh CD34<sup>+</sup> cells were directly immunoselected from LKP using the clinical Isolex 300i device (Baxter Healthcare, Deerfield, IL, USA). CD34<sup>+</sup> cells from controls 6–9 were secondarily isolated from thawed LKP MNC samples using a CD34 Microbead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

#### *Flow cytometry*

A cell fixation and permeabilization IntraStain Kit (Dako, Cytomation, Trappes, France) was used for cardiomyocyte progenitor detection. CD34<sup>+</sup> cells were incubated with a monoclonal antibody (MAb) against human cardiac Troponin T (cTroponin T; 1:200; clone 1C11; Clinisciences, Montrouge, France) or an anti-human desmin MAb (1:50; clone D33; Beckman Coulter, Roissy, France). IgG1 (BD, Le Pont de Claix, France) was used as a negative isotypic control. F(ab')<sub>2</sub> fragments of goat anti-mouse Ig (1:100; Dako) conjugated with fluorescein isothiocyanate (FITC) were added to cells labeled with unconjugated primary antibodies (Ab). Cells were first incubated with peridinin-chlorophyll protein (PerCP)-conjugated CD45 and phycoerythrin (PE)-conjugated CD34 MAb (BD) for surface staining, then measured by flow cytometry (FCM). The number of positive cells was compared with FITC-, PE- and PerCP-conjugated IgG isotype controls (BD).

For endothelial progenitor cell (EPC) detection, CD34<sup>+</sup> cells were incubated with PE-conjugated VEGFR-2 (clone 89106; R&D Systems, Lille, France), FITC–CD34 (clone 8G12; BD), allophycocyanin (APC)-conjugated CD133 (clone AC133; Miltenyi Biotec, Paris, France) and PerCP–CD45 (clone 2D1) MAb. Gates were established by non-specific immunoglobulin binding in each experiment.

#### *In vitro differentiation of mobilized PB CD34<sup>+</sup> cells*

We have settled a specific culture medium (MV06™) to allow cell differentiation into both the endothelial and cardiac muscle cell pathways. Iscove's modified by Dulbecco medium (IMDM; Fischer Scientific Bioblock

**Table I.** Inclusion criteria.

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Age $\leq 70$ years
Transmural AMI having occurred between 14 days and 6 months before CABG
Left ventricular ejection fraction (LVEF) $\leq 35\%$
Distinct area of akinetic left ventricular myocardium corresponding with the infarct localization
Distinct area of non-viable and non-perfused left ventricular myocardium at PetScan examination after successive intravenous injections of $^{18}\text{F}$ -FDG and of $^{201}\text{Tl}$ -chloride, respectively
Class IV exercise capacity according to the New York Heart Association (NYHA) criteria
Non-reperusable ischemic area
Indication for scheduled by-pass surgery on coronary arteries other than the obstructed vessel(s)
Absence of any valvular disease

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Invitrogen, Illkirch, France) was supplemented with 12.5% fetal calf serum (FCS), 2.5% horse serum, 1% glutamin, 1% antibiotics and a cocktail of growth factors (BMP2, FGF2 and VEGF; AbCyS, Paris, France), then displayed at a density of  $2 \times 10^6$  cells on fibronectin/gelatin-coated 12-well plates or  $3 \times 10^5$  cells on four-chamber slides (LabTek II, Nunc, Dominique Dutscher, Brumath, France). Cells were maintained in culture for 7 and 14 days.

#### *Reverse transcription-quantitative-polymerase chain reaction analysis*

CD34<sup>+</sup> cells at day 0 and cells adherent on 12-well plates were collected after 7 and 14 days. Total RNA extraction was realized using the RNeasy Extraction Kit (Qiagen, Courtabeuf, France). Reverse transcription (RT) analysis was carried out from 400 ng of total RNA using a cell-to-cDNA II kit (Ambion, Huntingdon, UK). Reverse transcription-quantitative (RTQ)-polymerase chain reaction (PCR) analysis was performed using a Quantitect-Probe Kit (Qiagen) with oligonucleotides and Taqman probes from assay-on-demand (Applied Biosystems, Foster City, CA, USA) corresponding to cTroponin T, Nkx2-5, endothelial NOsynthase (eNOS) and VEGFR-2. Gene expression was determined by the  $\Delta\Delta\text{Cp}$  method from PCR realized in duplicates for controls and patients.

#### *Immunofluorescence analysis*

CD34<sup>+</sup> cells spread out on glass slides at day 0 and cells adherent on LabTek II slides at 14 days of culture were permeabilized with methanol, blocked with phosphate-buffered saline (PBS)-5% bovine serum albumin (BSA)

and incubated with primary Ab for 2 h at 37°C. Secondary Ab were applied for 1 h at room temperature, in the dark. Slides were mounted with Vectashield medium and examined using an inverted fluorescence microscope.

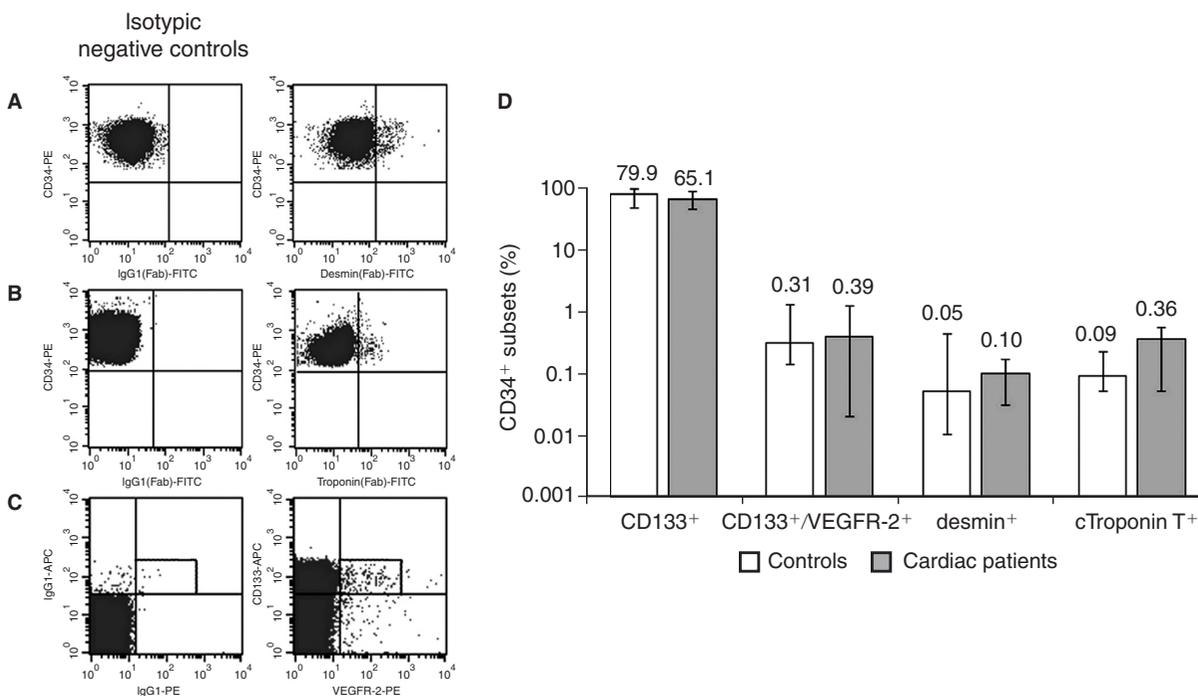
Ab used were specific for von Willebrand Factor (vWF) (rabbit polyclonal Ab; 1:100; Dako), cTroponin T (mouse MAb; 1:100; Clinisciences) and  $\alpha$ -sarcomeric actin (mouse IgM; 1:400; Sigma-Aldrich, Saint-Quentin Fallavier, France). Secondary anti-mouse IgG1-tetra methyl rhodamine isothiocyanate (TRITC), anti-mouse IgM-FITC and anti-rabbit Ig-FITC (1:100) were from Beckman-Coulter.

