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In Vitro and In Vivo Cardiomyogenic Differentiation of Amniotic Fluid Stem Cells

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Abstract Cell therapy has developed as a complementary treatment for myocardial regeneration. While both autologous and allogeneic uses have been advocated, the ideal candidate has not been identified yet. Amniotic fluid-derived stem (AFS) cells are potentially a promising resource for cell therapy and tissue engineering of myocardial injuries. However, no information is available regarding their use in an allogeneic context. c-kit-sorted, GFP-positive rat AFS (GFP-rAFS) cells and neonatal rat cardiomyocytes (rCMs) were characterized by cytocentrifugation and flow cytometry for the expression of mesenchymal, embryonic and cell lineage-specific antigens. The activation of the myocardial

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gene program in GFP-rAFS cells was induced by co-culture with rCMs. The stem cell differentiation was evaluated using immunofluorescence, RT-PCR and single cell electrophysiology. The *in vivo* potential of Endorem-labeled GFP-rAFS cells for myocardial repair was studied by transplantation in the heart of animals with ischemia/reperfusion injury (I/R), monitored by magnetic resonance imaging (MRI). Three weeks after injection a small number of GFP-rAFS cells acquired an endothelial or smooth muscle phenotype and to a lesser extent CMs. Despite the low GFP-rAFS cells count in the heart, there was still an improvement of ejection fraction as measured by MRI. rAFS cells have the *in vitro* propensity to acquire a cardiomyogenic phenotype and to preserve cardiac function, even if their potential may be limited by poor survival in an allogeneic setting.

Keywords Amniotic fluid · Stem cells · *In vitro* differentiation · Cardiomyocyte · Cell transplantation

Introduction

Recent animal models studies demonstrate that stem/progenitor cell transplantation, or mobilization from endogenous sources, plays a role in the functional recovery that follows acute myocardial infarct, mostly attenuating cardiac remodeling, which is responsible for organ failure [1]. Clinical studies have assessed cell-based therapeutic effects using adult bone marrow, skeletal or peripheral progenitor cells. As yet a consensus is difficult to form and the long-term benefit of such treatments still unknown [2]. It appears, however, that the use of a specific adult cell type in pursuing the so-called reverse remodeling, gives rise to different effects, namely increased neovascularization or attenuation of fibrosis [3]. Thus, the selection of cell type should be tailored to the primary clinical profile of the cardiac disease and its time-related progression. On the other hand, the regenerative potential of embryonic and fetal progenitor cells is possibly greater than the adult counterpart and comparable to that obtained with fetal/neonatal cardiomyocytes (CMs) [4]. The "immature" stem/progenitor cells display the valuable property of being able to differentiate into vascular endothelial and smooth muscle cells along with CMs. The interactions of these three cell types is essential for reconstructing the damaged or lost cardiovascular units that constitute the structural "building blocks" in the functionally efficient mammalian heart [5]. It is noteworthy that *in vitro* stem/progenitor cells are refractory to be transdifferentiated spontaneously to CMs and as such an event can artificially be induced by altering their DNA methylation pattern [6] or co-cultivation with fetal/neonatal CMs [7]. Even with this strategy however, the CM-potential of these cells *in vitro* and *in vivo* remains elusive.

Among the sources of "immature" stem cells, other than the ES cells, but potentially suitable for cardiac regeneration studies we have taken into account the amniotic fluid (AF). Cells present in this fluid, named Amniotic Fluid Stem (AFS) cells, possess self-renewal capacity, clonal properties and multi-lineage differentiation ability *in vitro* and *in vivo* [8]. So far several works have reported the myogenic potential of amniotic fluid stem cells: in a previous study our group showed that GFP-positive rat amniotic fluid-derived mesenchymal stem cells can differentiate into smooth muscle cells [9]; as well ovine amniotic fluid stem cells, collected both from the membranes or fluid, showed a smooth muscle phenotype under specific culture conditions [10]; recently Gekas and co-workers demonstrated that human ckit+ AFS cells, isolated according to [8], can acquire a myogenic-like phenotype *in vitro* with the expression of markers such as desmin and MyoD [11]. Additionally, similarly to the amnion [12, 13] and the chorionic mesoderm [14], unfractionated or c-kit-sorted human and rodent AF cells have been demonstrated to express, to various extent, cardiomyogenic and vascular-specific genes *in vitro* and to differentiate to cardiovascular structure when transplanted in models of heart injury of different species [15–19].

Importantly, cells derived from placenta, and the amnion in particular, lack immunogenicity because of a low expression of the major histocompatibility complex (MHC) class II antigen, in contrast with the, still controversial, expression of class I antigen [20–22]. Moreover, in allogeneic and xenogeneic mixed lymphocyte tests, these cells suppress the T-cell response [23], suggesting that they can be used in human transplantation, where a realistic utilization of cell therapy is in an allogeneic donor-to-host context. Unfortunately, our results with AFS cells suggest that these cells—in contrast to Zhao et al. [13] and despite a MHC profile similar to placenta and amnion—are not suitable for a discordant xenogeneic transplantation in the injured rat heart [16] whereas, in a syngeneic setting, unfractionated AF cells form CMs and capillaries [17].

The goal of the present study was to test the myocardial potential of GFP-labeled, c-kit-sorted rat AFS (GFP-rAFS) cells, *in vitro,* after a rCMs-induced differentiation commitment and, *in vivo,* after transplantation of undifferentiated GFP-rAFS cells in an allogeneic donor-to-host rat model of cardiac injury by ischemia/reperfusion (I/R), to ascertain their potential and suitability in tissue engineering applications.

The results obtained suggest that, despite a noticeable *in vitro* myocardial trans-differentiation, these cells might elicit an immuno-inflammatory reaction that brings about their rejection *in vivo*.

Materials and Methods

Cell Isolations and *In Vitro* Cultures

Isolation, Maintenance and Expansion of GFP-rAFS Cells

Samples of rat AF were collected from transgenic GFP-positive pregnant Sprague-Dawley rats, mean gestational age 16 days p.c. GFP-rAFS cells were isolated according to De Coppi et al. and Ditadi et al. [8, 24] to avoid problems of contamination with cells of different origins, as stated in the recent paper by Dobreva and co-workers [25]. The uterus was removed and the single fetuses with their membranes dissected under stereomicroscope (Leica Microsystems). Amniotic fluid samples were harvested by carefully removing the visceral yolk sac to expose the amniotic sac. A small opening was created in the exposed amniotic sac to collect the fluid. Briefly, AF samples were diluted with PBS and then spun at 311 x g; pellets were re-suspended in Chang's medium [αMEM (Invitrogen, Italy), 20% of Chang Medium (Chang B plus Chang C; Irvine Scientific, CA, USA), 15% of fetal bovine serum (FBS, Invitrogen, Italy), 1% of streptomycin and penicillin and L-glutamine] and seeded at a density of 2000 cells/cm^2. After a few days, non-adherent cells were discarded and the adherent cells cultivated until 80% pre-confluency. Adherent cells were detached using 0,05–0,02% w/v trypsin sodium-EDTA solution (Biochrom AG, Germany), immuno-sorted with rabbit anti-c-kit antibody (anti-CD117, H-300, Santa Cruz Biotechnology, CA) followed by anti-rabbit IgG CELLection Dynabeads M-450 (Dynal Biotech, Invitrogen, Italy) and then re-plated at a density of 2×10^3 cells/cm^2. Culture medium was changed 3 times a week. GFP-positive rat AFS cells were expanded and subsequently cloned by limiting dilution and kept growing in sub-confluent conditions.

rCMS Isolation

Neonatal rat cardiomyocytes (rCMs) were prepared according to Radisic et al. [26]. Briefly, rCMs cultures were obtained from 1 to 2-day-old neonatal Sprague-Dawley rats; ventricles were quartered, incubated overnight at 4°C in a 0.06% (w/v) solution of trypsin in Hank's Balanced Salt Solution (HBSS, Invitrogen, Italy), washed in Cardiac Growth Medium [CGM, made of DMEM (Gibco) containing 4.5 g/L glucose supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine and 100 units/ml penicillin], and then subjected to a series of digestions (4 min, 37°C) in 0.1% solution (w/v) of type II collagenase (125 U/mg, Wortinghton, USA) in HBSS. Cells were collected by centrifugation and then pre-plated for 1 h to allow for enrichment of cardiomyocytes. Finally, rCMs were seeded on 1% gelatine-coated petri dishes (Falcon, BD Biosciences, Italy). rCMs cultures were studied after 4, 6 and 9 days *in vitro*.

Phenotypic Characterization of GFP-rAFS and rCM Cells

GFP-rAFS cells antigenic profile was determined by immunostaining of cytocentrifugates (cytoplasmic antigens) and flow cytometry (cell membrane antigens). rCMs phenotype was also analyzed to confirm the purity of the primary culture isolation and determined by immunostaining of cytocentrifugates.

Cell cytospins were collected using a Shandon Cytospin 4 centrifuge (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cytospun cells were fixed in 4% PFA (Sigma, Italy) at room temperature, permeabilized with a 0.1% Triton X-100 (Sigma, Italy) solution and then incubated with primary and secondary antibodies.

