

Electrophysiological properties of mouse bone marrow c-kit⁺ cells co-cultured onto neonatal cardiac myocytes

Laura Lagostena^a, Daniele Avitabile^b, Elena De Falco^a, Alessia Orlandi^a, Francesca Grassi^c, Maria Grazia Iachininoto^b, Gianluca Ragone^b, Sergio Fucile^c, Giulio Pompilio^a, Fabrizio Eusebi^c, Maurizio Pesce^{a,*}, Maurizio C. Capogrossi^b

^aLaboratorio di Biologia Vascolare e Terapia Genica, Centro Cardiologico Monzino, Istituto di Ricovero e Cura a Carattere Scientifico, Milan, Italy

^bLaboratorio di Patologia Vascolare, Istituto Dermopatico dell'Immacolata, Istituto di Ricovero e Cura a Carattere Scientifico, Via dei Monti di Creta 104, 00167 Rome, Italy

^cLaboratorio di Biofisica, Dipartimento di Fisiologia Umana e Farmacologia "V. Erspamer", Centro di Eccellenza BEMM, Università La Sapienza, Rome, Italy

Received 11 May 2004; received in revised form 24 December 2004; accepted 18 January 2005

Available online 12 February 2005

Time for primary review 26 days

Abstract

Objective: Controversy about hematopoietic stem cells reprogramming into cardiac myocytes is currently supported by positive and negative findings. In fact, some reports have shown the ability of stem cells from the bone marrow (BM) to differentiate into cardiac myocytes and to contribute to myocardium repair, while others have reported the opposite.

Methods: C-kit⁺ cells from mouse bone marrow were co-cultured onto neonatal cardiac myocytes. Hematopoietic stem cell-derived cells were analyzed by investigating the expression of cardiac markers and ion channels and by single-cell electrophysiological recordings.

Results: Groups of undifferentiated c-kit⁺ cells displayed only outward currents. Co-cultured c-kit⁺ stem cells on neonatal cardiac myocytes expressed cardiac markers and Na⁺ and Ca²⁺ voltage-gated ion channels. However, Na⁺ and Ca²⁺ currents were not detected by electrophysiological patch-clamp recordings even if caffeine and cyclopiazonic acid treatment showed the presence of intracellular calcium stores. This suggests that these channels, although expressed, were not functional and thus do not allow the coupling between excitation and contraction that is typical of cardiac myocytes. Nevertheless, co-cultured cells had a more hyperpolarized resting membrane potential and, at least in a subset of cells, displayed voltage-gated inward rectifier currents and outward currents. Co-cultured c-kit⁺-derived cells were not connected to surrounding cardiac myocytes through gap junctions. To induce a more pronounced differentiation, co-cultured cells were treated with BMP-4 and TGF-β, two factors that were shown to trigger a cardiac myocyte differentiation pathway in embryonic stem (ES) cells. Even under these conditions, c-kit⁺ cells did not differentiate into functionally active cardiac myocytes. However, TGF-β/BMP-4-treated cells were hyperpolarized and showed an increased inward rectifier current density.

Conclusions: Our study shows that mouse BM hematopoietic stem cells exhibit a limited plasticity to transdifferentiate into cardiac myocytes in culture.

© 2005 European Society of Cardiology. Published by Elsevier B.V. All rights reserved.

Keywords: Cardiac myocyte; Stem cell; Reprogramming; Electrophysiology; Bone marrow; C-kit; TGF-β; BMP-4

1. Introduction

Cardiac myocytes bearing different excitation properties have been derived from multipotent embryonic stem (ES) cells by using different culture conditions [1,2]. In contrast, little evidence of in vitro cardiomyogenic conversion of

* Corresponding author. Laboratorio di Patologia Vascolare, Istituto Dermopatico dell'Immacolata, Istituto di Ricovero e Cura a Carattere Scientifico, Via dei Monti di Creta 104, 00167 Rome, Italy. Tel.: +39 6 6646 2428; fax: +39 6 6646 2430.

E-mail address: m.pesce@idi.it (M. Pesce).

adult-derived stem cells (particularly HSCs) has been provided, except for results obtained by culturing human peripheral blood (PB) endothelial progenitor cells (EPCs) onto neonatal cardiac myocytes.