Newborn rat cardiomyocyte primary cultures and a HUVEC cell line (PromoCell GmbH, Heidelberg, Germany) were used as positive controls.

### **Clinical study**

After ethic committee approval and patients' informed consent, we started a phase I clinical trial to assess the feasibility, safety and potential impact on cardiac function of G-CSF mobilization, collection, selection and intracardiac reinjection of autologous blood CD34<sup>+</sup> cells. The patient selection criteria are summarized in Table I. Patients were assessed before entering the trial and at 3, 6 and 12 months post-surgery, with three-dimensional echocardiography (Echo 3D; allowing left ventricular ejection fraction (LVEF) determination),  $^{201}\text{Tl}$  scintigraphy and positron emission tomographic scans (PetScan). Echo 3D examinations were always performed by the same investigator according to a previously validated protocol, and blindly reviewed by two cardiologists. Patient follow-ups were continued for as long as possible.

CD34<sup>+</sup> cell mobilization was started 7 days before CABG with subcutaneous injections of G-CSF (Granocyte®), 5  $\mu\text{g}/\text{kg}$  twice daily for 5 consecutive days. The apheresis procedure was performed from the sixth mobilization day using an AS 104 cell separator (Fresenius, Bad Homburg, Germany), with the goal of collecting at least  $1 \times 10^8$  CD34<sup>+</sup> cells recommended for further satisfactory cell selection procedures. When collection was poor, a second apheresis session was performed early on the morning of the 7th day. In any case, the bag containing the 6th day cell product was stored overnight at 4°C. The whole apheresis product was processed for CD34<sup>+</sup> immunoselection through the clinical Isolex 300i magnetic cell-separation device (Baxter Healthcare, Deerfield, IL, USA) according to the manufacturer's instructions. CD34<sup>+</sup> cells were then released from the beads, resuspended in



**Figure 1.** Flow cytometric determination of endothelial and cardiac muscle markers in CD34<sup>+</sup> cells purified from leukapheresis. Quadrant analysis of CD34<sup>+</sup> cells (A–C): the results are shown as bivariate dot-plots of CD34–PE fluorescence versus F(ab')<sub>2</sub>–FITC fluorescence desmin (A) or F(ab')<sub>2</sub>–FITC fluorescence cTroponin T (B) for muscle cell subpopulations. For immature endothelial progenitors, expression of VEGFR-2–PE was analyzed versus CD133–APC (C). Percentages of CD34<sup>+</sup> cell subpopulations were determined in the upper right quadrants of the right row of dot-plots, in comparison with isotypic negative controls (left row of dot-plots). Median percentages of CD34<sup>+</sup> cell subsets (D): data are presented as medians and ranges for seven cardiac patients and 12 controls. The difference between the two groups was not significant (Mann–Whitney test,  $P > 0.05$ ).

100 mL autologous plasma and concentrated by mild centrifugation at a final graft volume of 15–20 mL. An additional 5 mL sample was used for FCM quantification of total CD34<sup>+</sup> cells and their subpopulations, and for sterility studies.

CABG was begun when the cell graft was definitely available, and was done with a beating heart. The cell suspension was infused throughout the left ventricular (LV) wall ischemic area by longitudinal and parallel injections of 1.5–2 mL each, just before completion of the operation. The septum was never injected.

### Statistical analysis

Non-parametric tests were used to compare means between variables. Unpaired variables (expression of each Ag and gene on CD34<sup>+</sup> cell subpopulations) were tested with the Mann–Whitney tests. Expression of genes at different times of culture, and LVEF and left ventricular ejection diastolic volume (LVEDV) at different times before and post-surgery

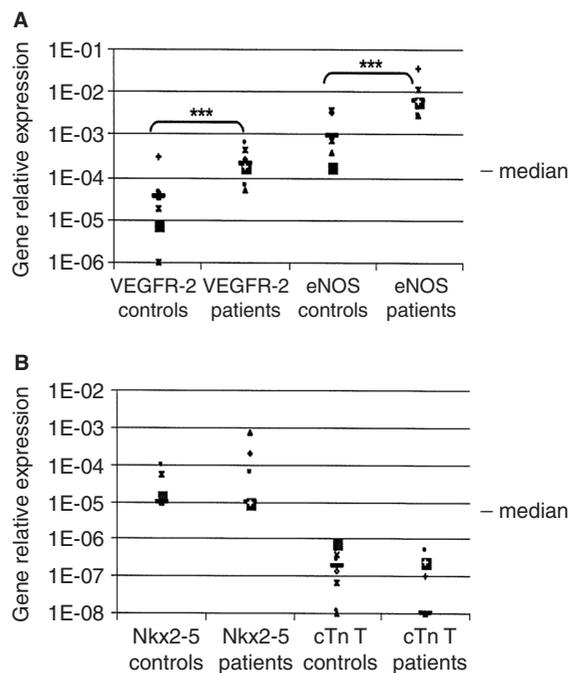
(i.e. 0 versus 3, 6, 12, 24 or 36 months post-surgery), were analyzed with the Wilcoxon-matched pairs test.