In the case of GFP-rAFS cells, characterization was carried out with the following primary antibodies: anti-SSEA4 (mouse monoclonal IgG, Chemicon, Italy), anti-Oct 3/4 (rabbit polyclonal IgG, Santa Cruz Biotech, CA), anti-c-kit (anti-CD117, rabbit polyclonal IgG, Santa Cruz Biotech, CA), anti-CD34 (mouse monoclonal IgG, Sigma, Italy) anti-CD29 (mouse monoclonal IgG, Chemicon, Italy) anti-CD105 (mouse monoclonal IgG, Cymbus Bioscience, UK), anti-CD90 (mouse monoclonal IgG Cymbus Bioscience, UK), anti-Stro-1 (mouse monoclonal IgG Iowa Hybridoma Bank, Iowa, USA), anti-Flk-1 (mouse monoclonal IgG, Santa Cruz Biotech, CA), anti-Smooth Muscle α-Actin (SMA, mouse monoclonal IgG Sigma, Italy), anti-NGF receptor (mouse monoclonal IgG, Pharmigen BD Biosciences, Italy), anti-pan-cytokeratin (mouse monoclonal IgG, Sigma, Italy) and anti-vimentin (mouse monoclonal IgG Dako, Italy). For cytospins of rCMs obtained from primary cultures the following antibodies were used: anti-c-kit (anti-CD117, rabbit polyclonal IgG, Santa Cruz Biotech, CA) and anti-cardiac troponin T (mouse monoclonal IgG, Abcam, UK). Goat anti-mouse Alexa Fluorescence 594-coniugated IgG (Molecular Probes, Invitrogen, Italy) or the swine anti-rabbit TRITC-coniugated IgG (Dako, Italy) were used as secondary antibodies. Three distinct preparations of cytocenrifugates from GFP-rAFS and rCMs cells were examined by two independent operators. Immunofluorescence observations were carried out using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and acquired by Leica IM 1000 software.

Flow cytometry characterization of the GFP-rAFS cells was performed in triplicate using cells re-suspended in PBS at concentration of 5×10^5 cells/100 μl using FITC-, PE- or Alexa Fluorescence 647-labeled monoclonal antibodies. The following antibodies were used: anti-CD45 (mouse monoclonal IgG Immunotech, MO, USA), anti-CD73 (mouse monoclonal IgG, BD Pharmigen, BD Biosciences,

Italy), anti-MHC I (mouse monoclonal IgG, AbD Serotec, UK) and anti-MHC II (mouse monoclonal IgG, Immunotech, MO, USA). Analysis was performed by a COULTER Epics XL-MCL cytometer (Beckman Coulter, Fullerton, CA, USA) and data were elaborated by means of EXPO™ 32 ADC Software. Data are expressed as number of cells/10^6 cytometric events.

In Vitro Differentiation of GFP-rAFS Cells Grown in the Presence of rCMS

Co-cultures of GFP-rAFS and rCMS Cells

Direct co-cultures were established according to Chiavegato et al. [16] by admixing neonatal rCMs and GFP-rAFS cells in a ratio of 4:1 (8×10^3 and 2×10^3 cells/cm^2 respectively) and seeding this cell mixture on 1% gelatine-coated glass coverslips. Cell viability after cell labeling was monitored by Blue Trypan exclusion test. Cells were cultured in CGM and the medium changed 3 times a week; co-cultured cells were analyzed at 4, 6 and 9 days. Co-cultures of cardiac fibroblasts and GFP-rAFS cells, used as control of induction potential by rCMs on GFP-rAFS, were set up as described for rCM cells. Fibroblasts were obtained as the first wave of cells spread out from neonatal cardiac explants (data not shown). The general pattern of CM antigen expression in the co-cultures was evaluated by immunofluorescence.

Indirect (non-contact) co-cultures were also established, seeding rCMs and GFP-rAFS in different wells of 6-well plate separated by Transwell® Membrane Inserts (Corning Life Sciences, UK). The semipermeable membrane of the insert (pore size 0.4 um) allows the diffusion of secreted factors but prevents the cells transporting from one chamber to the other, avoiding cell contact between the two sides of the chambers. rCMs were plated on the upper membrane insert and the GFP-rAFS cells in the lower bottom well (10^5 and 2×10^3 cells/cm^2 respectively) on 1% gelatine-coated glass coverslips.

In addition to this, GFP-rAFS cells were also cultured on different wells of 6-well plate at 2×10^3 cells/cm^2 density on 1% gelatine-coated glass coverslip and treated with rCMs-conditioned medium from a separate rCMs culture. rCMs—conditioned medium was collected every 48 h, centrifuged to exclude any debris and then used to treat GFP-rAFS cells.

Differentiation of co-cultured GFP-rAFS Cells

In order to study the differentiation pattern achieved by GFP-rAFS cells after co-culturing with rCMs and treatment with rCMs conditioned medium, cells were analyzed after 4, 6 and 9 days. The expression of CM antigens in these cells was assessed at protein (immuno-staining) and mRNA (RT-PCR) level. Immunofluorescence staining for CM differentiation in GFP-rAFS cells was performed on cell cytocentrifugates. Samples were fixed, permeabilized and incubated with primary and secondary antibodies as previously described. The following primary antibodies were used: anti-GFP (rabbit polyclonal IgG, Chemicon, Italy), anti-cardiac troponin T (cTnT; monoclonal mouse IgG, Abcam, UK) and anti-troponin I (cTnI; a gift of Prof. Stefano Schiaffino, Dept. of Biomedical Sciences, University of Padua, Padua, Italy), anti-sarcomeric α-actinin (mouse monoclonal IgM, Sigma, Italy), anti-sarcomeric myosin heavy chain (MF20, mouse monoclonal IgG, Iowa Hybridoma Bank, Iowa, USA). The secondary antibodies were the following: Alexa Fluorescence 594-conjugated (donkey anti-mouse IgG, Molecular Probes, Invitrogen, Italy) and Cy2-coniugated (goat anti-rabbit IgG, Chemicon, Italy), Alexa Fluorescence 488-conjugated (goat anti-rabbit IgG, Molecular Probes, Invitrogen, Italy) antibodies, diluted in a 1% PBS/BSA and rat or human serum solution. Finally, nuclei were stained by Hoechst dye (Sigma, Italy).

Co-cultures were continuous monitored for spontaneous beating via a Leica DC300 videocamera attached to a phase-contrast microscope Leica DMR microscope and the patterns obtained compared to those of control rCMs single cultures at the same post-seeding time.

For gene expression analysis, cells detached with a 0,05–0,02%w/v trypsin/EDTA solution, washed and re-suspended in PBS 1X. GFP-rAFS cells were sorted with a FACSAria cell sorter (BD Biosciences, Italy) equipped with blue, red and violet lasers. Cells were analyzed by forward scatter (FSC) vs side scatter (SSC) dot plot, selected and sorted using a 530 nm band pass filter and the argon ion laser (488 nm, 100 mW) for excitation. Cell sorters purity options at a rate of 5,000 events per second were used. Sorted populations were re-analyzed for GFP purity and viability, which resulted >95%. Total RNA was then isolated from single cultures of GFP-rAFS cells in Chang culture medium (untreated cells as control), sorted GFP-rAFS cells and control rCMs (all after 6 days *in vitro*) with RNAzol™ B (Tel-Test Inc.,Texas, USA). 1 μg of RNA was transcribed into first strand cDNA with Superscript II reverse transcriptase (Life Technologies, MD, USA) using oligo-dT primer (Invitogen, Italy), following the manufacturer's instructions. Both RT and PCR were done using a GeneAmp® PCR System 2700 (Applied Biosystem, Italy). For each PCR reaction, cDNA was used in a final volume of 25 μl with 200 nM dNTP, 10 pM of each primer, 0.3 U Taq-DNA-polymerase, reaction buffer and $MgCl_2$ (Invitrogen, Italy). Cycling conditions consisted of 94°C for 2 min, annealing at 63°C for 40 s and elongation at 72°C for 1 min. Cycle numbers varied between 27 and 30 cycles. The endogenous rat-specific house-keeping gene β-actin

was quantified to normalize differences in the added RNA and efficiency of reverse transcription. The rat specific primers used in this study were the following: β-actin (For: 5′-ATGCAGAAGGAGATTACTGCCCTG–3′, Rev: 5′-ATAGAGCCACCAATCCACACAGAG-3′; 98 pb), cardiac troponin I (For: 5′-ACGTGGAAGCAAAAGTCACC-3′, Rev: 5′-CCTTCTTCACCTGCTTGAGG-3′, 198 bp) and cardiac sarcomeric α-actinin (For: 5′-ATGATGCTCCCAG AGCTGTC-3′; Rev: 5′-TGTCGTCCCAGTTGGTGATA-3′, 174 bp). The primers were built using the website http://fokker.wi.mit.edu/primer3/input.htm and purchased from Invitrogen. PCR reactions were performed on 1% agarose gel electrophoresis and images taken by BioDoc It Imaging System UVP.

Single-Cell Electrophysiology of co-cultured GFP-rAFS Cells

Co-cultures were established by seeding neonatal rCMs and GFP-rAFS cells in a cell mixture at 10^5 and 10^3 cells/cm^2 density respectively on 1% gelatine-coated glass coverslips. Single cell electrophysiology was performed using the whole cell configuration of the patch-clamp technique. Action potentials were measured with an Axopatch 200B amplifier (Axon Instruments) using fire-polished pipettes with a resistance of 3–4 MΩ pulled from filamented borosilicated glass capillaries (Harvard Apparatus). Data were acquired using a Digidata 1322A interface (Axon Instruments) and analysed with pCLAMP (version 8) software (Axon Instruments). Recordings were made at room temperature.

