Human cord blood CD34+ progenitor cells acquire functional cardiac properties through a cell fusion process

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Avitabile D, Crespi A, Brioschi C, Parente V, Toietta G, Devanna P, Baruscotti M, Truffa S, Scavone A, Rusconi F, Biondi A, D'Alessandra Y, Vigna E, DiFrancesco D, Pesce M, Capogrossi MC, Barbuti A. Human cord blood CD34+ progenitor cells acquire functional cardiac properties through a cell fusion process. *Am J Physiol Heart Circ Physiol* 300: H1875–H1884, 2011. First published February 25, 2011; doi:10.1152/ajpheart.00523.2010.—The efficacy of cardiac repair by stem cell administration relies on a successful functional integration of injected cells into the host myocardium. Safety concerns have been raised about the possibility that stem cells may induce foci of arrhythmia in the ischemic myocardium. In a previous work (36), we showed that human cord blood CD34+ cells, when cocultured on neonatal mouse cardiomyocytes, exhibit excitation-contraction coupling features similar to those of cardiomyocytes, even though no human genes were upregulated. The aims of the present work are to investigate whether human CD34+ cells, isolated after 1 wk of coculture with neonatal ventricular myocytes, possess molecular and functional properties of cardiomyocytes and to discriminate, using a reporter gene system, whether cardiac differentiation derives from a (trans)differentiation or a cell fusion process. Umbilical cord blood CD34+ cells were isolated by a magnetic cell sorting method, transduced with a lentiviral vector carrying the enhanced green fluorescent protein (EGFP) gene, and seeded onto primary cultures of spontaneously beating rat neonatal cardiomyocytes. Cocultured EGFP+/CD34+-derived cells were analyzed for their electrophysiological features at different time points. After 1 wk in coculture, EGFP+ cells, in contact with cardiomyocytes, were spontaneously contracting and had a maximum diastolic potential (MDP) of −53.1 mV, while those that remained isolated from the surrounding myocytes did not contract and had a depolarized resting potential of −11.4 mV. Cells were then resuspended and cultured at low density to identify EGFP+ progenitor cell derivatives. Under these conditions, we observed single EGFP+ beating cells that had acquired an hyperpolarization-activated current typical of neonatal cardiomyocytes (EGFP+ cells, −2.24 ± 0.89 pA/pF; myocytes, −1.99 ± 0.63 pA/pF, at −125 mV). To discriminate between cell autonomous differentiation and fusion, EGFP+/CD34+ cells were cocultured with cardiac myocytes infected with a red fluorescence protein-lentiviral vector; under these conditions we found that 100% of EGFP+ cells were also red fluorescent protein positive, suggesting cell fusion as the mechanism by which cardiac functional features are acquired.

CD34; cord blood; stem cells; cardiac repair; cell fusion

THE ABILITY OF PLURIPOTENT stem cells [embryonic stem cells (ESCs), induced pluripotent stem cells, spermatogonial-derived pluripotent cells] to differentiate into functional cardiomyocytes is well established, both in vitro and in vivo, but the clinical research and therapeutic use of these cell types are hampered by their high teratogenic potential and, in the case of ESCs, by ethical issues (10, 21, 32, 46). Mesoderm-derived adult stem cells, such as cardiac-derived stem cells, mesenchymal stem cells, skeletal myoblasts, hematopoietic stem cells (HSCs), and endothelial progenitor cells, represent a more suitable cell source for cell therapy intervention. These cells have been reported to differentiate into cardiomyocytes when injected in vivo into animal models of myocardial infarction (26, 37) or in vitro by coculturing with neonatal cardiac myocytes (5, 27, 31); however, controversy still exists about the differentiation potential of these cells (25). The ability of HSCs to generate functional cardiomyocytes has been challenged by the hypothesis that they may acquire cardiac features through cell fusion, when coming in contact with cardiac myocytes (34, 1, 6, 33), and a possible molecular mechanism for the fusion process has been provided (47).

Recently, the physiological properties of mouse bone marrow (BM)-derived c-kit+ cells and human cord blood CD34+ cells, cocultured with rodent neonatal cardiac myocytes, have been investigated (29, 36) by us. However, in those two reports, the electrophysiological properties of cells reisolated from the coculture and the role of cell fusion in the acquisition of a molecular repertoire and a typical excitable/contractile phenotype were not investigated. In the present study, we used gene marking techniques to follow the fate of CD34+-derived cells when cocultured with newborn myocytes. We provide strong evidence that in vitro acquisition of cardiac features by these cells is mediated by cell fusion.

MATERIALS AND METHODS

Neonatal cardiomyocytes isolation and culture. The procedures employed in this work conform to guidelines for the care and use of laboratory animals as established by State (D.L. 116/1992) and European directives (86/609/CEE). Animal protocols were reviewed and approved both by the Institutional Review Board of the Biological Departments, University of Milan and by the Italian Ministry of

Health. Hearts were quickly removed from 2-day-old rats, ventricles were isolated and minced in small pieces in phosphate-buffered saline (PBS), and five consecutive enzymatic digestions were performed by gentle shaking at 37°C. Collagenase I (136.8 U/ml, Whorthington) and pancreatine (0.6 mg/ml, Sigma) were added to a $1\times$ ADS solution (in mM: 116.4 NaCl, 5.4 KCl, 1 $NaH_2PO_4H_2O$, 0.8 $MgSO_4H_2O$, 5.5 glucose, 20 HEPES, pH 7.4). The cell suspension was layered on top of a discontinuous Percoll (Sigma) gradient (40.5%, 58.5% in ADS $1\times$) and centrifuged at 1,750 g for 30 min at room temperature (RT) to separate cardiomyocytes from fibroblasts and blood cells. Myocytes were then plated at a density of 4×10^5 cells/ml in DMEM/medium-199 (4:1 vol/vol, EuroClone/Sigma) with 10% horse serum, 5% FBS, L-glutamine (2 mM), penicillin, and streptomycin (100 U/ml and 0.1 mg/ml, respectively; EuroClone). After 24 h, the plating medium was switched to maintenance medium: DMEM/medium-199 (4:1 vol/vol) with 5% horse serum, 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin-0.1 mg/ml streptomycin, and Ara-C (2.5 μM, Sigma).