### Results

#### Already dedifferentiated endothelial and cardiac progenitor cells are present in G-CSF-mobilized CD34<sup>+</sup> cells

Phenotypically, the majority of CD34<sup>+</sup> cells purified from control LKP co-expressed CD133, marking for immature cells (median 79.9%, range 47–94.2), and CD45 Ag; among CD34<sup>+</sup> CD45<sup>+</sup> cells, a median of 0.31% (range 0.14–1.25) co-expressed CD133<sup>+</sup> VEGFR-2, 0.05% (range 0.01–0.43) co-expressed Desmin and 0.09% (range 0.05–0.22) co-expressed cTroponin T (Figure 1).

Cardiac patient results were not significantly different from those recorded for controls, except for the cTroponin T<sup>+</sup> cell subpopulation, which was four times larger in this group than in controls (median 0.36%, range 0.11–0.54). However, this difference was not statistically significant



**Figure 2.** Expression of endothelial and cardiac muscle genes in  $CD34^+$  cells purified from LKP. VEGFR-2 and eNOS (A), Nkx2-5 and cTroponin T (B) transcript levels were measured by RTQ-PCR. RNA was isolated from  $CD34^+$  cells harvested from patients with hematologic disease (controls) or with acute myocardial infarct (patients). Quantification was related to GAPDH transcript levels. Baselines were  $10^{-6}$  for VEGFR-2,  $10^{-5}$  for eNOS and Nkx2-5, and  $10^{-8}$  for cTroponin T. Data are presented as medians and ranges for seven cardiac patients and nine controls. The difference between both groups was significant for endothelial genes expression (Mann-Whitney test,  $***P < 0.01$ ).

( $P > 0.05$ ), probably because of the low numbers of controls/patients studied.

Gene expression of VEGFR-2 and eNOS for endothelial cells, and Nkx2-5 and cTroponin T for cardiac cells, was analyzed by RTQ-PCR in  $CD34^+$  cells from the seven cardiac patients and nine controls (Figure 2). The median VEGFR-2 relative expression was significantly higher in patients than in controls ( $2.1 \times 10^{-4}$ , range  $5.1 \times 10^{-5}$ – $6.5 \times 10^{-4}$ , versus  $3.3 \times 10^{-5}$ , range  $1 \times 10^{-6}$ – $2.9 \times 10^{-4}$ ;  $P < 0.01$ ). It was the same when considering the median eNOS relative expression (patients,  $5.8 \times 10^{-3}$ , range  $2.8 \times 10^{-3}$ – $3.5 \times 10^{-2}$ ; controls,  $8.8 \times 10^{-4}$ , range  $1.9 \times 10^{-4}$ – $3.6 \times 10^{-3}$ ;  $P < 0.01$ ) (Figure 2A). Cardiac gene expression was recorded near detection levels in patient and control  $CD34^+$  cells, but rarely both together (Figure 2B). Median Nkx2-5 relative expression was  $1 \times 10^{-5}$  in both

groups (range  $1 \times 10^{-5}$ – $7.7 \times 10^{-4}$  and  $1 \times 10^{-5}$ – $9.1 \times 10^{-5}$ , respectively) and the median cTroponin T was  $1 \times 10^{-8}$  (range  $1 \times 10^{-8}$ – $9.7 \times 10^{-8}$ ) in patients and  $1.9 \times 10^{-7}$  (range  $1 \times 10^{-8}$ – $7.4 \times 10^{-7}$ ) in controls. Such discrepancies might be related to the small number of cells expressing cardiac genes and variations in cell differentiation stages depending on each individual person.

Altogether, FCM and RTQ-PCR data showed that G-CSF-mobilized  $CD34^+$  cells contained small but significant amounts of cell subsets already differentiated into either endothelial or cardiac muscle progenitors. There was no difference in the numbers of putative  $CD34^+$  endothelial and cardiac muscle progenitors between frozen/thawed and non-frozen control LKP. These results are in accordance with our previous report showing that freezing/thawing processes do not alter the number of total  $CD34^+$  HSC in LKP from hematologic patients [19]. Endothelial VEGFR-2 gene expression was higher in patients than in controls, although it did not correspond to higher amounts of  $CD34^+$  cells expressing VEGFR-2.

### Endothelial and cardiac *in vitro* differentiation potential of G-CSF-mobilized $CD34^+$ cells

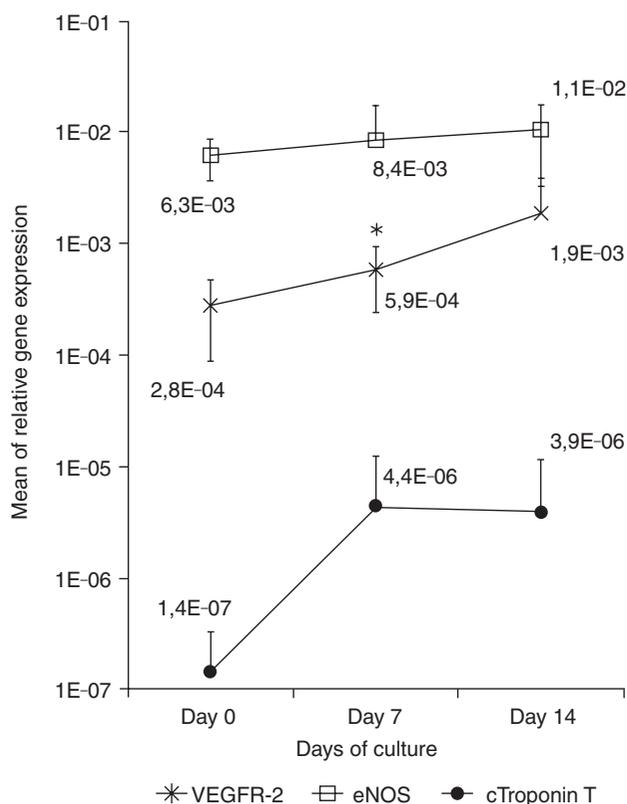
#### *In vitro* cultures

Immunoselected  $CD34^+$  cells from six cardiac patients were cultured on the MV06™ medium. After 14 days of culture, two cell populations remained adherent to the fibronectin/gelatin coating. The largest one was composed of small, round cells, the smallest was represented by large and spreading cells, in amounts varying from one patient to another. As endothelial and cardiac muscle cell populations both have an adherent phenotype, gene expression was assayed only on adherent cells. *In vitro* cell growth was not different when considering frozen/thawed or non-frozen  $CD34^+$  cells.