For patch clamp analysis GFP-rAFS cells and rCMs were cultured alone or were co-cultured in the relative proportion reported above. Cell suspensions in CGM were seeded on 1% gelatin-coated glass coverslips (13 mm, BDH). Action potential recordings of GFP-rAFS cells and rCMs cells were obtained by injecting current with a 5 ms pulse at 1 Hz for a minute. For cells with pacemaking activity no current was injected. We measured the resting membrane potential (E_m, taken as the most hyperpolarized potential in cells with intrinsic pacemaker activity), the maximal depolarization from this potential (ΔV), and the durations for 50% (APD_{50}) and 90% repolarization of the membrane potential (APD_{90}) measured from the point of sharp upstroke of the voltage trajectory. GFP-rAFS cells were identified by epifluorescence in the co-cultures. The extracellular solution was (mM): NaCl 135, KCl 5.4, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 0.33, HEPES 5, glucose 10 (pH=7.4). The intracellular solution was (mM): K-gluconate 110, KCl 20, NaCl 10, $MgCl_2$ 1, Mg-ATP 2, EGTA 2, GTP 0.3 (pH=7.35) (all from Sigma-Aldrich, UK). Drugs were applied by a gravity driven perfusion system.

In Vivo Differentiation of GFP-rAFS in a Model of Cardiac Ischemia/Reperfusion Injury

Animals and Set up of the Ischemia/reperfusion(I/R) Model

The animal study was approved by the Ethics Committee of the University College London London, UK. All surgical and pharmacological procedures were performed in accordance with regulations expressed in the Animals Act 1986 (Scientific Procedures), following the rules about research and testing using animals established by the Home Office, Science, Research and Statistics Department, UK. Wild-type Wistar rats (Harlan UK Limited) weighing about 250–300 g and 8-weeks-old, housed and maintained in a controlled environment, were randomly assigned to four experimental groups: Group I (ischemia/reperfusion injury + cell transplantation, n=5), Group II (ischemia/reperfusion injury + injection solution, n=4), Group III, (Sham injury + cell transplantation, n=5) and Group IV (Sham injury + injection solution, n=5). Animals, anesthetized with an intraperitoneal injection of 50 mg/kg body weight of ketamine hydrochloride (Vetalor, Parke Davis, NJ) were maintained on a heating blanket during surgery. Body temperature was kept constant during the procedure. An endotracheal tube was inserted into the trachea and artificial respiration with pure oxygen was provided via a Respirator (Harvard Apparatus Lt., U.K.; 70 strokes/min, tidal volume 8–10 ml/kg). ECG was acquired via subcutaneous electrodes (PowerLab with Chart5 software, ADInstruments, USA). The myocardial infarction was performed as follows: the left pectoris major muscle and muscles below were dissected and a cardiac access procured via thoracotomy performed in the 4th intercostal space. The pericardium was removed and the left anterior descending coronary artery was occluded (LAD ligation) close to its origin with a snare occluder for 30 min (see Fig. 4).

The efficacy of infarct induction was confirmed by visually inspecting the myocardium for pallor following LAD occlusion and controlled indirectly via S-T elevation on the ECG recorded during the surgery. Only animals with observable pallor and ECG changes (T-inversion or S-T elevation) were included.

After 30 min the occlusion was removed and the myocardium re-perfused, thus inducing a “reperfusion” injury (I/R). Animals were then fully recovered and analgesic (buprenorphine, Vetergesic, 0.25 mg/Kg Alstoe Ltd, UK) and antibiotics (Baytril, Bayer, UK, 0,5 ml/Kg) were supplied by intraperitoneal injection.

Cells Labeling and in Vitro MRI Validation

GFP-rAFS cells were cultured *in vitro* for 48 h in Chang medium and then labeled with Endorem solution (Guerbet

Laboratories Ltd, UK) prior to *in vivo* transplantation. Cells to be transplanted were incubated with Endorem super paramagnetic iron oxide particles (20 μl/ml of cell medium) for 24 h at 37°C and then detached using a 0,05–0,02% w/v trypsin sodium-EDTA solution. Cell viability after cell labeling was monitored by Blue Trypan exclusion test. A calibration for the MRI signal intensity versus iron oxide particle concentration for iron labeled Endorem-GFP-rAFS cells was performed according to [27].

Cells Transplantation

In vivo cell transplantation was performed as following. In Groups I and III, after obtaining the cardiac lesion (as shown in Fig. 4), the heart was injected with 5×10^6 Endorem-labeled GFP-rAFS cells re-suspended in 100 μl PBS 1X distributed in three sites (33 μl per site) in the periphery of the damaged area. In the heart of Group II and IV injections of cells was replaced by PBS. The chest was closed and the respiration tube removed. Animals were monitored until they fully recovered from anesthesia.

Cell Tracking and MRI Determination of the Ejection Fraction

Animals were subjected to MRI assessment after 3 weeks following surgery. Rats were anesthetized with isoflurane 4% (in pure oxygen), maintained at 2% and placed together with a heating blanket, supine on an animal holder. A respiration sensor and a cardiac phased array coil (Rapid Biomedical GmbH, Germany) were placed on the chest. Needle electrodes were inserted subcutaneously into the front limbs to record the electrocardiogram (ECG). For cardiac and respiratory gating a MR monitoring and gating system (SA Instruments, NY) was used. Cardiac imaging was performed with a 9.4 T (400 MHz) horizontal bore system (Varian Inc. Palo Alto, CA) with a shielded gradient system (400 mT/m). A short axis image series perpendicular to the long axis orientation was acquired. In order to cover the whole left ventricle from apex to base, 15–20 short axis slices were acquired without a gap. A double gated segmented gradient echo sequence was used with the following imaging parameters: echo time ~1.7 ms, repetition time ~7.5 ms, flip angle 15°, field of view 40×40 mm², slice thickness 1 mm, Matrix size 192×192. Twenty time frames were recorded for every cardiac cycle. A short axis slice was obtained in approximately 45 s leading to a total scan time for one heart of 10 to 15 min. Segment (http://segment.heiberg.se) was used to analyze the data and calculate the ejection fraction [28]. The same sequence and settings were used for cell tracking but only a single frame at the end diastolic time point was acquired.

Characterization of Transplanted Cells and Cellular Response to Transplants

Hearts were harvested 3 weeks from surgery. Hearts, embedded in OCT and snap frozen in 2-metylbutane and liquid nitrogen, were cut into 8 μm-cryostat sections. Sections were processed by standard histology with hematoxylin and eosin staining and immunofluorescence protocols as described above for cardiac troponin T, SMA, and vWf as well as macrophages (CD163, Serotec, Italy), pan T-lymphocytes (Cymbus, Southhampton, UK), CD4 and CD8 (Serotec) and NK cells (CD161, Abcam) markers.

Statistical Analysis

The GFP-rAFS cells differentiation value was determined at time point analysis by paired or unpaired Student's *t*-test vs untreated GFP-rAFS cells using Graph Pad Instat and Prism 4 softwares. Single cell electrophysiological data and MRI was evaluated using one way ANOVA with a Bonferroni multiple comparison test. All results are given as mean±S.E.M. Results were considered statistically significant if $p<0.05$.

Results

In Vitro Studies

Antigenic Profile of GFP-rAFS and rCM Cells

The differentiation antigenic profile of GFP-rAFS cells was determined by immunofluorescence staining of cytocentrifugates and by flow cytometry analysis (Table 1).

GFP-rAFS cells (Fig. 1a) consistently expressed the "embryonic stem cell" marker SSEA4 and, to a different extent, Oct 3/4, CD105 and CD29, NGF receptor, Flk-1, CD90, CD73, and SMA, whereas both MHC I and MHC II were expressed at very low level and CD34 and CD45 were not detectable. Additionally, the mesenchymal cell marker vimentin was present in all the cells examined but Stro-1 and pan-cytokeratin were absent.

Freshly isolated rCMs (Fig. 1b) were found positive for expression of the cardiac-specific differentiation marker cTnT in about 65–70% of the whole cell population. rCMs did not express the stem marker c-kit, showing that contamination of resident cardiac progenitor cells from the neonatal heart could be excluded [29, 30]. This pattern did not change substantially after 4–9 days of cell growth *in vitro* of primary GFP-rAFS cells.