CD34$^+$ cell isolation from human umbilical cord blood. Human umbilical cord blood (UCB) was collected, using a clinically approved method, into heparin containing bags immediately after delivery, upon written approval by the mothers. Isolation, characterization, culture, and lentiviral infection procedures were carried out as previously described in detail (38). Briefly, cord blood was diluted at a ratio of 1:3 in PBS (Gibco). Cell suspension was carefully layered over 20 ml of lymphoprep (Axis-Shield PocAS) before centrifugation in a swinging bucket rotor (400 g, 30 min, RT, without brake). The upper layer, containing the mononuclear cell fraction, was collected. Mononuclear cells were washed twice in wash buffer (WB; PBS supplemented with 5% FCS and 2 mM EDTA) by centrifugation (400 g, 30 min, RT) and then resuspended in 300 μl of WB. CD34$^+$ cells were positively selected by immunomagnetic separation (MACS) using the mini CD34 progenitor cell isolation kit (Miltenyi Biotec), according to the manufacturer's instructions. Briefly, Fc receptor-blocking reagent (100 μl/10^8 cells) was added to the cell suspension before monoclonal mouse anti-human CD34 antibody-coupled magnetic beads (IgG1 isotype, 30-min incubation, 4°C, under continuous shaking) were added. Cells were washed again as described above, resuspended in 500 μl of WB, and applied to an activated column (miniMACS, Miltenyi Biotec) on a magnet. The column was rinsed with WB (3 × 500 μl), and CD34$^+$ cells were eluted after the column was removed from the magnet. CD34$^+$ cells were cultured overnight in serum-free expansion medium (EM) consisting of StemSpan (SFEM medium, StemCell Technologies) supplemented with IL-3 (20 ng/ml, Biodesign), IL-6 (20 ng/ml, Endogene), SCF (100 ng/ml, Endogene) Flt-3 ligand (100 ng/ml, Biodesign), and next transduced by lentiviral vector. For cocultures, 1.2×10^5 CD34$^+$ cells were seeded in each 35-mm dish containing a monolayer of neonatal rat ventricular cardiomyocytes. In a series of experiments, CD34$^+$ cells were incubated with 10 μM 5-azacytidine (5-AZA) for 24 h in the maintenance medium (see above). 5-AZA was diluted at the desired concentration, directly in the culture medium from a 0.5 mM stock. Medium was replaced after 24 h and every 2–3 days. Cells were analyzed at *day 6.*

Plasmids. The third-generation packaging constructs pMDLg/pRRE and pRSVRev pMD.G-VSV-G, the VSVG envelope encoding plasmid pMD.G, and the self-inactivating transfer vectors were cotransfected with transfer vector to produce high-transduction efficiency viral stocks. The plasmids pCCL.SIN.cPPT.hPGK.EGFP. WPRE were used as enhanced green fluorescent protein (EGFP) labeling system and as backbone for cloning. Transfer vector carrying the red fluorescence protein (RFP) gene was cloned as follows: pLentiBidirez-RFP-DeltaNGFR (a kind gift of A. Bertotti, IRCC, Candiolo, Italy) was digested with *Pme*I/*Nhe*I (NEB), and RFP gene was ligated into the backbone vector previously digested with *Bam*H I/*Sal*I (NEB) and blunt ligated (Quick ligase, NEB). All digestion enzymes and the ligase were from NEB.

Vector production. Vector stocks were produced by calcium phosphate cotransfection of packaging and transfer vectors into 293T cells.

The calcium phosphate-DNA precipitate was allowed to stay on the cells for 16 h, after which the medium was replaced, collected after 48 h posttransfection, and filtered through 0.22-μm-pore nitrocellulose filter (Nalgene). To obtain high-titer viral stocks, vectors were prepared by ultracentrifugation (50,000 g, SW28 rotor, 140 min, RT), resuspended in PBS, and stored at −80°C until use. Vector titer was determined by adding serial dilutions of viral stocks to 10^5 HeLa cells in a Costar six-well plate (Corning) and determining the proportions of green fluorescent protein (GFP) expressing cells by fluorescent-activated cell sorting (FACS) analysis 72 h later. Typical titers of supernatants were in the range of $10^6/10^7$ transducing units (TU)/ml, while titers of concentrated stocks were usually in the range of $10^9/10^{10}$ TU/ml.

Cell infection. CD34$^+$ viable cells were counted by Trypan blue exclusion method, 24 h post-MACS separation. Depending on the experiment, a number of cells ranging from 3×10^5 to 5×10^5 CD34$^+$ were transduced with Lenti-GFP vector at multiplicity of infection of 50 or 100 in the cytokine-containing EM (see above), as previously reported (36), in the presence of polybrene (8 μg/ml, Sigma-Aldrich) and incubated at 37°C and 5% CO_2 atmosphere for 24 h. Next, cells were harvested and extensively washed three times in 10 ml of PBS supplemented with 5% FCS. Lenti-RFP vector supernatants, at a ratio of 1:1 between viral supernatant and rat cardiomyocyte maintaining medium, were used to obtain RFP-labeled neonatal rat cardiomyocytes. Twenty-four hours postinfection, adherent cells were vigorously washed three times with PBS, and fresh medium was added to the cell culture dishes. Transduction efficiency ranged between 70 and 80%, as evaluated by FACS analysis.

FACS analysis. MACS-sorted cells were cultured for 2 days in EM and then assayed for cell differentiation surface marker expression. Cells were resuspended in 100 μl of PBS containing 0.5% BSA and incubated with 10 μl or 0.5 μg per 10^5 cells of direct FITC or phycoerythrin-conjugated mouse anti-human CD34, CD133, CD31, CD14, CD146, CD105 (BD-Biosciences Pharmigen), CD144 (eBioscience), and KDR (R&D Systems) monoclonal antibodies. Cells were incubated in the dark for 10 min at RT, or 30 min in ice when expressly specified by the manufacturer. After incubation, cells were washed with 1 ml of PBS containing 0.5% BSA and then resuspended in 300 μl of the same buffer. Phycoerythrin- or FITC-conjugated mouse IgGs or secondary FITC-conjugated antibody (all from BD-Biosciences Pharmigen) were used, at the same concentration as specific antibodies, as an isotype control during FACS analysis. FACSCalibur fluorescence activated cell sorter (Beckton-Dickinson) was used to acquire 1×10^4 gated events per sample, and Cell Quest software (Variety Software House) to analyze data. In a subset of experiments, EGFP$^+$/RFP$^+$ cocultures and cultures of RFP$^+$ only cardiomyocytes were detached after 6 days, centrifuged, and resuspended in 250 μl of PBS with 10% FBS and 5 mM EDTA. Cells were then analyzed by a FACSAria flow cytometer (Beckton-Dickinson).

Immunofluorescence experiments. Cocultures were fixed in paraformaldehyde (4%), permeabilized in Triton X-100 (0.3%), and blocked in BSA (1%). Cells were then labeled with anti-Cx43 antibodies (1:50, Chemicon) and rhodamine-conjugated phalloidin (1 unit/coverslip, Molecular Probes); secondary antibody was anti-mouse Cy5 (1:1,000). Nuclei were stained by 4,6-diamidino-2-phenylindole (Vectashield Mounting medium). Immunolabeling images were acquired using a confocal microscope (Leica).