#### RTQ-PCR analysis

Mean VEGFR-2 expression continuously increased in adherent cells from day 0 ( $2.8 \times 10^{-4}$ ) to day 7 ( $4.9 \times 10^{-4}$ ) and day 14 ( $1.9 \times 10^{-3}$ ). In contrast, mean eNOS expression was not significantly modified ( $6.3 \times 10^{-3}$  at day 0 and  $1.1 \times 10^{-2}$  at day 14) (Figure 3).

On the cardiac side, the mean expression of cTroponin T increased (2.8 log) from day 0 ( $1.4 \times 10^{-7}$ ) to day 14 ( $3.9 \times 10^{-6}$ ). However, even if it expressed a strong trend, this increase was not statistically significant because of the low number of cases.



**Figure 3.** Expression of endothelial and cardiac muscle genes into adherent cells after culture on MV06<sup>TM</sup> medium. VEGFR-2, eNOS and cTroponin T transcript levels were measured by RTQ-PCR. RNA was isolated from CD34<sup>+</sup> cells before and after 7 and 14 days of culture. Data are presented as means and standard deviations for cells from six cardiac patients. The difference between day 0 and day 7 was significant for VEGFR-2 expression (Wilcoxon-matched pairs test, \*P<0.05).

These results suggested that the MV06<sup>TM</sup> medium was capable of enhancing cardiac muscle differentiation as well as early but not terminal endothelial differentiation, and not cellular proliferation.

#### Immunofluorescence analysis

During the period of culture, immunofluorescence staining revealed the presence of adherent cells growing in the culture on fibronectin/gelatin coatings, which strongly expressed the vWF protein. Several of them, either scattered or gathered into small clusters, around day 14 acquired a spindle-shaped aspect typical of endothelial cell morphology (Figure 4). In parallel, the presence of disseminated adherent cells, stained positively with anti- $\alpha$ -sarcomeric actin and cTroponin T Ab, became obvious at day 14, in 6/6 and 5/6 patients, respectively.

Thus the expression of endothelial and cardiac muscle genes at RNA and protein levels indicated that CD34<sup>+</sup> cells adherent to fibronectin/gelatin after culture may generate progenies capable of differentiation along endothelial and cardiomyocytic pathways.

#### Clinical study

Seven patients (five males and two females), aged 33–70 years (average 53), had undergone the entire procedure between December 2002 and December 2006. Their clinical data are detailed in Table II. Patients 2, 4 and 6 would have been considered for heart transplantation, because of their worse prognosis, but they lacked a readily available donor.