Table 1 Immunophenotyping of GFP-rAFS cells

Antigen	GFP-rAFS cells
c-kit	–
OCT ¾	+
SSEA4	++++
CD34	–
CD45	–
CD29	++++
CD105	++++
CD90	+/–
CD73	+/–
Stro-1	–
NGFr	+++
Flk-1	++++
αSMA	+++
Vimentin	++++
Pan-cytokeratin	–
MHC I	+/–
MHC II	+/–

Percentage of cells expressing the antigen was evaluated as follows: – 0%; +/– <10%; + 10–30%; ++ 31–60%; +++ 61–90%; ++++ >90%

Antigenic Profile of GFP-rAFS Cells in co-culture with rCMS

After 4 days of co-culture, some GFP-rAFS cells were detected in CM-enriched beating areas, where they were assembled in small clusters (Fig. 1c); however, only a minority of these GFP-rAFS-containing clusters expressed a spontaneous contractile activity as detected by the video recording (Movie 1, GFP-rAFS cell in co-culture, in the supplementary data). Some of them were positive for the myocardial antigenic markers cTnI and MyHC (Fig. 1d-i). After 6 days *in vitro* GFP-rAFS cells were in closer contact to rCMs and beating areas markedly increased; cytocentrifugates of 9 day old co-cultures expressed in addition to the other markers cardiac sarcomeric α-actinin (Fig. 1j-l). Few bi-nucleated GFP-rAFS cells expressing cardiomyocyte markers were detected (Fig. 1g-l), possibly suggesting cellular fusion. The GFP-rAFS cells myocardial differentiation efficiency was about 5.67±1.59% and 16.58±7.13% after 6 and 9 days of co-culture respectively (Fig. 1m).

In single cell electrophysiological experiments (Fig. 2), cells were grown in co-culture 4 days prior to the experimental procedure. Electrical activity was recorded from control GFP-rAFS cells cultured alone (Fig. 2a-b), from beating rCMs cultured alone (Fig. 2c) and on cells in co-cultures containing synchronized beating areas (Fig. 2d). The rCMs showed pacemaking activity, with an APD_{50}= 95.6±12.9 ms and APD_{90}=174.6±33.2 ms (Fig. 2c). Control GFP-rAFS cells, cultured alone, had a depolarised membrane potential (−10 to −20 mV) and were not able to develop an action potential when stimulated with a current pulse (Fig. 2a). This lack of excitability remained when cells were held at more hyperpolarised membrane potential (around −70 mV, Fig. 2b). In contrast, when GFP-rAFS cells (identified by epifluorescence) were co-cultured with rCMs, they developed electrical excitability (Fig. 2d-e) and only those cells in close contact with beating rCMs had electrical activity. When GFP-rAFS cells were challenged with 10 μM isoprenaline there was a marked reduction of APD_{50} and APD_{90}, and acceleration of the pacemaking activity (Fig. 2f). The action potential recorded in co-cultured GFP-rAFS cells fell into two categories, that we named "immature" and "mature", according to their electrophysiological profile, as shown in Table 2. "Immature" GFP-rAFS cells had a membrane potential (Em) of −30.0±2.8 mV (n=7) and depolarization during the action potential (delta V) of 42.2±3.4 mV. In "mature" GFP-rAFS cells, Em was −53.1±3.2 mV (n=9), and delta V was 82.1±5.5 mV. The membrane potential recorded from rCMs was −59.3±5.0 mV (n=6) and the depolarization during the AP was 90.6±12.2 mV. The "immature" GFP-rAFS cells have a more depolarized membrane potential and are not able to reach, during the firing of the AP, a depolarization comparable to the rCMs. Both parameters, Em and delta V, recorded from immature GFP-rAFS cells were significantly different compared to the mature GFP-rAFS cells (### p <0.001 for Em, ## p< 0.01 for delta V, Table 2 and Fig. 2g) and to the rCMs (*** p <0.001 for Em, ** p<0.01 for delta V, Table 2 and Fig. 2g). The data values of delta V are scattered into two groups that would reflect immature AP for the lower values, and mature AP for the higher values. The same cell populations also presented significantly lower/higher resting membrane potential. On this basis, cells were split into two groups (Table 2, Fig. 2g), and the characteristics of their AP (APD50, APD90, deltaV, and Em) were statistically analyzed using two way ANOVA analysis with a Bonferroni multiple comparison test. However, Em and delta V were not significantly different between the mature GFP-rAFS cells and the rCMs (Table 2 and Fig. 2g). The APD_{50} and APD_{90} of "immature" and "mature" GFP-rAFS cells were not significantly different between the two groups, however these durations were significantly longer than the one obtained with rCMs (*** p<0.001 for mature GFP-rAFS cells, ** p< 0.01 and * p<0.05 for immature GFP-rAFS cells).

GFP-rAFSs cells from 6 days of co-cultures were studied for myocardial-specific antigen expression of cTnI and cardiac sarcomeric α-actinin by first FACS sorting the whole cell population (Fig. 3a). To confirm specificity of the sorting and to exclude any contamination of rCMs, the sorted GFP-rAFS cells were also analyzed by immunoflu-

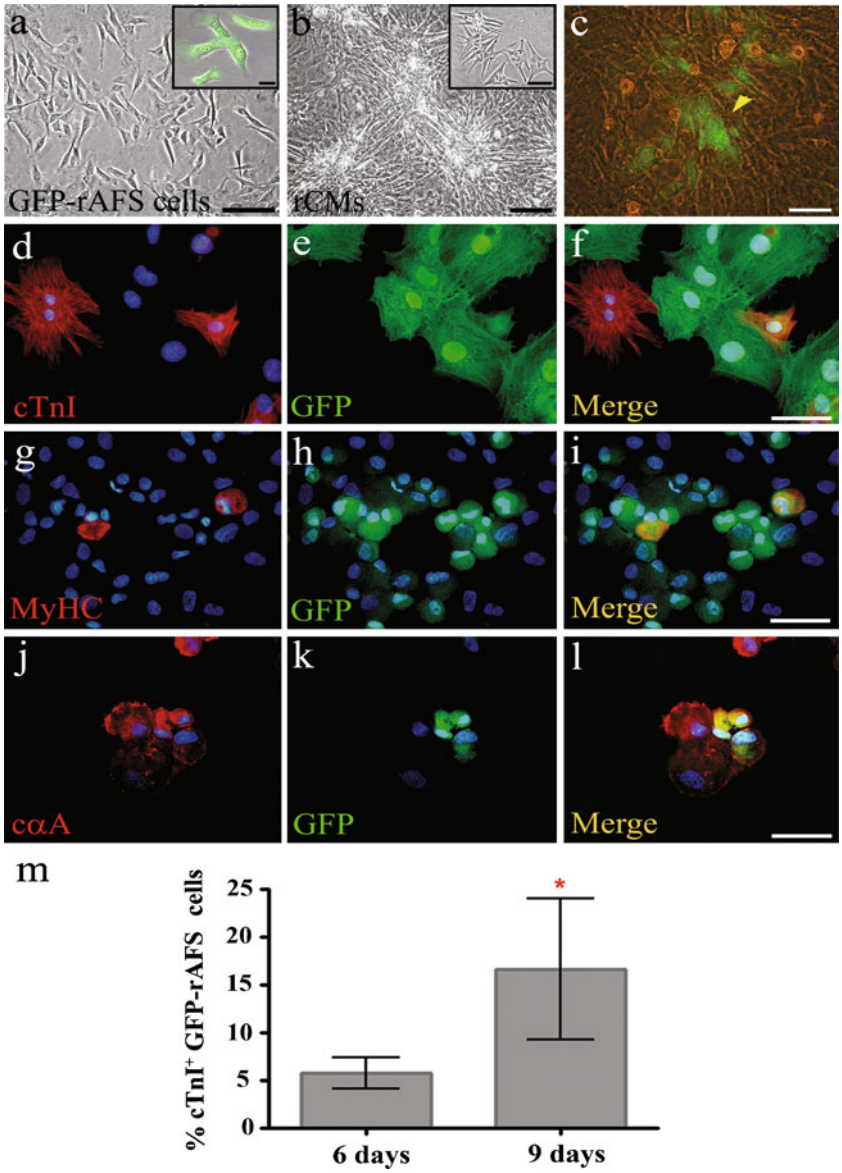

Fig. 1 (**a**) Primary GFP-rAFS cells in culture after 4 days *in vitro*; bar, 250 μm. In the inset, GFP-positive cells under UV exposition; bar, 75 μm. (**b**) Confluent control rCMs after 4 days *in vitro*; bar, 100 μm. In the inset, single rCM; bar, 75 μm. (**c**) Overlay of phase contrast and fluorescence of a GFP-rAFS-containing rCMs cluster in co-culture 4 days after seeding and characterized by a marked beating activity, yellow arrowhead; bar 100 μm. (**d**–**l**) Immunofluorescence of GFP-rAFS cells and rCMs co-cultures after 6 and 9 days *in vitro*. (**d**–**f**) cTnI expression (*in red*); in the Merge panel (**f**) a GFP-rAFS cell expressing the CM-specific marker is shown in yellow, bar 75 μm. (**g**–**i**) MyHC expression (in red) in a cytospun spot; GFP-rAFS cells expressing this sarcomeric marker are shown in yellow in the Merge picture, bar 75 μm. (**j**–**l**) Immunofluorescence of GFP-rAFS cells and rCMs co-cultures after 9 days *in vitro*. Cardiac sarcomeric α-actinin (cαA) expression (in red); GFP-rAFS cells expressing this sarcomeric marker are shown in yellow in the Merge picture, bar 75 μm. (**m**) Percentage of GFP-rAFS cells expressing the CM-specific marker cTnI after 6 and 9 days of co-culture with rCMs in comparison to total number of GFP-rAFS. Values are expressed as the mean±SEM (*, $p<0.05$)

orescence staining, soon after collection (Fig. 3b-g). The purity of the GFP-positive sorted population was confirmed by the fact that the positive fraction obtained from the sorting process was represented by cells all expressing GFP (Fig. 3b-d). Some sorted GFP-rAFS cells showed to have acquired cardiomyocyte markers expression after co-culture with rCMs as they were found positive for the expression of both GFP (in green) and cardiac Troponin I (in red, Fig. 3e-g), confirming the results previously shown by the analysis on the whole co-cultured cell population. No contaminating rCMs were found in the sorted GFP-rAFS cells (Fig. 3c-d), in which no GFP-negative cell expressing cardiac troponin I was found. In light of these evidences we can confirm that sorted GFP-rAFS cells did not contain any rCMs and that acquisition of cardiomyocyte markers, at gene and protein expression level was due to AFS cells plasticity. Figure 3(h) shows the gel electrophoresis analysis of RT-PCR products from FACS-sorted GFP-rAFS cells (lane 2) grown in co-culture with rCMs in comparison with control rCMs (lane 3) and untreated GFP-rAFS cells (lane 1). mRNAs for cTnI and sarcomeric α-actinin were indeed detectable in GFP-rAFS cells extracts from co-cultures.