Electrophysiology and data analysis. Action potentials (APs) and membrane ion currents were recorded with the whole-cell patch-clamp technique. Cells were superfused with Tyrode solution (in mM: 140 NaCl; 5.4 KCl; 1.8 $CaCl_2$; 5.5 D-glucose; 5 HEPES-NaOH; pH 7.4) at 37°C. Patch-clamp pipettes had a resistance of 3–6 MΩ when filled with an intracellular-like solution containing (in mM) 130 potassium-aspartate, 10 NaCl, 5 EGTA-KOH, 2 $CaCl_2$, 2 $MgCl_2$, 2 ATP (Na-salt), 5 creatine phosphate, 0.1 GTP (Na-salt), 10 HEPES-KOH, pH 7.2. To record the hyperpolarization-activated current (I_f),

1 mM $BaCl_2$ and 2 mM $MnCl_2$ were added to normal Tyrode to block contaminating currents.

I_f was activated by a standard activation protocol (15). Normalized tail currents measured at -125 mV were used to plot activation curves, which were fitted to the Boltzmann distribution function: $y = 1/1 + \exp[(V - V_{1/2})/s]$, where V is voltage, y is fractional activation, $V_{1/2}$ the half-activation voltage, and s is the inverse slope factor. Measured values are reported as means ± SE. Statistical analysis was performed by t-test for independent populations and one-way ANOVA. Pairwise multiple-comparison procedures (Bonferroni's t-test) were performed to isolate the group or groups differing from the others. Level of significance was set to $P = 0.05$.

RESULTS

Electrophysiological features of CD34$^+$/GFP$^+$ cells cultured onto neonatal rat cardiac myocytes. Purified CD34$^+$ cells from human UCB were obtained using a magnetic cell sorting system (MINI-MACS) (38). Purity and antigen characterization of immunoselected cells are shown in Supplemental Fig. S1. (The online version of this article contains supplemental data.) As expected, sorted cells expressed high levels of stem cell antigens CD34 and CD133, the endothelial marker CD31 (platelet endothelial cell adhesion molecule-1), and lower levels of stromal derived factor-1 receptor, the CXCR4 molecule. They did not express the mature endothelial marker CD144 (VE-cadherin) and the monocyte marker CD14. To distinguish CD34$^+$ cells from the myocytes in cocultures, they were infected with a lentivirus that provides for stable EGFP expression. Supplemental Fig. S2A shows a typical FACS analysis to assess the transduction efficiency of lentiviral infection after 72 h, resulting in ~70% EGFP$^+$/CD34$^+$ cells. The day after lentiviral infection, CD34$^+$ cells were seeded onto spontaneously beating rat neonatal myocyte primary cultures. Although it was previously shown that CD34$^+$ UCB cells in coculture with mouse neonatal cardiomyocytes functionally integrate within the substrate, no data about the time course of this integration were previously described (36). Here we show that, when in contact with neonatal rat cardiomyocytes, the small and rounded EGFP$^+$ cells adhered to the myocyte layer and progressively changed their morphology. At this stage (*day 1*), neonatal rat cardiomyocytes contracted spontaneously and showed APs (Fig. 1A), while the EGFP$^+$/CD34$^+$ cells did not contract and were electrically inactive and depolarized (Fig. 1B and Supplemental Video S1). Figure 2A and Supplemental Video S2 show the membrane potential recording and the contractility of an EGFP$^+$ cell at *day 6* after seeding onto cardiac myocytes, respectively. At this stage, CD34$^+$-derived EGFP$^+$ cells did contract and fired spontaneous APs, in accordance with our laboratory's previous findings (36). Recordings from five additional EGFP$^+$ cells in coculture produced similar results; in these cells, the mean MDP was
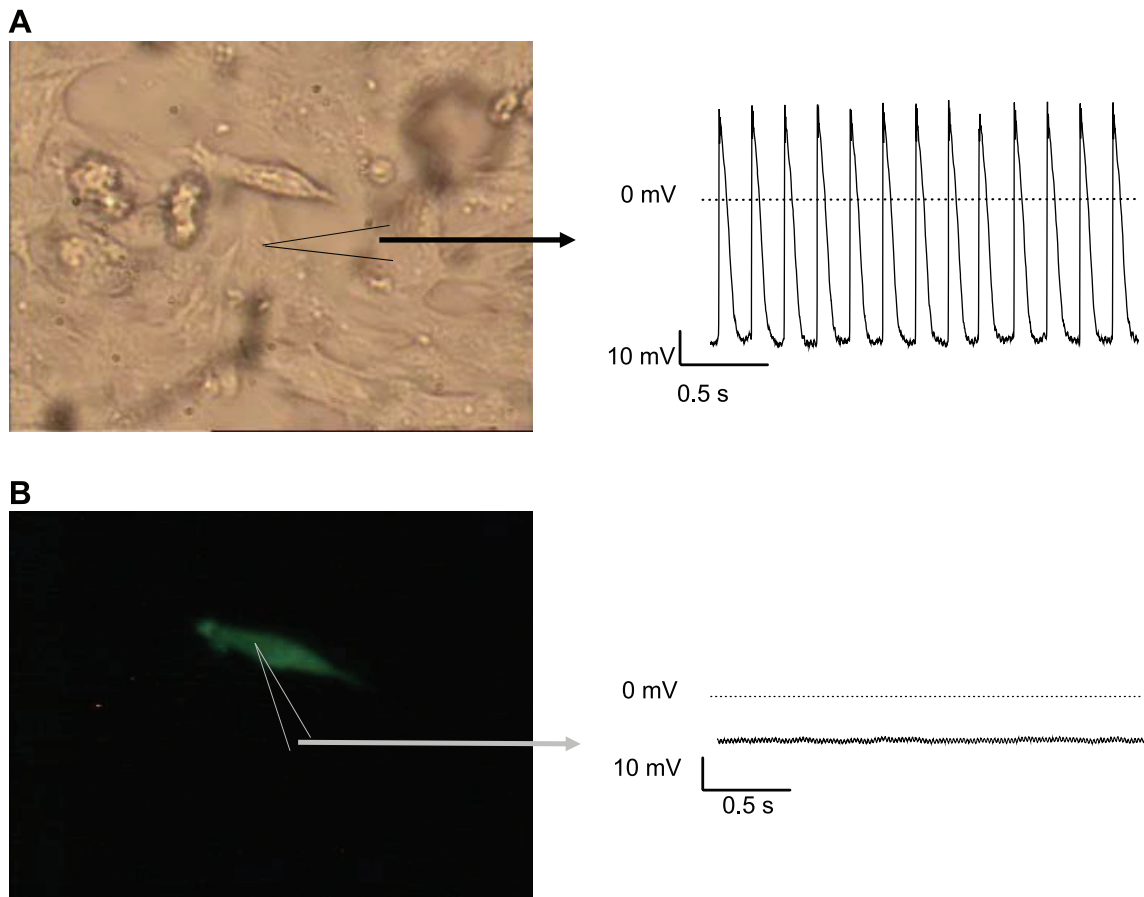