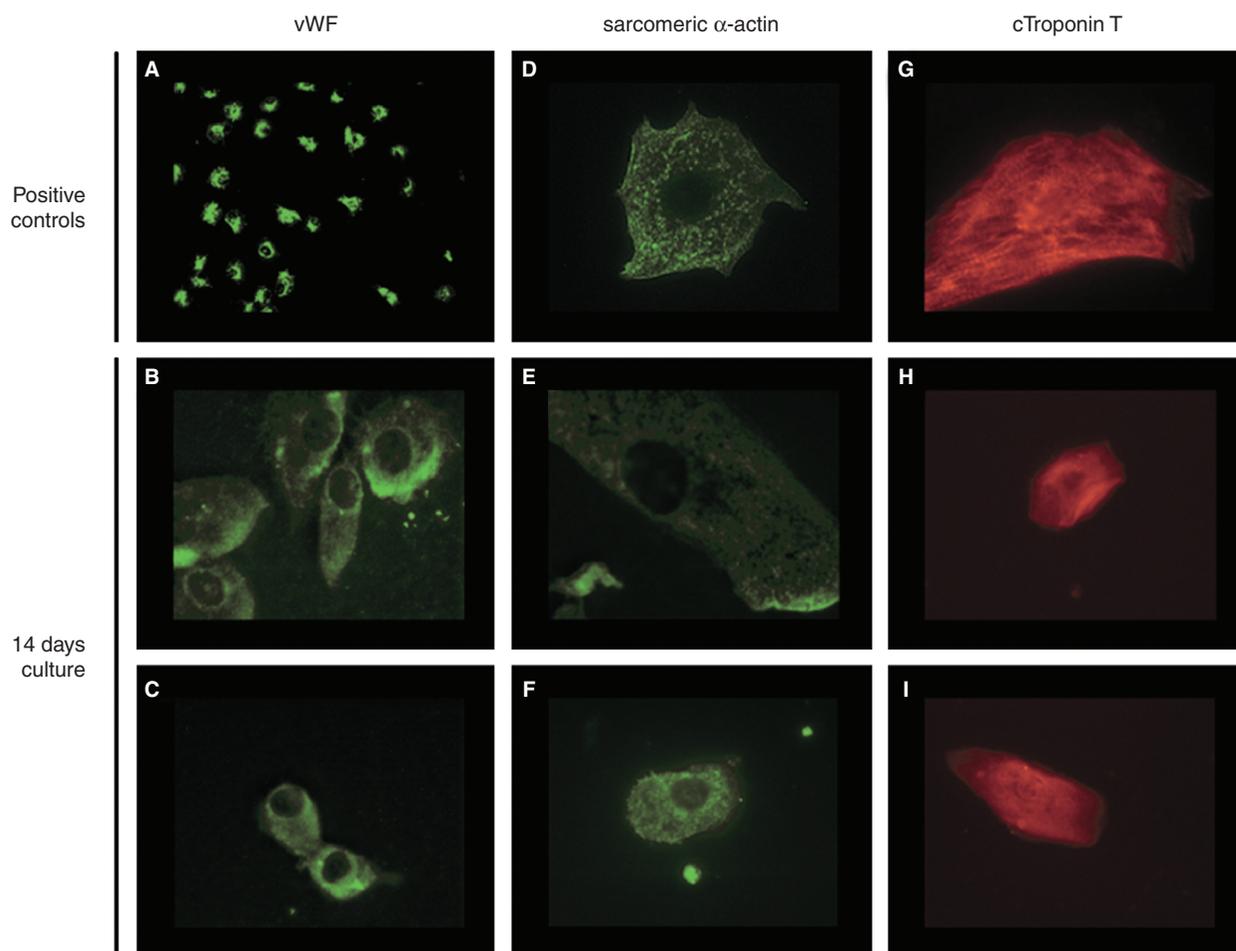
#### Procedural safety

After a 5-day G-CSF administration, total white blood cells (WBC) increased counts attested the mobilization of progenitor cells in all patients. The maximum admitted level of  $50 \times 10^6/L$  WBC was reached in only two patients at the end of the mobilization period, which did not necessitate disrupting the G-CSF administration before its scheduled end-point. G-CSF-induced leucocytosis was never associated with relevant clinical symptoms, electrocardiographic changes or myocardial enzyme release, and apheresis did not cause any hemodynamic compromises. Only transient thrombocytopenia was recorded after apheresis, two patients needing platelet transfusions before surgery. Platelet and WBC counts always returned to baseline values from day 3 to day 4 post-surgery. All patients were discharged from hospital to the rehabilitation program around the 7th day.

No immediate or later specific side-effects were recorded after cell reinjection. In particular, no significant bleeding or malignant supra-ventricular arrhythmia was observed. Only patient 2 developed a relevant pericardial effusion 1 month after CABG, easily managed without any sequelae.

#### Patient outcome

All patients were alive at the time of preparation of this paper, with a rather long and unexpected average follow-up (Fu) of 49 months (range 24–76) (Table III). They all showed a significant improvement (although to varying degrees) of cardiac parameters, except patient 1 who had undergone AMI 8 years before CABG and presented a totally calcified infarcted zone at the time of cell therapy, which retrospectively was clearly not a good indication in



**Figure 4.** Detection of endothelial and cardiac muscle protein into adherent cells after culture on MV06™ medium. Indirect immunofluorescence staining of positive controls (A, D, G) and 14-day cultured adherent cells (magnification  $\times 1000$ ) (B, C, E, F, H, I) using anti-vWF (FITC) (A–C), anti-sarcomeric  $\alpha$ -actin (green) (D–F) and anti-cTroponin T (red) (G–I) labeling. HUVEC (magnification  $\times 200$ ) (A) and newborn rat cardiomyocyte primary cultures (magnification  $\times 1000$ ) (D, G) were used as endothelial and cardiac muscle cell positive controls, respectively.

this case. We thus decided to limit the period of inclusion to 6 months post-AMI for the following patients. Altogether, the patients' LVEF values progressively increased, gaining on average 11, 14, 16 and 18 points from the baseline values at 6, 12, 18 and 24 months Fu, respectively (+38, +50, +57 and +64% average improvement rates, respectively) (Figure 5). The LVEF increase was correlated in patients 2–7 with a striking regeneration and revascularization of the infarcted zone at PetScan examination (Figure 6), recovery of contractility and important improvement of NYHA grade. LVEDV also decreased in 5/7 patients, from an average of  $177 \pm 34$  mL pre-operatively to  $125 \pm 33$  mL at 12 months, but, although expressing an interesting trend, this decrease was statistically non-significant ( $P > 0.05$ ) because of the low number of patients. Heart transplantation

was once more considered for patient 4, who had required implantation of an automatic defibrillator before CABG, when his LVEF value decreased to 15% at 3 month Fu. But from the 6th month all evaluation parameters began to obviously improve, and the patient did not require a heart transplant, being in a much improved clinical condition (+16 points of LVEF from month 3 to month 24). LVEF rapidly improved from 27 to 40% in patient 5 (who had previously undergone an inferolateral AMI 10 years before the present anteroseptal one), when a supraventricular arrhythmia access occurred on the 9th month, associated with a drastic drop of LVEF value, requiring implantation of a defibrillator. He was then well, with his LVEF value progressively reincreasing. Furthermore, PetScan examination showed, from 1 year Fu, a significant regeneration

**Table II.** Patients and procedure-related baseline data.

Variables	1	2	3	4	5	6	7	Average/ratio
Age (years)	39	49	61	33	70	63	57	53,4
Gender	M	F	F	M	M	M	M	5/2
Infarct time (weeks)	420	6	12	6	25	12	8	68
NYHA	IV	IV	IV	IV	IV	IV	IV	–
CA stenosis (n)	LAD, LCx, Mg	LAD, LCx, RCA	LAD, LCx, RCA	LAD, RCA	LAD/D <sub>1</sub> junction, LCx	LAD, LCx, Mg <sub>1</sub>	LAD, LCx, LCx	–
Target area	Ant. septal	Ant. apex	Ant. later apex	Ant. dorsal apex	Ant. septal apex	Ant. septal apex	Ant. septal apex	–
Infarct size (% left anterior wall)	30	22	16	60	32	40	48	36
Apheresis (n)	2	1	2	1	1	2	2	1,5
Number of CD34 <sup>+</sup> cells harvested (×10 <sup>6</sup> ) <sup>a</sup>	94	109	127	245	148	119	162	143
Cell doses reinjected (×10 <sup>6</sup> ) <sup>b</sup>								
CD34 <sup>+</sup>	29.1	40.3	43.8	107.6	41.0	46.5	55.1	51.9
CD34 <sup>+</sup> CD133 <sup>+</sup>	18.0	35.3	36.5	68.3	18.3	30.3	36.8	34.8
CD34 <sup>+</sup> CD133 <sup>+</sup> VEGFR-2 <sup>+</sup>	0.006	0.16	0.22	0.17	0.46	0.57	0.34	0.28
CD34 <sup>+</sup> Troponin T <sup>+</sup>	0.16	ND	0.22	0.21	0.21	0.02	0.07	0.14
Number of bypasses <sup>c</sup>	1 (LCx)	1 (D <sub>1</sub> )	1 (LAD)	1 (LAD)	3 (LAD, D <sub>1</sub> , LCx) <sup>a</sup>	1 (D <sub>1</sub> )	2 (LAD, LCx)	
Early complication (0–6 months)	0	Pericardial effusion (day 30)	0	0	0	0	0	
Hospital stay post-CABG (days)	7	7	6	8	11	7	6	7.5

<sup>a</sup>The target cell count before immunoselection was achieved in more or less all patients.

<sup>b</sup>After CD34<sup>+</sup> selection.

<sup>c</sup>Vessels grafted. LAD, left anterior descending artery; LCx, left circumflex artery; Mg, marginal artery; D<sub>1</sub>, LAD diagonal artery; RCA, right coronary artery; LMC, left main coronary artery; ND, not detected.