GFP-rAFS cells in non-contact co-culture with the rCMs seeded on the Transwell® insert or cultured using the rCMs-conditoned medium after 6 and 9 days showed no cardiac differentiation as they did not express any cardiomyocyte markers as cardiac troponin I or cardiac sarcomeric α-actinin by immunostaining (Suppl Fig. 2a-f) or gene expression analysis (Supplement Fig. 2 g).

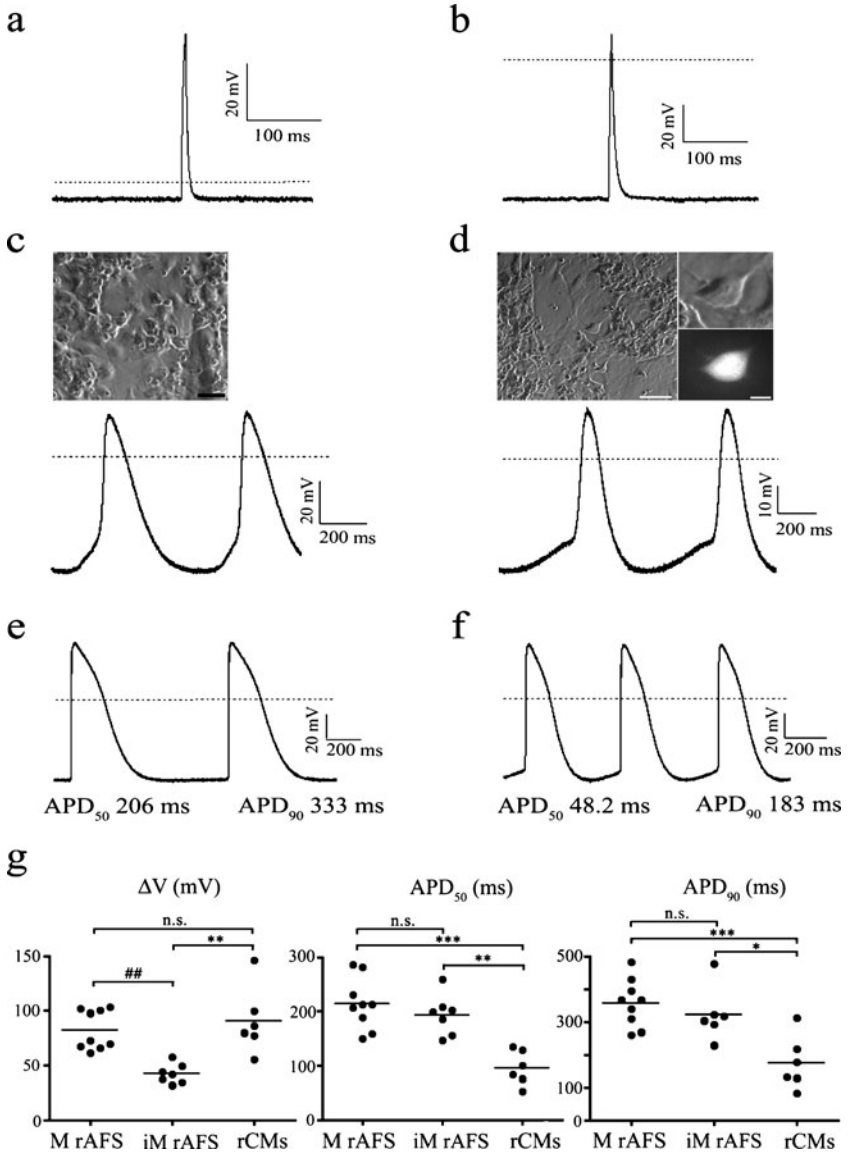

Fig. 2 Single cell electrophysiology. (**a, b**) Representative trace of GFP-rAFS cells cultured alone. Cells have a depolarized membrane potential (−10 to −20 mV). A 5 ms current pulse was not able to elicit an AP and the membrane potential of the cell passively follows the current injection: (**a**) at the resting membrane and even when the membrane potential was held at −70 mV (**b**). (**c**) Rat neonatal cardiomyocytes in culture (bar 100 μm), representative trace of a spontaneous pacemaker activity. (**d**) GFP-rAFS cells in co-culture with rCMs (left brightfield, bar 250 μm; right brightfield and GFP, bar 75 μm). In these culture conditions, a pacemaking activity developed in GFP-rAFS cells which examples of which are shown in the traces. (**e, f**) effect of 10 μM isoprenaline on a GFP-rAFS cells in co-culture with rCMs. Isoprenaline led to an acceleration of the pacemaker activity, with shortening of APD_{50} and APD_{90} (**f**). For all traces dotted line represents zero mV membrane potential. All experiments were done in current clamp, using the whole cell mode of the patch-clamp technique at room temperature. In (**g**) statistical analysis to compare immature (iM rAFS) and mature (M rAFS) GFP-rAFS cells with rCMs in co-culture for the depolarization during the action potential (ΔV, delta V) and the APD_{50} and APD_{90} parameters. The analysis was done using one way ANOVA with a Bonferroni multiple comparison test. A p-value of <0.05 was taken to be statistically significant. Mature GFP-rAFS cells have a shorter APD_{50} and APD_{90} compared to rCMs, *** $p<0.001$, whereas their ΔV was comparable to rCMs, $p>0.05$ (n.s.). As well immature GFP-rAFS cells have a shorter APD_{50} and APD_{90} compared to rCMs (** $p<0.01$ and * $p<0.05$) and a smaller ΔV compared to rCMs (** $p<0.01$) and mature GFP-rAFS (## $p<0.01$). Immature and mature GFP-rAFS cells show no statistically difference in their APD_{50} and APD_{90} parameters (n.s.)

In Vivo Studies

To ascertain the myocardial differentiation potential of GFP-rAFS cells *in vivo,* we set up a cardiac model of ischemia/reperfusion injury (Fig. 4). Three weeks after surgery and cell transplantation, cardiac MRI was performed. Rats were sacrificed to assess the antigenic profile of survived cells, the distribution pattern in the cardiovascular tissues and the

Table 2 General feature of the electrical parameters recorded on GFP-rAFS cells and rCMs

	APD_{50} (ms)	APD_{90} (ms)	ΔV (mV)	Em (mV)
rCMs $n=6$	95.6±12.9	174±33.2	90.6±12.2	−59.3±5.0
GFP-rAFS $n=16$	199±11.7	343±18.3	59.6±6.8	−42.5±3.5
Immature GFP-rAFS $n=7$	193.7±14.0 **	322±33.6 *	42.2±3.4 ** ##	−30±2.8 *** ###
Mature GFP-rAFS $n=9$	213.6±15.7 ***	357±24.3 ***	82.1±5.5	−53.1±3.2

Membrane potential (Em), membrane depolarization during the action potential (ΔV), time to 50% repolarization (APD_{50}) and time to 90% repolarization (APD_{90}) of the action potential are shown. According to these parameters, GFP-rAFS cells were subdivided into two categories: "immature" ($n=7$) and "mature" ($n=9$) cell phenotypes. Statistical analysis to compare immature and mature rAFS cells with rCMs was done using one way ANOVA with a Bonferroni multiple comparison test. A p-value of <0.05 was taken to be statistically significant. Immature cells have a more depolarized Em compared to rCMs. *** $p<0.001$ and to mature GFP-rAFS cells, ### $p<0.001$ and a smaller ΔV during the firing of the AP compared to rCMs, ** $p<0.01$ and to mature GFP-rAFS cells, ## $p<0.01$. Statistical analysis also revealed that immature and mature rAFS cells have a shorter APD_{50} and APD_{90} compared to rCMs, respectively ** $p<0.01$ and *** $p<0.001$ and * $p<0.05$ and *** $p<0.001$

cellular immuno-inflammatory response. MRI demonstrated that following myocardial infarction the left ventricular ejection fraction (LVEF) in the control animals (Group II) significantly decreased ($p<0.05$) from ($n=5$: 67±2%) to ($n=5$; 39±9%). Animals injected with $5x10^6$ GFP-rAFS cells following myocardial infarction (Group I) showed an LVEF of ($n=2$; 55±3%), indicating a trend toward control values. Rats with injection of 5×10^6 GFP-rAFS cells without myocardial infarction (Group III) did not show a decrease in LVEF ($n=2$; 69±1%), thus suggesting that the GFP-rAFS cell treatment did not have a detrimental effect on cardiac function.