Fig. 1. Morphology and action potential (AP) recordings of enhanced green fluorescent protein (EGFP)$^+$/CD34$^+$ cells after 1 day in coculture onto neonatal rat cardiac myocytes. A: phase-contrast picture showing a coculture of EGFP$^+$/CD34 with neonatal rat cardiac myocyte layer and AP recording from the cardiomyocyte indicated. B: fluorescence microscopy image of the same field as in A, and membrane potential recording from an EGFP$^+$ cell settled on the neonatal rat cardiac myocyte layer (see also Supplemental Video S1). Note the absence of APs and the relatively depolarized resting potential (V_r; -13.5 mV).
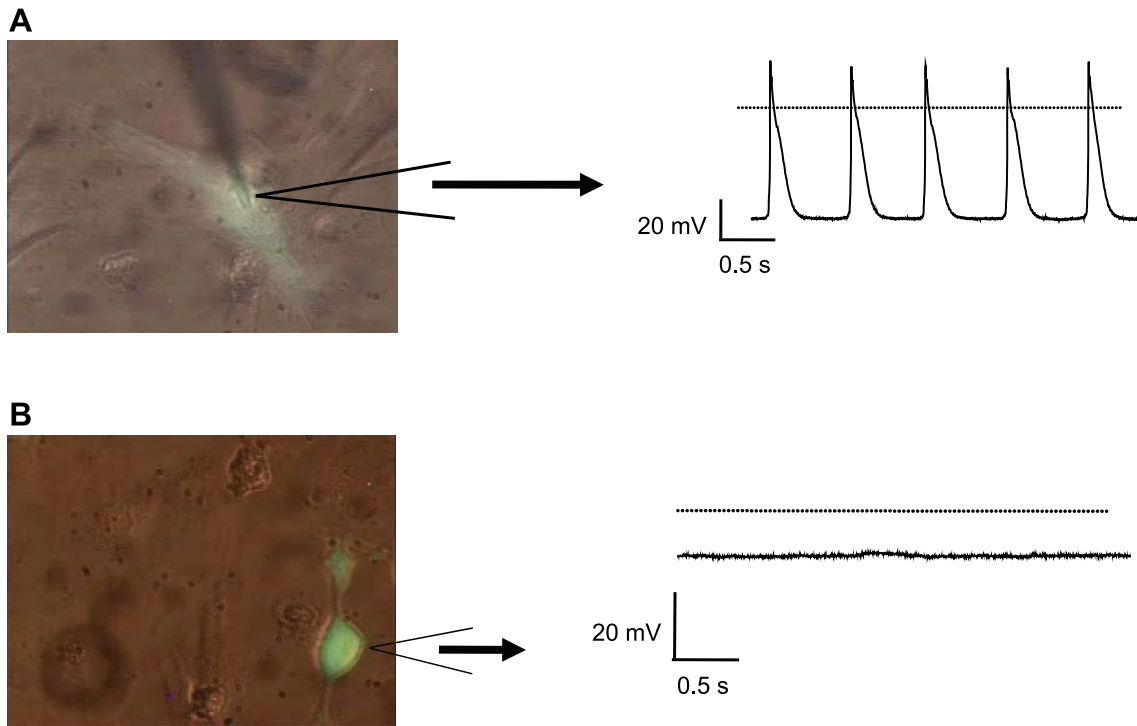
Fig. 2. Morphology and AP recordings of EGFP$^+$/CD34$^+$-derived cells after 6 days in coculture onto neonatal rat cardiac myocytes. *A*: phase-contrast and overlapped EGFP fluorescence images showing the integration of an EGFP$^+$/CD34$^+$-derived cell into the rat cardiac myocyte layer and the corresponding AP recording in the same cell. This cell was also synchronously beating with surrounding cardiac myocytes (see Supplemental Video S2). *B*: EGFP$^+$/CD34$^+$-derived cells that did not contact the cardiomyocyte layer remained essentially round-shaped, lacked electrical activity, and maintained a relatively depolarized V_r (-13.5 mV).

-53.1 ± 3.2 mV ($n = 6$). In some dishes, we found few round-shaped EGFP$^+$ cells that were not in contact with the surrounding myocytes. Voltage-clamp recordings from these single EGFP$^+$ cells, directly in the coculture dish, failed to show either spontaneous activity (Fig. 2*B*) or contractility (data not shown). In addition, as reported in Fig. 2*B*, they maintained a depolarized resting potential (V_r) (-11.4 ± 3.9 mV; $n = 7$) similar to that of CD34$^+$ cells at the beginning of the coculture protocol.

The presence of APs in EGFP$^+$ cells indicates gap junction-mediated electrical coupling with the underlying cardiac myocytes layer, without necessarily implying the acquisition of a cardiac phenotype. To confirm this observation, we performed multicolor confocal microscopy experiments to detect the expression of connexin-43, the most highly expressed cardiac subunit of gap junctions, in EGFP$^+$ cells stained with rhodamine-conjugated phalloidin, which labels polymerized actin (F-actin) and 4,6-diamidino-2-phenylindole. As shown in Fig. 3 (white
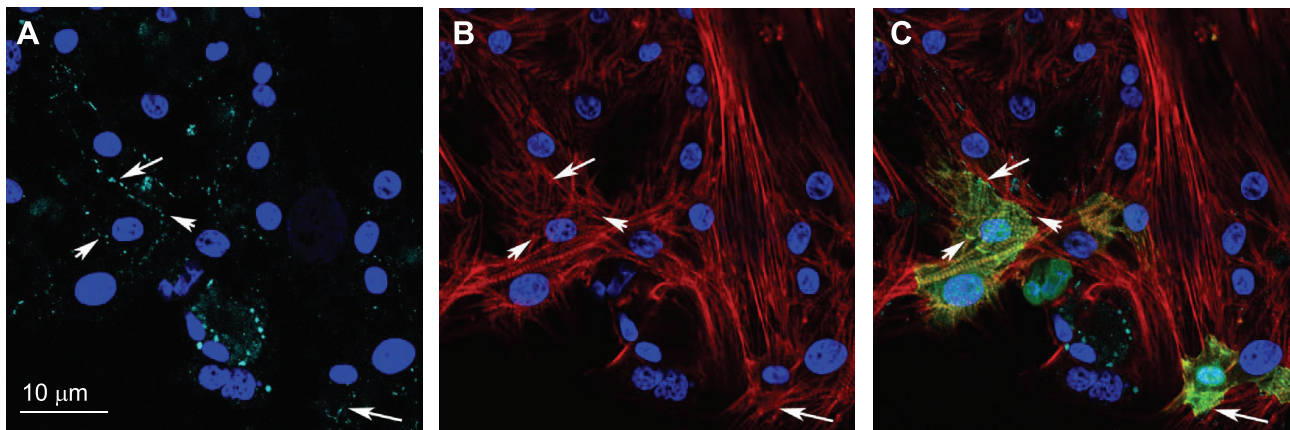