**Table III.** Patient outcome at 1-year post-cell transplant.

Patient	LVEF (%) day 0 <sup>a</sup> / 12 months <sup>b</sup>	LVEDV (mL) day 0 <sup>a</sup> / 12 months <sup>b</sup>	Damaged segment area	PetScan			Functional class NYHA grade day 0 <sup>a</sup> /12 months <sup>b</sup>
				Viability (no. seg. improved) <sup>c</sup>	Perfusion (no. seg. improved) <sup>c</sup>	Area kinesis <sup>d</sup> (- to +++)	
1	34/38	140/153	Anteroseptal	0/8	0/8	-	IV/III
2	30/44	157/118	Anterior	6/8	5/8	+++	IV/I
			Apex	1/1	1/1	+	
3	33/61	125/100	Anterolateral	6/8	6/8	+++	IV/I
			Apex	0/1	0/1	-	
4	21/23	238/327	Anterodorsal	4/8	3/8	+	IV/II
			Apex	0/1	0/1	-	
5	27/32	188/158	Anteroseptal	8/10	7/10	++	IV/II
			Apex	0/1	0/1	-	
6	20/53	226/83	Anteroseptal	6/8	6/8	++	IV/I
			Apex	1/1	1/1	+	
7	30/42	190/168	Anteroseptal	5/8	5/8	++	IV/I
			Apex	0/1	0/1	-	

<sup>a</sup>Before CABG surgery.

<sup>b</sup>After CABG surgery.

<sup>c</sup>Viability/perfusion: the first number indicates the number of myocardial segments showing restored viability or perfusion after surgery; the second number indicates the number of myocardial segments initially damaged by the ischemic event.

<sup>d</sup>Area kinesis ranging from persisting total akinesis (-) to totally restored kinesis (+++).

of the ischemic zone that had been treated by cell injection, contrasting with the 'dumb' scar of the first non-treated inferolateral AMI, which thus appeared as an internal negative control (Figure 6G–J).

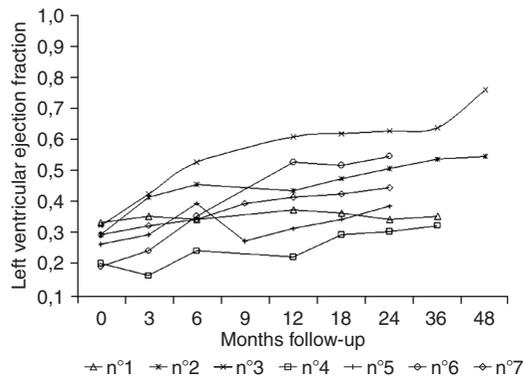
## Discussion

Very recently, the concept of regenerative medicine has been applied to heart regeneration attempts. A consensus of opinion currently suggests that, at least in the immediate future, adult stem/progenitor cells will be used [20]. However, the type(s) and amount of stem/progenitor cells to be used, the cell suspension volume to be delivered, the time and the route of cell reinjection remain controversial.

Different sources of adult stem cells have been proposed. In-scar transplantation of *in vitro*-expanded skeletal myoblasts after AMI did not reliably reproduce a similar left-ventricular function improvement in humans in the way it was observed experimentally in animals, and was additionally hindered by the frequent occurrence of severe supraventricular arrhythmia [21]. Multipotent adult progenitor cells (MAPC) only emerge from BM mesenchymal stromal cells (MSC) after more than 120 population doublings in *in vitro* culture, suggesting that their

phenotype might be cell-culture related: this unsettling point makes their clinical use difficult for the moment [22]. Once reinjected into a damaged myocardium, BM MSC only reduced the stiffness of the scar and attenuated post-infarction remodeling, but did not regenerate contracting cardiomyocytes; such mild effects are limited further by poor cell viability after transplantation [23].

Several investigators have identified novel pluripotent stem cells from both mice and human BM, which might be a small subpopulation of CD34<sup>+</sup> cells, precisely separated from the majority by their adherent properties to plastic [24]. They also adhere to fibronectin and fibrinogen, and might interact with BM-derived stromal fibroblasts [25]. These rare cells, 1% of the total CD34<sup>+</sup> population, ≈0.02% of BM MNC [24,25], are morphologically and immunophenotypically different from HSC and MSC. They can self-reproduce in culture without loss of multipotency, or differentiate into cells expressing genes corresponding to multiple tissue types issued from all three germ layers [25]. They are also found in cord blood [26], can be mobilized into blood by growth factors [24,26] and can be expanded clonally [24,27]. Thus these cells might be considered 'embryonic-like' cells, maybe deposited early



	Months					
	0	3	6	12	18	24
LVEF Average	0,28	0,32	0,39	0,42	0,44	0,46
Standard Deviation	0,05	0,09	0,08	0,12	0,10	0,11
Enhancement from baseline	-	16%	38%	50%	57%	64%
p values	-	Not significant	<0,05	<0,05	<0,05	<0,05

**Figure 5.** LVEF. Progressive increase of LVEF values over time in 6/7 cardiac patients. Data were analyzed with the non-parametric Wilcoxon-matched pairs test.

during development in the BM, and could be a source of pluripotent stem cells for tissue/organ regeneration [28].

Our data show on the same lines that G-CSF-mobilized CD34<sup>+</sup> cells contain cells featuring immunophenotypic and gene characteristics of both endothelial and cardiac muscle progenitor cells. Distinct CD34<sup>+</sup> VEGFR-2<sup>+</sup> cell subsets displaying phenotypic characteristics of either mature vascular endothelial (eNOS and vWF) cells or endothelial progenitors (co-expressing AC133, but not eNOS and vWF), have been identified in circulating blood and after G-CSF mobilization [15]. When injected intravenously in nude rats after experimental AMI, these cells directly induced vasculogenesis and angiogenesis in the infarct bed. However, very little effect on local contractility was observed in clinical assays of intracardiac and intracoronary infusion of relatively large amounts of EPC immunoselected from BM harvests [10,29] or LKP [30], although a pronounced improvement of myocardial perfusion was noted. These little clinical effects could imply that these cells facilitate neoangiogenesis but not cardiac muscle regeneration [10].