In the present study, we used co-culture onto neonatal cardiac myocytes to assess whether c-kit⁺ cells acquire a cardiac phenotype. Our results demonstrated the expression of GATA-4, α -sarcomeric actin and MEF-2C cardiac markers and Na⁺ and Ca²⁺ voltage-gated subunits in co-cultured stem cells, but no electromechanical coupling, action potentials and Ca²⁺ transients.

Recent reports have shown that ES cells can be induced to differentiate into cardiac myocytes by embryonic activation pathways like those elicited by BMP-4 and TGF- β molecules [3] or visceral endoderm tissue [2]. Additionally, HSCs are affected in differentiation and proliferation/self-renewal pathways by treatment with TGF- β and BMP-4 in culture [4,5]. In the presence of TGF- β and BMP-4, c-kit⁺ cells had a more negative membrane potential and the amplitude of inward rectifier currents was increased compared to untreated co-cultured cells. However they still exhibited little differentiation ability.

2. Methods

2.1. Isolation of neonatal cardiac myocytes

We performed animal experimentation in compliance with NIH ethical rules. Neonatal cardiac myocytes were obtained as described in [6]. Cardiac myocytes were plated in 35 mm TC dishes coated with fibronectin (FN) (20 μ g/ml).

2.2. Isolation of c-kit⁺ cells

C-kit⁺ cells were separated from adult mice bone marrow by magnetic cell sorting (MACS) (Miltenyi Biotech) according to a protocol established by us previously [7].

2.3. Co-culture experiments

C-kit⁺ cells were co-cultured for 7 days onto cardiac myocytes in DMEM containing 10% FBS in the presence or the absence of 100 ng/ml of BMP-4 and 10 ng/ml of TGF- β (both from R&D systems) [6]. After this period, co-cultured cells were fixed for immunohistochemistry or analyzed for physiological parameters.

2.4. Immunofluorescence staining

Fixed cells were stained for α -sarcomeric actin (5C5, Sigma), myocyte enhancer factor 2C (MEF-2C, C-21, Santa Cruz Technologies), GATA-4 (H-112, Santa Cruz Technologies), anti CX-43 antibody (Sigma), anti Ca_v1.2a (cardiac L-type voltage gated Ca²⁺ channel Ca_v1.2a, Alomone) and

anti-pan-Na_v (all isoforms of voltage-gated Na⁺ channels α subunits, Alomone), followed a Rhodamine/Texas red or Cy5-conjugated secondary antibodies staining. Nuclei were stained with Hoechst 33258 and PI. Cells were analyzed with a Zeiss Axioplan2 Fluorescence microscope or a Zeiss LSM510 META confocal microscope.

2.5. RT-PCR

RNA was extracted from c-kit⁺ cells using Trizol reagent (Invitrogen). Primers sequence for GATA-4 mRNA amplification were: sense 5'GCCTGTATGTAATGCCTGCG 3'; antisense 5'CCGAGCAGGGAATTTGAAGAGG 3'. Primers for mouse β -actin amplification were: sense 5' CACCTTCTACAATGAGCT 3' and antisense 5' GAAGGTAGTCTGTGAGGTCCC 3'. Primers for mouse MEF-2C amplification were: sense 5' GATTACGAGGATAATGATGAG 3' antisense 5' GTACACCAAACACTGTATGGCTG 3'.