Fig. 3. Confocal microscopy analysis of EGFP$^+$/CD34$^+$-derived cells and surrounding cardiac myocytes. *A*: arrows indicate the Cx-43 staining (cyan) in two EGFP$^+$ cells. *B*: rhodamine-conjugated phalloidin staining in the same cells as in *A*. Note the sarcomeric structure of the two EGFP$^+$ cells. *C*: merge of all fluorescence signals, showing that EGFP$^+$/CD34$^+$ cells acquired a cardiac myocyte phenotype and were connected to surrounding myocytes by gap junctions. Nuclei were labeled with 4,6-diamidino-2-phenylindole (blue).

arrows), EGFP$^+$ cells displayed a cytoskeleton organized in sarcomeric structures, connected to each other and to surrounding cells by gap junctions, thus suggesting that cells are coupled and that electrical activity can propagate across contiguous cells. The percentage of CD34$^+$ cells surviving after 6 days in coculture was also evaluated. To do this, we have counted both EGFP$^+$ and EGFP$^-$ cells in 10 different confocal microscopy fields (of 7 different cocultures), and we have found that 7.7 ± 1.6% of the total cells were EGFP$^+$. Comparing this value with the 13% of CD34$^+$ cells in coculture at *day 0* (1.2 × 10^5 CD34$^+$ seeded onto 8 × 10^5 myocytes), we can estimate that ~50% of UCB cells survived.

Acquisition of cardiac features by isolated CD34$^+$-derived cells. In our laboratory's previous study (36), it was not investigated whether electrical activity recorded from EGFP$^+$/CD34$^+$-derived cells cocultured with cardiac myocytes is simply a propagated, passive activity triggered by adjacent myocytes, or, in fact, the result of acquisition of cardiac properties. To assess this, cells were enzymatically isolated after 6 days of coculture and plated at low density to avoid intercellular contacts. Figure 4*A* shows the phase contrast and the corresponding EGFP fluorescence images of a single resuspended EGFP$^+$ cell. Patch-clamp recordings showed the presence of spontaneous APs (Fig. 4*A*, *bottom*), characterized by the presence of a slow diastolic depolarization (14, 23, 40). Cells showing spontaneous electrical activity were able to contract, although with an irregular frequency (Supplemental Video S3). Cell count revealed that 16% of isolated EGFP$^+$ cells (12/74 over three different cocultures) contracted spontaneously, while the remaining cells were quiescent.

The I_f current has a major role in the generation of the slow diastolic depolarization driving AP firing in embryonic/neonatal cardiomyocytes (8, 45) and adult sinoatrial nodal cells (14, 7). I_f is a mixed (Na$^+$/K$^+$) inward current activated upon membrane hyperpolarization. To assess whether I_f was present in spontaneously beating EGFP$^+$ cells, we applied a standard hyperpolarizing two-step protocol (15) to measure the current activation curve, as shown in Fig. 4*B*. The *top* panel shows traces recorded from one cell during steps to the range −45 to −135 mV, from a holding potential of −35 mV, followed by a fully activating step to −135 mV, and the *bottom* panel shows the corresponding steady-state activation curve fitted to the Boltzmann equation. In $n = 6$ cells, mean $V_{1/2}$ and inverse slope factor (s) were −73.8 ± 2.9 and 8.7 ± 0.9 mV, respectively. These values were similar to $V_{1/2}$ and s values of I_f in isolated neonatal ventricular myocytes (−78.2 ± 2 mV and 7.8 ± 0.5 mV, $n = 4$, data not shown, see also Refs. 11, 41). The I_f current density (recorded at −125 mV) was also similar in spontaneously beating EGFP$^+$ cells and neonatal myocytes (EGFP$^+$ cells: −2.24 ± 0.89 pA/pF, $n = 8$; myocytes: −1.99 ± 0.63 pA/pF, $n = 7$, $P > 0.05$ by Student's t-test).

Finally, in Fig. 5, we compared the cell capacitance (C_m) and the V_r or the MDP in EGFP$^+$ cells before coculture with the following: *1*) EGFP$^+$ cells, reisolated after 1 wk in coculture showing APs only when electrically stimulated (EGFP$^+$ stim); *2*) spontaneously beating reisolated EGFP$^+$ cells (EGFP$^+$ AP); *3*) nonexcitable reisolated EGFP$^+$ cells (EGFP$^+$ no AP); and *4*) rat neonatal myocytes. Figure 5*A* shows that CD34$^+$ cells before coculture had a significantly smaller C_m compared with EGFP$^+$ cells after coculture and to cardiomyocytes ($P < 0.05$ by ANOVA). C_m values were 3.6 ± 0.8 pF ($n = 3$, undifferentiated CD34$^+$ cells); 40.7 ± 5.4 pF ($n = 7$, stimulated EGFP$^+$ cells); 37.8 ± 5.2 pF ($n = 11$, spontaneously beating EGFP$^+$ cells); 35.6 ± 5.2 pF ($n = 14$, nonexcitable EGFP$^+$ cells); and 31.0 ± 2.4 pF ($n = 10$; cardiomyocytes). Figure 5*B* reports the mean V_r or MDP values of the same subsets of cells
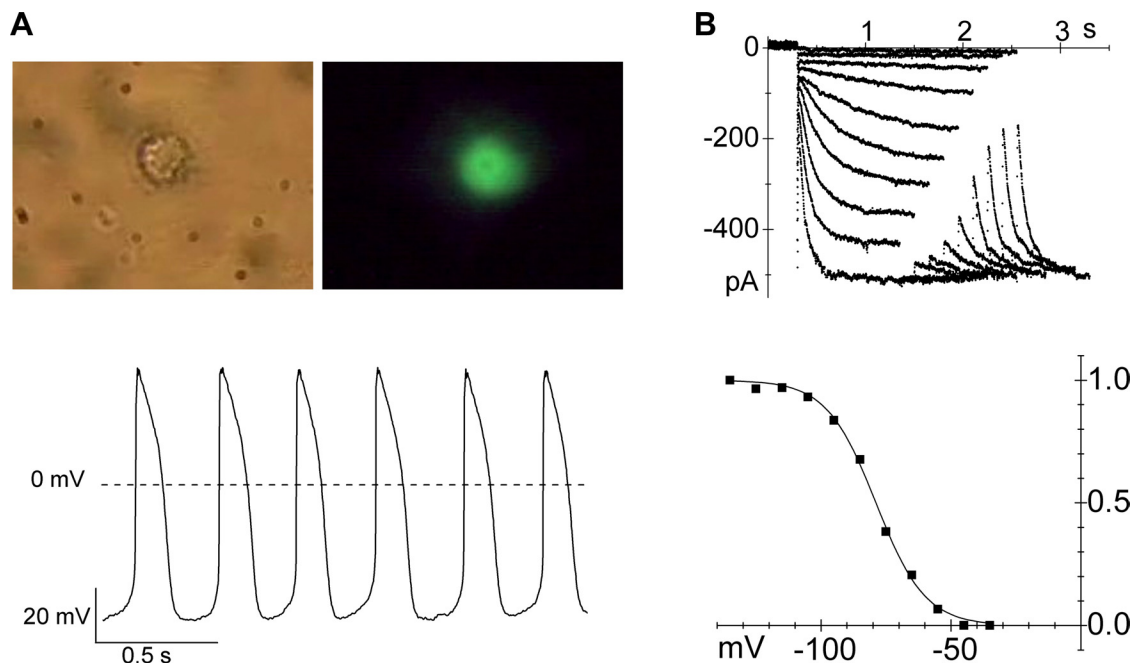