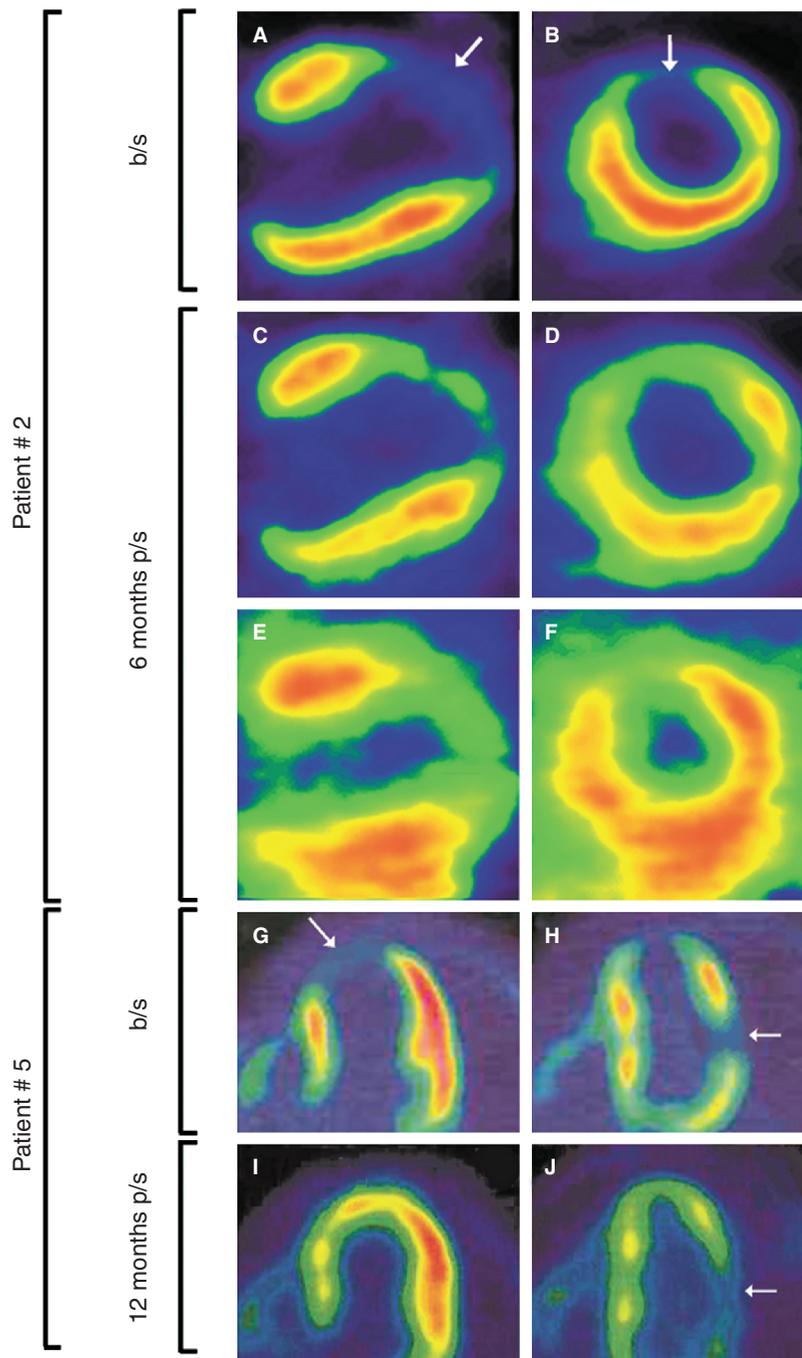
In contrast, the ability of HSC to actually transdifferentiate into cardiomyocytes is still strongly debated. Conflicting results obtained from different groups might relate to differences in the profile of the cells studied. In mice, for example, highly purified Lin<sup>-</sup>c-kit<sup>+</sup> Sca-1<sup>+</sup> HSC cells failed to undergo cardiomyogenic differentiation once

transplanted into recipient injured hearts [31,32]. On the other hand, investigators who reinjected human BM and PB CD34<sup>+</sup> cells in SCID mice injured hearts have suggested that these cells or their progeny actually transdifferentiate, either *in vitro* [16] or *in vivo* [17], into cardiomyocytes, endothelial cells or smooth muscle cells.

In fact, the results of these studies might be much less contradictory than they seem to be. Indeed, while human HSC, phenotypically characterized as CD34<sup>+</sup> CD45<sup>+</sup> CD38<sup>-</sup>, only represent a low percentage of the total CD34<sup>+</sup> population [33], none of the above investigators who reinjected human CD34<sup>+</sup> cells into mice hearts investigated other CD34<sup>+</sup> subpopulations before reinjection. Thus, considering both experimental mice-to-mice studies and our own data in humans, it is likely that the cells responsible for cardiac regeneration, if any, would not be 'true' HSC but CD34<sup>+</sup> subsets already dedicated to other cell lineages.

Here, we clearly demonstrate that CD34<sup>+</sup> cell subpopulations, representing approximately 0.5% of the total CD34<sup>+</sup> cells mobilized by G-CSF and expressing molecular and phenotypic characteristics of both immature and more committed cardiomyocyte progenitors, are present besides EPC in LKP. Additionally, *in vitro* culture of still undifferentiated CD34<sup>+</sup> cells in our MV06<sup>TM</sup> medium induces further development of adherent cell progenies expressing endothelial and cardiac lineages characteristics, as revealed by RTQ-PCR analysis and immunocytochemistry, suggesting initiation of endothelial and cardiac muscle cell differentiation pathways.

The actual contribution of these CD34<sup>+</sup> cell subpopulations to post-AMI structural and functional repair remains to be determined, among other possible factors. The therapeutic impact on LVEF of CABG performed after AMI is extremely weak. For example, the average changes in LVEF recorded in control groups (CABG alone) of two randomized studies comparing CABG alone versus CABG+intracardiac reinjection of BM progenitor cells, were 5.7±2.6 and 3.4±5.5%, respectively, within 1 month of therapy [6,10]. This slight improvement was maintained but did not increase significantly more over a 6-month Fu period in both studies. It was also consistently lower than that observed after CABG+BM cell injection. An increase in the release by the BM of endogenous CD34<sup>+</sup> cardiac and endothelial progenitor cell subpopulations into the blood circulation has been reported within the first hours and for several days following AMI [34]; a return