Cells labeled with iron oxide nanoparticles produce hypo-intensities or dark regions on an MRI image (Fig. 5a and b, the latter showing the three dimensional reconstruction of the injected cells localization,in red), which can be correlated to the number of cells present [31], as the signal void detected via MRI is proportional to the amount of iron oxide present in a reference volume [32]. Our initial observation following injection of cells indicated a correlation between the signal void and the number of cells injected, which supports our in vitro calibration (Supplement Fig. 1) demonstrating a linear relation between iron particles concentration and T2 (as long as the T2 values are between 20 and 90 ms).

Comparing the signal void in images for the I/R plus cell transplantation (Group I) with the sham operated group receiving cells (Group III) indicates that Group I retained approximately twice as many cells as Group III. However, this does not indicate if cells are alive, thus interpretation of the absolute values will need to take the limitations of this quantification into account [33].

The following notable features emerged from the histological, histochemical and immunofluorescence study of the explanted hearts obtained from animals in this experiment: I/R rats injected with PBS (Group II) showed a marked necrotic myocardial region with an interstitial fibrosis, cellular infiltration and neovascularization, involving the peri-infarct and the proper infarct region (Fig. 5c); I/R (Group I) and sham-operated (Group III) rats transplanted with GFP-rAFS cells displayed cellular infiltration especially evident in sub-epicardial region (Fig. 5d).

The unexpected presence of numerous mononuclear cells in the heart of animals of Group I and III prompted the characterization of such infiltrates. Immunofluorescence analysis of cell composition of such infiltrated is reported in Fig. 6. Abundant NK cells (Fig. 6a-c), T-lymphocytes (Fig. 6d-f) and macrophages (Fig. 6g-i) were accumulated in these hearts.

Cell tracking analysis on hearts from animals of Group I revealed while rare GFP-AFS cells survived, they indeed expressed cTnT in the peri-infarcted area (see Fig. 7a-c). Along with myocardial-like cells, GFP-rAFS transplanted cells gave rise to smooth muscle-like cells (expressing SMA) as well as endothelial-like cells (expressing vWf) in the new arterioles and capillaries in the newly formed blood vessels in the ischemic infarcted area (Fig. 7d-i).

Discussion

In this study we showed that c-kit-sorted, GFP-positive rat AFS (GFP-rAFS) cells co-cultured with rCMs acquired both phenotypic and physiological characteristics of rCMs when evaluated using immunofluorescence, RT-PCR and single cell electrophysiology. After transplantation in the hearts of animals with ischemia/reperfusion injury a small number of GFP-rAFS cells acquired an endothelial or smooth muscle phenotype and to a lesser extent CMs. Despite the low GFP-rAFS cells count in the heart, there was still improvement of ejection fraction as measured by MRI.

Various types of stem/progenitor cells have been used to generate CMs *in vitro* or *in vivo*, but the results obtained remain quite unsatisfactory, both in qualitative and quantitative terms. Besides the recently discovered iPS cells, the only source that has given unambiguous results about its

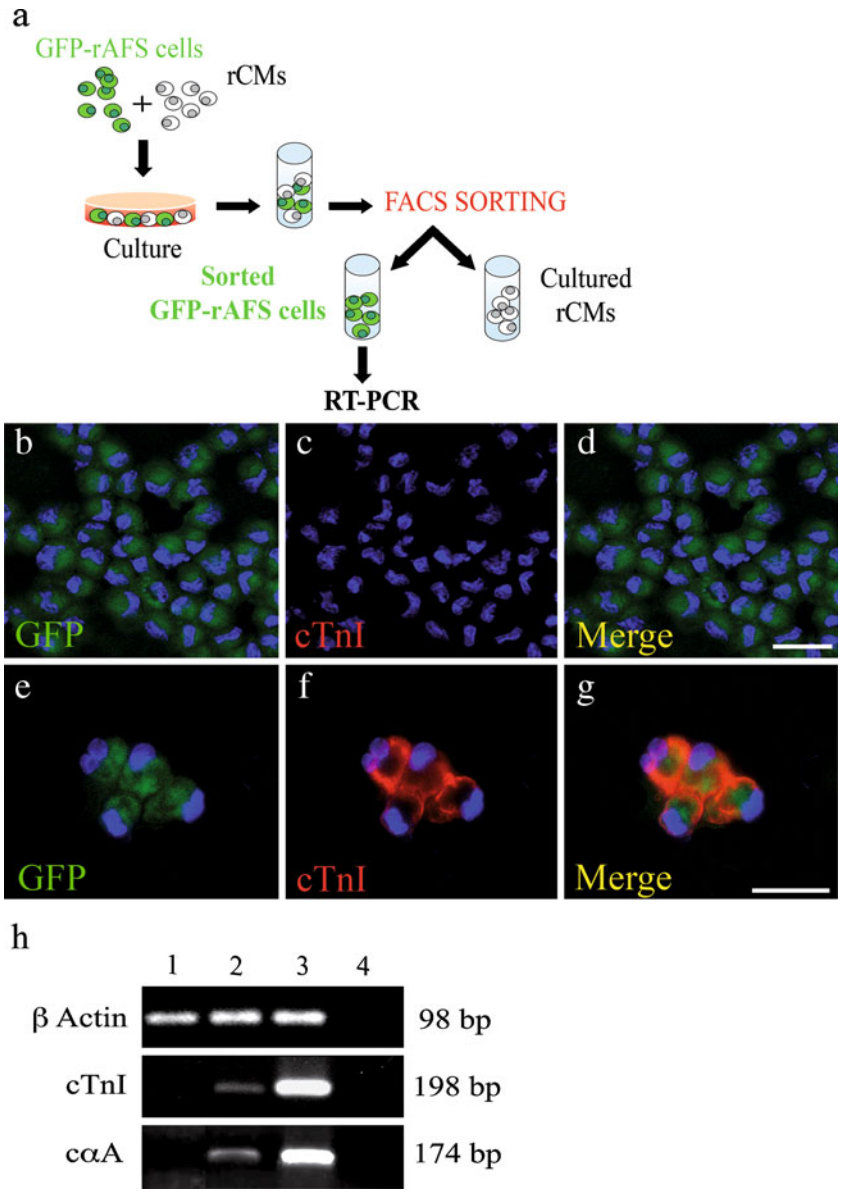

Fig. 3 Gene expression analysis of GFP-rAFS cells after 6 days of co-cultivation with rCMs. (**a**) Cartoon showing the procedure to isolate GFP-rAFS cells from rCMs. (**b**–**g**) Immunofluorescence analysis on cytocentrifugates of FACS-sorted GFP-rAFS cells after co-culture with rCMs. All the sorted rAFS cells were GFP positive (in green, **b**–**d**), bar 100 μm. Some of them were also expressing the cardiomyocyte marker cardiac troponin I (cTnI, in red) as merged in yellow (**f**–**g**), bar 75 μm. (**h**) Gel electrophoresis of RT-PCR products of control untreated GFP-rAFS cells (control GFP-rAFS cells, lane 1), sorted GFP-rAFS cells (lane 2), control rCMs (lane 3) and H_2O (negative control, lane 4) for the housekeeping gene β-Actin and the cardiac genes troponin I (cTnI) and sarcomeric α-actinin (cαA) expression is investigated. GFP-rAFS cells co-cultured with rCMs and FACS-sorted are positive for expression of cardiomyocyte genes (lane 2) compared to control undifferentiated GFP-rAFS cells (lane 1)

cardiogenic potential are embryonic stem cells (ESC). Unfortunately their marked propensity to form teratomas upon transplantation into the immuno-deficient host and the immune response that may be elicited, have hampered their use in a clinical context.

We reasoned that cells from amniotic fluid (AF) could circumvent these problems and provide effective cell therapy for cardiac disease.