Fig. 4. EGFP$^+$/CD34$^+$-derived cells acquire spontaneous activity and ion currents typical of autorhythmic neonatal cardiomyocytes. *A*: phase-contrast and EGFP fluorescence in a CD34$^+$-derived cell isolated after 6 days of coculture. Spontaneous APs recorded from the spontaneously beating EGFP$^+$ cell are shown below (see also Supplemental Video S3). *B*: a series of hyperpolarization-activated current traces (*top*) recorded from a representative spontaneously beating EGFP$^+$ cell and the corresponding activation curve (*bottom*).
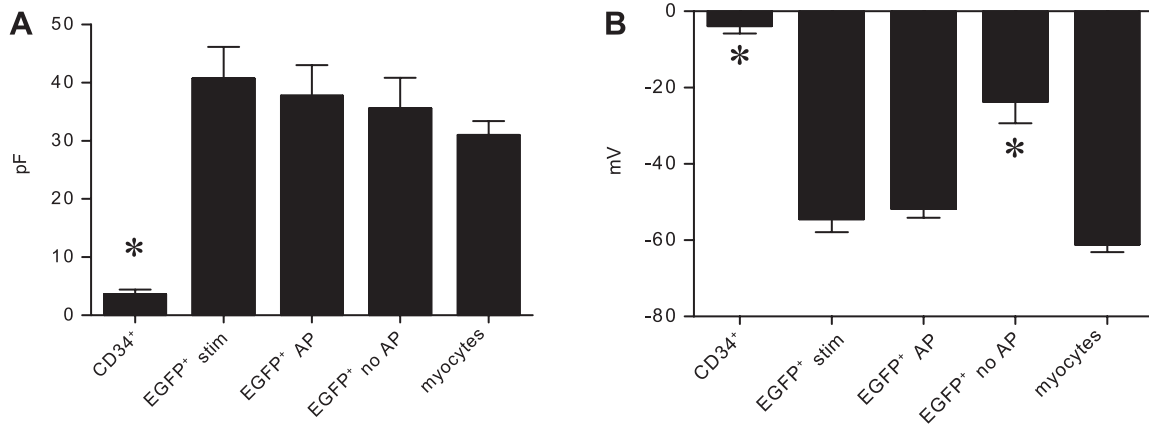
Fig. 5. Cell capacitance (C_m) and membrane potential analysis in isolated CD34 cells before and after the coculture. Bar graphs are shown of the mean values of C_m (A) and V_r or maximum diastolic potential (B) in undifferentiated CD34$^+$ cells, different types of reisolated EGFP$^+$/CD34$^+$-derived cells (see text for details), and neonatal rat cardiac myocytes. Values are means ± SE. *Significant different means by one-way ANOVA, $P = 0.05$.

as in Fig. 5A. CD34$^+$ before coculture and quiescent EGFP$^+$ cells had relatively depolarized V_r values (-3.9 ± 2 and -23.7 ± 5.6 mV, respectively). These values were significantly different ($P < 0.05$) from values recorded in excitable EGFP$^+$ cells (-54.4 ± 3.4 mV), in spontaneously beating EGFP$^+$ cells (-51.8 ± 2.3 mV), and in myocytes (-61.1 ± 2.0 mV).

Previous studies reported that cell incubation with DNA demethylating agents, such as 5-AZA, is able to induce a cardiogenic program in various cell types (28, 30). We, therefore, incubated CD34$^+$ cells with this drug for 24 h (see METHODS for details), followed by culture for 6 days in a drug-free medium. Electrophysiological analysis at *day 6* revealed that 5-AZA-treated CD34$^+$ cells maintained a relatively depolarized V_r (-15.9 ± 2.7 mV, $n = 10$), and neither expressed sodium or pacemaker (f) currents (data not shown), suggesting the lack of cardiac differentiation. Moreover 5-AZA-treated CD34$^+$ cells had a mean C_m of 24.6 ± 7.8 pF ($n = 12$). These values are not significantly different from either those of day-matched untreated CD34$^+$ cells ($V_r = -15.7 \pm 7.9$ mV, $C_m = 35.1 \pm 1.4$ pF, $n = 3$) or from those of CD34+ cells not in contact with cardiomyocytes (see above). These data indicate that 5-AZA is not sufficient to induce cardiac differentiation of UCB CD34$^+$ cells, in line with previous reports indicating that such treatment does not induce cord blood progenitor differentiation, but improves their expansion capacity and stemness (4, 43).

Acquisition of cardiac features in GFP$^+$ cells is a result of fusion to cardiac myocytes. Several studies have called for caution about possible interference of cell fusion in cardiomyocyte differentiation of BM-derived stem cells and HSCs and have provided evidence for mechanisms underlying the fusion process (18, 34, 35, 44, 47, 48). To assess the role of cell fusion into acquisition of neonatal cardiomyocyte properties of CD34$^+$-derived cells, we cocultured EGFP$^+$-transduced CD34$^+$ cells (Fig. 6) with layers of rat neonatal cardiac myocytes previously infected with a lentiviral vector containing RFP (for a description of the RFP lentiviral gene transfer in cardiac myocytes, see Supplemental Fig. S2B).