**Figure 6.** Patients' PetScan data. Dark-blue: no viability or perfusion. Green, yellow, red: increasing viability or reperfusion. Patient 2 (A–F), left vertical row (A, C, E): longitudinal heart axis. Right vertical row (B, D, F): transversal heart axis. (A, B) Scans completed before surgery (b/s) and CD34<sup>+</sup> cell reinjection: arrows show location of the extended ischemic dead zone (dark-blue). (C, D) Six months post-surgery (p/s): evaluation of myocardial viability after intravenous injection of <sup>18</sup>Fi-FDG. Significant restoration of myocardial viability (green-colored) in the previously dead ischemic zone, except in the apex. (E, F) Striking improvement of perfusion (green-colored) of the initially non-perfused ischemic zone, visualized after intravenous injection of <sup>201</sup>Ti-chloride. Patient 5 (G–J): this patient had undergone a first AMI (inferolateral) 10 years previously. Scans completed after intravenous injection of <sup>18</sup>Fi-FDG. (G, I) Lateroseptal heart section (LV wall on the right, septum on the left): before surgery (b/s) the arrow shows the location of the recent anteroseptal infarct (dark-blue) (G). Almost complete restoration of myocardium viability 12 months after cell injection (yellow-red) (I). (H, J) Inferolateral section. The arrows show the 10-year-old inferolateral myocardial infarction (H), which did not receive cell injection and remained unchanged 1 year after surgery (J).

to normal circulating rates occurs within 1 week, supposedly related to the homing and consumption of these cells within the ischemic lesion [35]. As this phenomenon appears to be a physiologic response to AMI, its impact on ischemic scar formation/limitation is not measurable, but is clearly unable to compensate for the loss of infarcted cardiomyocytes. Experimental mouse models have suggested that post-AMI endogenous circulation and intracardiac homing of endothelial and cardiomyocytic progenitor cells might be enhanced by G-CSF administration and prevent left ventricular remodeling and dysfunction [36]. Our results, showing a four-times larger number of cTroponin T cells in cardiac compared with control LKP, might support these experimental data, even if the chemotherapies previously received by all controls would also have had a negative impact on cell mobilization. However, administration of G-CSF alone, without cell collection/reinjection, did not result in any clinical heart function improvement in a non-human primate model [37] or in humans [38]. Despite G-CSF mobilization of CD34<sup>+</sup> cells in large numbers into the systemic circulation, it is likely that homing signals are not so powerful that they can preferentially attract and retain in the heart enough cells to engraft and improve cardiac function. As a comparison, even when delivered intracoronarily, less than 10% CD34<sup>+</sup> cells, measured by radio-immunolabeling, were retained into the irreversible ischemic myocardial area 24 h after reinfusion, the remaining radioactivity being distributed mainly to the liver and spleen [29]. Thus, neither CABG alone nor spontaneous or G-CSF-enhanced CD34<sup>+</sup> cell mobilization seems able, by themselves or in combination, to explain reliably the imaging, functionally and clinically striking improvements we observed in 6/7 patients in our present phase I study.

After 6 months Fu, our patients' average LVEF improvement was indeed much more significant compared with those reported in the literature, several of them being even negative for this primary endpoint [3,5,39]. It is true that our patients were more ill than most of those enrolled in the other studies, with a numerically lower initial mean LVEF (28% versus 42–48%), making it easier to evaluate any improvement. We also reinjected much larger amounts of CD34<sup>+</sup> cells than the others, which could facilitate myocardium repair.

Even more interestingly, the heart function parameters continued to improve progressively for years post-transplant, becoming compatible with a normal quality of life, when proof of regeneration of myocardial viability and reperfusion was provided by PetScan examination. Considering the presence in cardiac patients' LKP of a

mixture of both already committed cardiac and endothelial CD34<sup>+</sup> cell subpopulations and immature CD34<sup>+</sup> cells capable of either self-renewal or further differentiation, we might thus hypothesize a two-phase mechanism to explain the progressiveness of clinical improvement. Inspired by the model of hematopoietic regeneration described after PB HSC transplantation [33], we could suggest that, after direct in-scar delivery, already committed cells initiate the processes of myocardium regeneration/revascularization. However, their low amounts (several ten to hundred thousands) would not allow a rapid replacement of the 1–2 billion cardiac cells destroyed during the ischemic event. Consequently, the clinical benefit induced by this initiating phase would be weak. Achievement of a larger and sustained restoration of myocardium viability would require a secondary *in situ* differentiation of immature CD34<sup>+</sup> cells into expanding cell progenies capable, under cytokine interaction, of progressive repair of the damaged area, clinically expressed by a progressive improvement of cardiac function. This secondary CD34<sup>+</sup> cell differentiation would be mediated by the contact with the ischemic scar, as demonstrated experimentally [16,17].

Our pilot study has also demonstrated the feasibility and safety of G-CSF administration in patients with severe heart failure. Apheresis procedures and direct intracardiac reinjection of cell suspension volumes 5–10-fold larger than those proposed by most investigators [6,7,11] were well tolerated, with no observed immediate procedure-related complications.

We and others [24] have demonstrated that the relevant stem cells are only a tiny minority of the total CD34<sup>+</sup> cells mobilized into the blood. One can then speculate that stem cell engraftment in the damaged heart would be better enhanced by direct delivery to the injured myocardium of high concentrations of these relevant stem cells rather than by intracoronary perfusion, which does not guarantee the arrival of adequate numbers of cells into the damaged area [29]. It seems logical to suggest that these relevant cells might be issued from CD34<sup>+</sup> embryonic-like cells resident in the BM [25,26] and do not correspond to the actual transdifferentiation of HSC. Considering their FCM, molecular, immunocytochemistry and *in vitro* culture characteristics, we can reasonably postulate that they are probably involved in the significant clinical benefit observed in most of our patients, depending on the associated release, either by themselves or scar cells (or maybe both), of a complex blend of cardioactive cytokines [40].

However, although the results of this study are somewhat unprecedented, we acknowledge that they suffer from several limitations, which must be taken into account when interpreting them. First, expression of cardiac markers by progenitor cells does not make them functional cardiomyocytes: co-culturing these progenitor cells with adult cardiomyocytes could help demonstrate their complete differentiation [16]. Second, the link between the proposed two-phase myocardium regeneration mechanism and clinical outcome remains speculative, as one presently lacks any efficient and safe long-term labeling of human stem cells for *in vivo* application. And finally, the clinical part of the study is limited by the modest number of patients enrolled and the absence of a control group, and needs to be confirmed by a phase II randomized study.

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### Disclosure

All authors declare that they have no conflicts of interest.

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