In the field of pediatric cardiology congenital heart malformations often required surgical treatment, therefore it would be very advantageous to isolate an autologous source of fetal cardiomyogenic progenitors during the pregnancy and to transplant them back into the patient shortly after birth. Stem cells with a therapeutic potential in cardiovascular disease have been recently identified in several fetal tissue and membranes [34–39]. Along these, amniotic fluid represent a very attractive source of stem cells with suitable potential for therapeutic applications as they can be easily collected during amniocentesis, a well established technique for prenatal diagnosis, with low risk both for the foetus and the mother [8, 40, 41]. AF cells can be readily available in the autogenic setting via cell banking of the amniocentesis samples. Their peculiar properties, such as survival at lower oxygen tension and withstanding protracted cryopreservation without loss of self renewal potential, make them suitable for cell therapy and tissue engineering for diseases or malformations diagnosed prenatally [42–47]. Besides, human AFS cells have also been investigated for genetic modifications [48] and the generation of induced pluripotent stem cells for autologous gene therapy [49]. In light of all of these considerations, AFS cells may represent a novel source of progenitor cells,

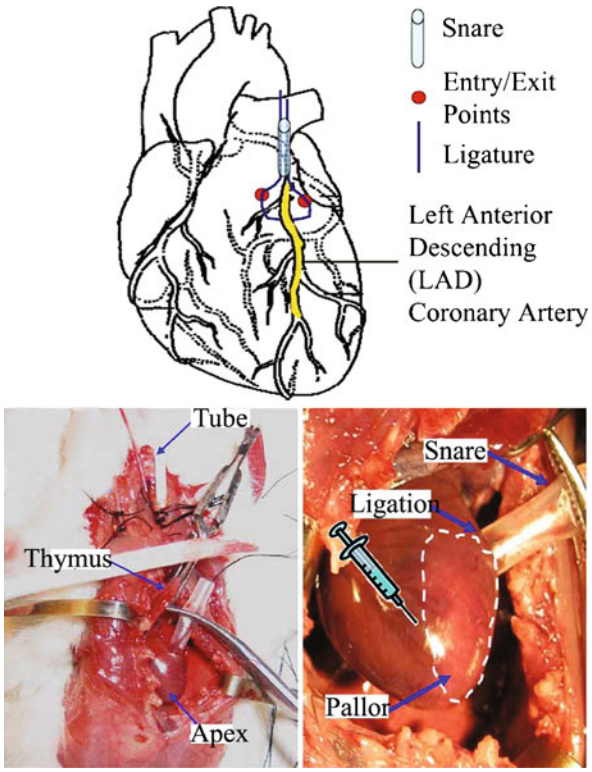

Fig. 4 Schematic representation of the surgical procedure to induce cardiac ischemic injury by I/R model. The acute effect consequent to the injury (pallor) and the sites of GFP-rAFS cells injection (three, exemplified by a syringe) are shown in the bottom right panel. Note that in the bottom left panel, the thoracic chest was on purpose cut to better visualize the surgical field

especially in congenital birth defects where prenatal diagnosis is often required and the unnecessary cells from the sampling can be used to isolate the stem subpopulation.

To fully explore the AFS cells cardiovascular capacity we have undertaken this pilot study, which suggests the *in vitro* and *in vivo* transdifferentiation potential of these cells for a cardiomyogenic phenotype, after contactual effects elaborated by rCMs. However, this may be confounded by an inflammatory process when not used in an autologous setting and this aspect needs to be elucidated by further experiments.

Potentially, human AFS cells sorted for c-kit possess one interesting property that fulfills the reconstruction of the "cardiovascular units" (the indispensable building block of the mechanically efficient heart, based on the combination of CM-capillary-extracellular matrix [6, 50]), namely the ability for differentiation into multiple cell types. Indeed, cloned human AFS cells can be induced *in vitro* to differentiate into cell types representing each embryonic germ layer, including cells of endothelial and myogenic lineages [8]. But also unsorted AFS cells from porcine [15] and rat [17] AF displayed a transdifferentiation potential and can give rise to *in vitro* endothelial, vascular (and non-vascular, possibly by fusion [9]) smooth muscle cells and CMs. Besides, in this study we showed that a proportion of undifferentiated GFP-rAFS cells do already express *in vitro* smooth muscle markers, as smooth muscle α-actin, demonstrating a cardiovascular lineage potential that has to be triggered by specific conditions.

In vitro, we have found that c-kit+ rAFS cells co-cultured in the presence of neonatal rCMs, possibly as donors of specific cardiogenic factors [3], can be converted into structurally and functionally CMs as witnessed by appearance of sarcomeric, cardiac-specific cTnT and cTnI, MyHC and α-actinin in 16.58±20%, after 9 days of culture.

The microenvironment plays a crucial role in determining stem cells differentiation and as the co-culture with neonatal CMs is a widely applied, established technique to achieve stem cells cardiomyocyte differentiation *in vitro*, we decided to use this method in our study. Indeed, several works have highlighted the critical relevance of the direct cell-to-cell contact and the physical stimulation of the rCMs on the stem cells in influencing their transdifferentiation showing how this method can provide a system more effective than modified media or demethylating agents [51–54]. This means that stem cells differentiation in co-culture may also relate to the physical interaction and contraction of the surrounding cardiomyocytes, as shown in our supplement data (Suppl Movie 1), representing a GFP-rAFS cell contracting synchronously with surrounding beating rCMs. A proof of the contactual effect elaborated by the rCMs on the stem cells is represented by the electrical excitability developed by the GFP-rAFS cells in close contact with the contractile rCMs. This suggests that the physical contact between the rCMs and GFP-rAFS cells is required to drive the transdifferentiation, potentially transmitting physical/electrical stimuli. Moreover, to determine if putative soluble factors secreted into medium could be alone sufficient to induce the AFS cells differentiation and to establish the role of the physical cell-cell interactions in the co-culture system, we have also provided experiments culturing GFP-rAFS with rCMs-conditioned medium or maintaining the two cell populations in co-culture physically separated by using membrane inserts. Here we demonstrated that the direct cell-to-cell interaction with the beating rCMs is a key factor influencing the differentiation of the GFP-rAFS cells as cells in non-contact culture with the rCMs, or cultured using the rCMs-conditoned medium, showed no cardiac differentiation and no expression of cardiomyocyte markers by immunostaining or gene expression analysis, as documented in Supplement Figure 2 and in accordance to several previous works [51–53, 55].

GFP-rAFS cells in co-culture with rCMs were responsive to the β adrenergic agonist isoprenaline, with an increased beating rate and a shortening of APD_{50} and APD_{90}. Analysis of the spontaneous pacemaking and action potential parameters of the GFP-rAFS cells in co-culture

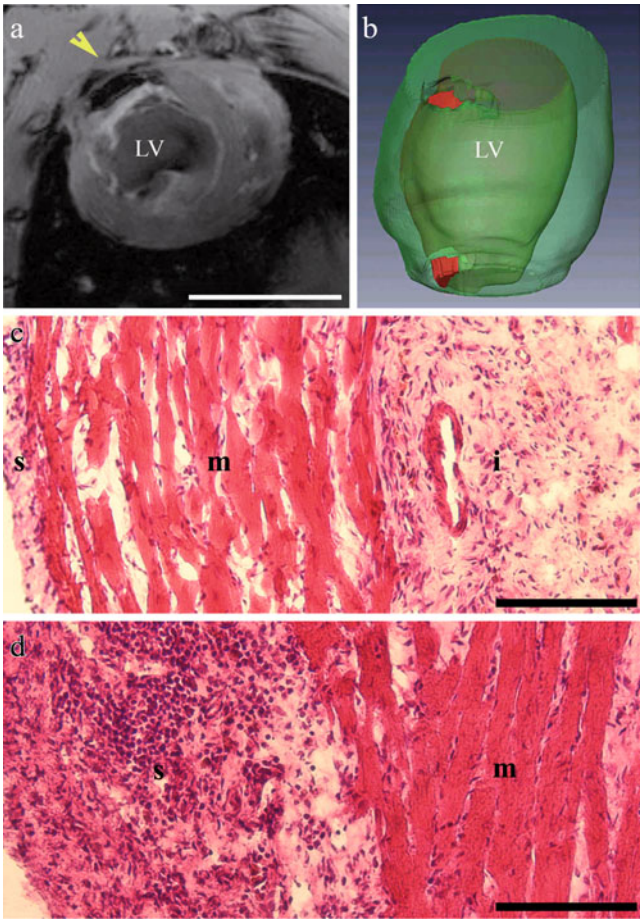

Fig. 5 In vivo MRI cell tracking of GFP-rAFS cells 3 weeks after injection in I/R rats (**a**) Three dimensional reconstruction of injected cells localization (in red, **b**). Histology by hematoxylin and eosin staining of I/R injected with PBS, Group II (**c**) and I/R hearts injected with GFP-rAFS cells, Group I (**d**) after 3 weeks from transplantation. i, Infarct; s, sub-epicardium region; m, myocardial tissue. Bars 10 mm (**a**); 75 μm (**c, d**)

with rCMs prompts us to divide them into two categories: the "immature" and "mature" cells. The "immature" GFP-rAFS cells were characterized by a more depolarized membrane potential, along with a smaller depolarization during the firing of the action potential. However APD_{50} and APD_{90} were not significantly different in the two groups. The membrane potential and the depolarization during the action potential of "mature" GFP-rAFS cells were not significantly different from the rCMs. However, APD_{50} and APD_{90} of the "mature" rAFS cells were longer when compared to the rCMs. Though we favor the hypothesis that co-culture of GFP-rAFS cells in the presence of rCMs may have tip the balance from "immature" to "mature" cell phenotype, we cannot exclude that this electrophysiological cell heterogeneity is due to a cell fusion between the two partners in vitro and the variable outcome in terms of cardiomyogenic expression in the hybrid cells [56, 57].

After validating the in vitro cardiomyogenic potential of the GFP-rAFS cells, we transplanted them into a myocardial infarct rat model to analyze their *in vivo* potential in the undifferentiated state. Several works reported the beneficial effect of using undifferentiated stem cells in cardiac cell therapy: Nassiri and co-workers showed that there is no need for prior differentiation induction of BM-MSCs before transplantation as untreated MSCs can efficiently regenerate the infarcted myocardium and improve cardiac function [58], equally Mazo and co-workers reported that, in a chronic model of myocardial infarction, transplantation of untreated adipose-derived stem cells induced a significant enhancement in heart function and tissue viability, increasing angiogenesis and decreasing fibrosis, whereas transplantation of cardiac pre-differentiated adipose stem cells did not translate into a significant improvement [59].