Figure 6B shows a layer of RFP$^+$ cardiac myocytes onto which an EGFP$^+$ cell adhered and elongated (Fig. 6C). Supplemental Video S4 shows that EGFP$^+$ cells contracted in phase with the underlying RFP$^+$ cardiac myocytes. Cells were then enzymatically isolated at *day 6* after the beginning of the coculture and plated at a low density. In Fig. 7, representative images collected with an epifluorescence microscope (A–F and Supplemental Video S5) or with a confocal microscope (G–I) show that EGFP$^+$ cells resuspended after 1 wk of coculture were also RFP$^+$ cells. The analysis showed that all isolated EGFP$^+$ cells, either beating ($n = 8$) or quiescent ($n = 4$), were also RFP$^+$, suggesting the occurrence of fusion events of CD34$^+$-derived cells and cardiac myocytes (Fig. 7). To quantify the fraction of EGFP$^+$ progenitors fusing with RFP$^+$
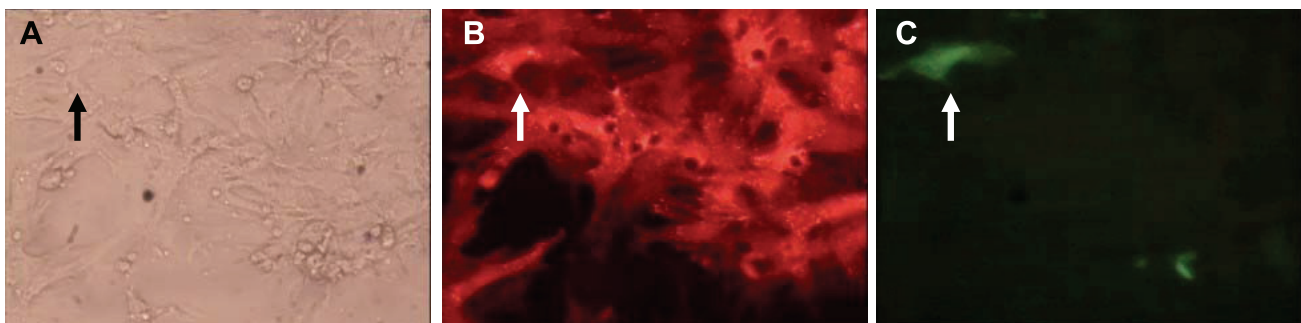

Fig. 6. Coculture of EGFP$^+$/CD34$^+$ cells onto red fluorescence protein (RFP)$^+$ cardiac myocytes. A: phase-contrast image showing the coculture. B and C: RFP and EGFP staining in the same field as in A. Note that essentially all myocytes are stained in red. Arrow indicates the position of an EGFP$^+$ cell (C) that was also RFP$^+$ (B; see also Supplemental Video S4).
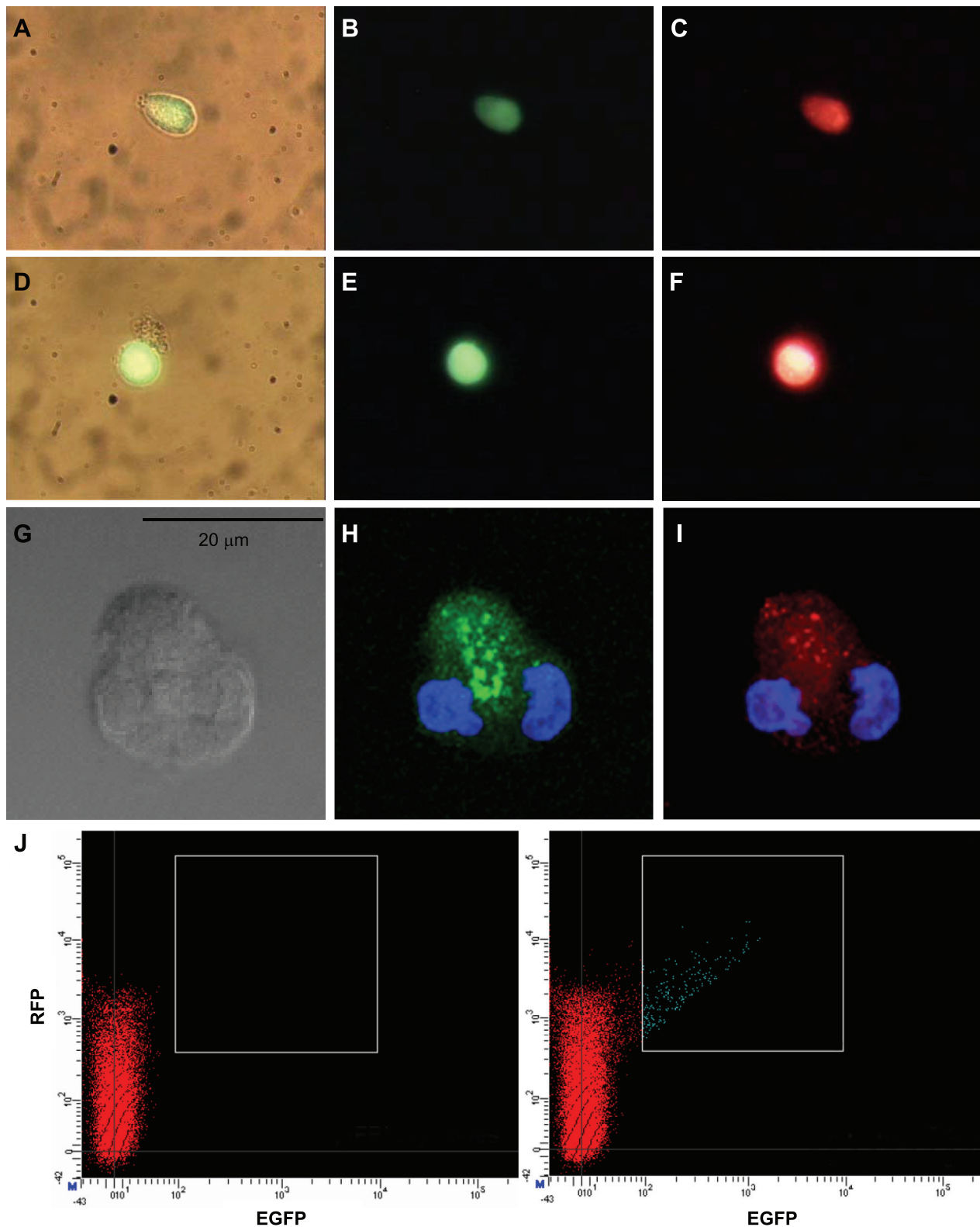
Fig. 7. EGFP$^+$ cells isolated after 6 days of coculture display RFP positivity. *A–F*: bright-field/EGFP fluorescence merging (*A* and *D*), EGFP fluorescence (*B* and *E*), and RFP fluorescence (*C* and *F*) of isolated, spontaneously beating cells (see also Supplemental Video S6 showing beating of the cell in *A*). The presence of both staining indicates the occurrence of cell fusion. *G–I*: single confocal section of an isolated EGFP$^+$ (*H*)/RFP$^+$ (*I*) cell. The presence of two nuclei (stained by 4,6-diamidino-2-phenylindole; *H* and *I*) indicates cell fusion. *J*: dot plots showing EGFP (*X*-axes) and RFP (*Y*-axes) fluorescence intensities (arbitrary units in logarithmic scale) of cells isolated from a 6-day-old coculture (*right*) or a day-matched culture of cardiomyocytes only (*left*). The white square identifies the double-positive (EGFP$^+$/RFP$^+$) population.

cardiomyocytes, we carried out flow cytometry analysis. In four independent experiments, flow cytometry analysis showed the absence of EGFP$^+$/RFP$^-$ cell population (Fig. 7J). This confirmed that 100% of EGFP$^+$ cells were also RFP$^+$, indicating the lack of cell-autonomous transdifferentiation of CD34$^+$ cells into cardiac myocytes.

DISCUSSION

In two previous reports (29, 36), it was shown that human cord blood-derived CD34$^+$ progenitor cells are able to integrate and connect with primary cultures of spontaneously beating neonatal cardiomyocytes. The main difference between mouse BM-derived c-kit$^+$ cells and human cord blood CD34$^+$ cells consisted in a different degree of functional integration with the myocyte monolayer (29, 36). In fact, while c-kit$^+$ cells in contact with neonatal cardiac myocytes presented inward and outward K$^+$ currents, but did not show any evidence of electromechanical coupling with surrounding cells (absence of APs), cord blood CD34$^+$ cells exhibited spontaneous calcium transients and functional gap junctions (36). In the latter study, however, the question as to whether human cord blood-derived CD34$^+$ progenitor cells simply express proteins (connexins) that allow them to electrically connect with the substrate or whether they may actually acquire a cardiac phenotype and, in such case, if it was the result of a trans-differentiation or a cell fusion process was not addressed. Furthermore, while several other reports have described in vitro differentiation (or lack of differentiation) of HSC/progenitor cells into cardiac cells on the basis of morphological and marker analysis, relatively little information is available about the relationship between functional differentiation (i.e., ability to generate APs), or potential electrophysiological integration into a contractile and electrically active tissue, such as the myocardium, and the fusion process. These issues are of relevance in the light of studies describing the modification of electrical impulse propagation by stem cells cocultured with cardiac myocytes (12) and calling for caution about the generation of potential heart rhythm disturbances following cellular administration (especially using skeletal myoblasts) to the ischemic myocardium, both in animal models and in patients (16, 17, 22).

Several sources of stem cells have been used so far in the search of a cell type suitable for cardiac regenerative interventions; pluripotent stem cells of either embryonic [ESCs (21)] or adult origin [induced pluripotent stem cells and spermatogonial-derived cells, (20, 46)] can easily and reproducibly generate electrically active cardiomyocytes and can integrate in a host myocardium, both in vitro and in vivo. It is less certain whether other types of stem cells derived from adult tissue, and in particular HSC/progenitor cells, can be used for the same purpose, despite a wealth of evidence for an improved cardiac function following injection of HSCs in the ischemic myocardium (reviewed in Ref. 2) and for their myocardial differentiation based on morphological, marker (5, 13, 19, 27, 31) and electrophysiological evidence (42). It is also still controversial whether in vivo cell fusion events do occur. A major criticism is that an apparent occurrence of fusion may result from methodological artifacts in experiments (especially histological analyses), addressed to localize injected stem cells into ischemic tissues (reviewed in Ref. 3). In addition, a mechanism involving the interaction of integrin-$\alpha_4\beta_1$ and the VCAM-1 cell adhesion molecule has been recently found to be responsible for fusion events of hematopoietic progenitors and cardiomyocytes in culture (47).

Assessment of cell fusion is not easily accomplished. Systems based on the Cre-LoxP system, for example, have been used mainly in vivo to demonstrate (1, 31, 35) or exclude (9) that stem cells injected in the myocardium undergo cell fusion with myocytes. This technique, however, is not suitable for quantification of fusion in vitro due to a relatively low efficiency of Cre enzyme that does not allow expression of reporter genes as a result of recombination of LoxP sites in 100% of cells (M. Pesce, unpublished observations).

In this study, we provide evidence that acquisition of cardiac features is a consequence of cell fusion. Using a two-color labeling system based on transduction with lentiviral vectors carrying EGFP and RFP, we observed green (hCD34$^+$-derived)/red (rat cardiac myocytes-derived) hybrid cells showing spontaneous contractility (Fig. 7). We decided to use this system to avoid possible *trans*-infection, as may occur, for example, by using adenovirus-based gene transfer (our unpublished results and Ref. 31) and possible unspecific cell labeling due to leakiness of vital fluorescent dyes, such as 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate/ 3,3'-dioctadecyloxacarbocyanine perchlorate labeling [DiI/DiO (5, 18)]. In addition, this system has been already used to assess the effects of cell fusion in studies in which different cell types were cocultured with cardiac myocytes (31, 39) or to show fusion of BM-derived stem cells and cardiac myocytes in vivo (24).

In agreement with our laboratory's previous study (36), we have confirmed that human CD34$^+$ hematopoietic progenitors integrate within the primary culture of rat neonatal cardiomyocytes. Here we show that this coculture system induces a change in morphology of CD34$^+$/EGFP$^+$ cells, within the first day, which is not immediately accompanied by electrical coupling, as demonstrated by their relatively depolarized V_r. The electrical coupling mediated by the connexin-43 proteins is then accomplished in the first week, as demonstrated by the hyperpolarization of the V_r of EGFP$^+$ cell and by their ability to propagate APs.

FACS analysis run on transduced cells, isolated after 6 days of coculture, and showing that 100% of the EGFP$^+$ cells recovered were also RFP$^+$ (Fig. 7) strongly suggests that acquisition of cardiac features is the result of fusion events between cardiac myocytes and CD34$^+$-derived cells. Furthermore, electrophysiological recordings did not show any difference between EGFP$^+$ cells and neonatal cardiac myocytes, supporting the hypothesis that these EGFP$^+$ cardiomyocytes originate indeed from the fusion between electrically passive CD34$^+$ cells and cardiac myocytes. In accordance with this observation, CD34$^+$/GFP$^+$ cells that did not contact neonatal cardiomyocytes during the coculture period did not show the functional properties of excitable cells (see Figs. 2B and 5B).

Finally, incubation with 5-AZA, a treatment reported to induce differentiation of mesenchymal progenitor cells toward a cardiogenic lineage (28, 30), failed to induce any electrophysiological change in CD34$^+$ cells. These data, in agreement with the previously reported lack of upregulation of human cardiac genes in CD34$^+$ cells during coculture (36), rule against a paracrine effect or a nuclear reprogramming process, induced by soluble factors deriving from the coculture conditions.

In summary, the present study shows that direct culture of human CD34$^+$ cells with neonatal cardiac myocytes determines cell fusion events, leading to the acquisition of cardiac physiological features rather than inducing differentiation. Although it remains controversial whether similar fusion events between hematopoietic-derived progenitors and adult cardiac myocytes also occur in vivo in the ischemic myocardium, the lack of cell autonomous differentiation in vitro suggest that CD34$^+$ UCB cells do not represent a suitable cell type to derive functional myocardium for cardiac regenerative interventions.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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