We have previously examined *in vivo,* in cardiac ischemic injury models, the cardiovascular cell potential of AFS cells observed *in vitro* and contrasting results were obtained: while unsorted AFS cells were converted to CMs and capillaries (syngeneic setting [17]) or capillaries and arterioles (autogeneic setting [15]), sorted AFS cells failed to survive in a xenogeneic environment [16], whereas Yeh and co-workers reported that unsorted human amniotic fluid-derived mesenchymal stem cells transplanted into a xenogeneic model resulted in angiogenesis and acquisition of cardiomyogenic phenotype [60].

To analyze the AFS cells *in vivo* potential, here we preferred to avoid the use of immunosuppressant drugs, as several controversial results have been recently reported regarding their influence on stem cells differentiation potential [61–63]. In this work a marked cellular response characterized by an infiltration of T-cells, NK cells and macrophages occurs in transplanted animals, independently from the presence or absence of the I/R injury. This result suggests that the immuno-rejection is essentially evoked to the antigenic properties of injected cells and we cannot rule out that a macrophage-activated, innate immuno-response to Endorem-released particles is involved in the GFP-rAFS cells rejection as reported by Terrovitis et al. [64]. As well, as the rAFS cells used in this study were permanently genetically labeled with GFP, we can not exclude either that the host immune system might have been stimulated by the presence of this transgenic protein. The level of GFP expression in gene-modified stem cells has been demonstrated to be critical for their *in vivo* immunogenicity after transplantation, with controversial results in immunocompetent and in partially immunosuppressed recipient [65, 66]. In addition, a recent study in a rat model of myocardial infarction and AFS cells transplantation showed that an endogenous inflammatory response, with formation of chondro-osteogenic masses in the cardiac tissue, may occur following ligation of the left anterior descending coronary

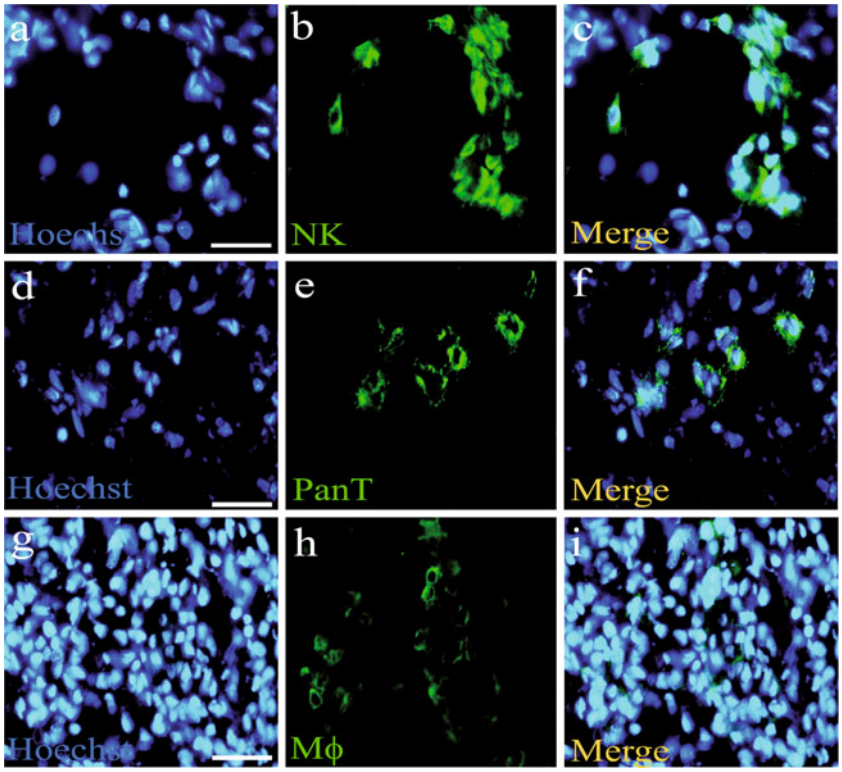

Fig. 6 Cell composition of cellular infiltrates found in the hearts of I/R rats transplanted with GFP-rAFS cells three weeks after injection. (**a–c**), NK cell labeling; (**d–f**) pan-T lymphocytes staining; (**g–i**), macrophage's antigen detection. **Left panels**, nuclear staining; **middle panels**, immunofluorescence with specific antibodies; **right panels**, merge. Bar 75 μm

artery. This effect has been shown to correlate only to the infarction size and the model itself and to be totally independent of AFS cells treatment [67].

Considering rAFS cells mesenchymal stem cell-like antigenic profile (see Table 1) and their very weak expression of both class I and II MHC, i.e. a profile compatible with a low cellular antigenicity, it is quite surprising that these cells can undergo immuno-rejection. Their antigenic phenotype is in accordance to what previously reported on mouse and human embryonic stem

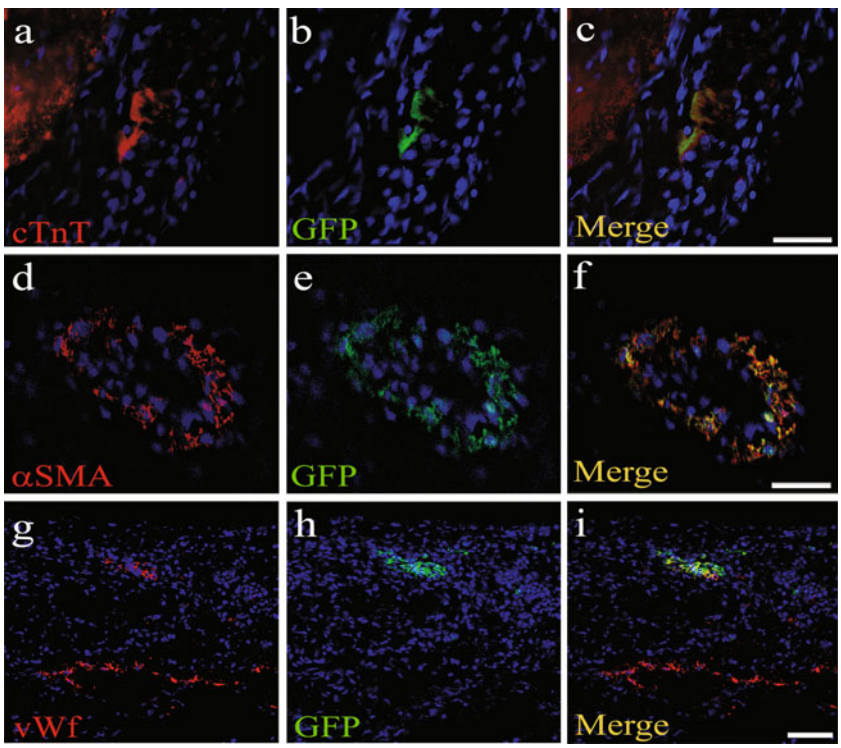

Fig. 7 Cardiovascular antigens expressed by transplanted GFP-rAFS cells in the heart of I/R rats. (**a–c**) Rare injected cells expressing cTnT in the infarct area are shown in yellow in the Merge picture; bar, 75 μm. (**d–f**) Injected cells co-stained for αSMA as shown in yellow in the Merge picture, bar 75 μm. (**g–i**) Some injected cells positive for vWf as reported in the Merge picture in yellow, bar 100 μm

cells, which express little to no MHC class I antigen in the undifferentiated state [68–71]; as well, multipotent cells isolated from fetal membranes and placenta, second-trimester amniotic fluid and amnion membrane showed to be negative for MHC II expression [23, 72, 73] and fetal membranes-derived progenitor cells demonstrated not to induce a cytotoxic response and inhibit lymphocyte proliferation in a mixed allogeneic lymphocyte test [74].

We have also recently demonstrated that both mouse and human c-kit$^+$ cells from amniotic fluid are indeed capable of forming hematopoietic cells, including those in the myeloid lineage and, hence, the myeloid dendritic cells [24]. If, as reported by for transplanted ESC [75], differentiation of GFP-rAFS cells in the ischemic myocardium is accompanied by an increased immunogenicity, it becomes feasible that a mechanism of direct rejection is activated, as happens in the xenogeneic, discordant human-to-rat c-kit-sorted cell transplantation to the heart [16]. Moreover, pluripotent stem cells have recently been shown to become targets for T lymphocytes even if the expression level of MHC class I molecules is below the detection limit of flow cytometry and rejected after transplantation into immunocompetent hosts [76, 77].

Therefore it might be useful in future to test the allogeneic potential of AFS cells at different times of pregnancy to study possible differences in the immuno-rejection potential. Such a study is motivated by the fact that in the stem cells from the amnion (collected at term) both the mesenchymal and epithelial layers seem to be endowed with immuno-modulatory properties [22, 23] and are suitable for xenogeneic [13] and allogeneic [12] transplantation for the cell therapy of cardiac diseases.

In conclusion, AFS cells display an interesting cardiogenic potential *in vitro* and *in vivo* and their use could be endowed in the autologous setting (i.e. tissue engineering approaches to treat paediatric congenital cardiovascular malformations), [78] but their use for cell therapy in an allogeneic context (i.e. I/R injury) need further evaluation [79].

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Conflict of Interest and Disclosures None to declare.

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