2.6. Electrophysiological recordings

Undifferentiated or co-cultured cells (c-kit⁺-GFP labeled cells and mouse neonatal cardiomyocytes) were bathed in NES (NES, mM: 140 NaCl, 2.8 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 10 glucose at pH=7.4) for recordings and observed under an inverted microscope (Zeiss Germany) with a 32 \times objective. Only healthy cells, showing clear cytoplasm and smooth membrane, were chosen for investigation. Whole-cell patch-clamp recordings were performed at room temperature (22–25 °C) using the Axopatch 200A amplifier (Axon Instruments, CA, USA). Patch pipettes were made using borosilicate capillaries (Hilgenberg, Germany), pulled using a vertical pipette puller (List Medical, Germany) and filled with a solution containing (mM): 135 KCl, 1 MgCl₂, 1 EGTA, 20 HEPES, 4 ATP, buffered with KOH at pH=7.3. Final pipette resistance was 3–5 M Ω . For investigations on Na⁺ and Ca²⁺ currents, intracellular KCl was substituted by the same amount of CsCl. Data processing was performed using pClamp9.0 (Axon Instruments, CA, USA). Whole-cell currents were low-pass filtered at 5 kHz and digitized at 10 kHz by the analog-to-digital interface DigiData 1200 (Axon Instruments). Series resistance was compensated for by 50–60%. Cell capacitance and membrane resistance were measured, using the Membrane Test option of pClamp 9.0, by applying brief voltage pulses around the holding potential. The total charge (Q_T) displaced by the step was estimated from the fit of the transient portion of the current response and the area under the curve. Membrane capacitance was calculated as the ratio between Q_T and the amplitude of the voltage step. Current magnitudes were normalized to cell capacitance (pA/pF). Where indicated extracellular solutions were modified by equimolar substitution of 20 mM NaCl by TEACl, KCl or 4-AP. Data are given as mean \pm standard error. Significance ($p < 0.05$) was determined by one-way ANOVA test.

2.7. Dye transfer and Ca^{2+} imaging recordings

In these experiments, the fluorescent tracer Alexa Fluor 594 hydrazide (Molecular Probes, Eugene OR, USA), was added to the intracellular solution to monitor gap junction permeability.

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using a microscopy system driven by Axon Imaging Workbench software (Axon Instruments), as described in [8]. Cells were loaded for 45 min with the Ca^{2+} indicator X-rhod-1 (λ_{excit} 560 nm; $\lambda_{emission}$ 602 nm, 4 μ M; Molecular Probes, USA) to avoid overlapping with GFP fluorescence. The changes of $[Ca^{2+}]_i$ were estimated from the ratio $\Delta F/F_0$. For ratiometric $[Ca^{2+}]_i$ measurements, cells were incubated with the cell membrane permeant fura-2 AM (4 μ M) for 45 min. Variation of $[Ca^{2+}]_i$ was expressed as time-resolved ratio, R , between fluorescence images obtained at 340 nm and 380 nm λ_{excit} . The reported amplitude of $[Ca^{2+}]_i$ transients (ΔR) was measured as the difference between peak and basal R values, and averaged from all the cells included in a single optical field [8].

3. Results

3.1. Marker and functional analysis of c-kit⁺ cells before and after co-culturing onto neonatal cardiac myocytes with or without BMP-4 and TGF- β

To detect whether c-kit⁺ cells express early cardiac markers prior to being co-cultured, we performed RT-PCR for MEF-2C and GATA-4 transcription factors. Although we did not detect GATA-4 expression, we noticed MEF-2C early cardiac transcription factor signal in c-kit⁺ cells (Fig. 1A). The expression of MEF-2C mRNA was specific, as we did not observe its expression in peripheral blood mononuclear cells. C-kit⁺ cells were then seeded onto layers of neonatal beating cardiac myocytes isolated from 1 day newborn mice. Three different labeling approaches were used to recognize seeded cells: (1) infection with a retrovirus carrying green fluorescent protein (GFP) [9], (2) sorting c-kit⁺ cells from mice expressing GFP [10] and (3) labeling cells using DiI [6].

Co-culture of skeletal myogenic cells [11], endothelial cells [12] and human PB [6] or cord blood (CB) [13] EPCs onto beating cardiac myocytes enhanced the expression of cardiac markers. We investigated the expression of the cardiac markers MEF-2C, GATA-4 and α -sarcomeric actin by immunohistochemistry. All these markers were expressed in both DiI- and GFP-labeled cultured cells (Fig. 1B–K).

Undifferentiated c-kit⁺ cells were relatively small and spherical, similar to cardiac stem cells [14], muscle-derived stem cells [15], and neuronal stem cells [16]. Under current-clamp conditions, the resting membrane potential (V_r) of undifferentiated c-kit⁺ cells was -21.0 ± 3.8 mV ($n=28$). No

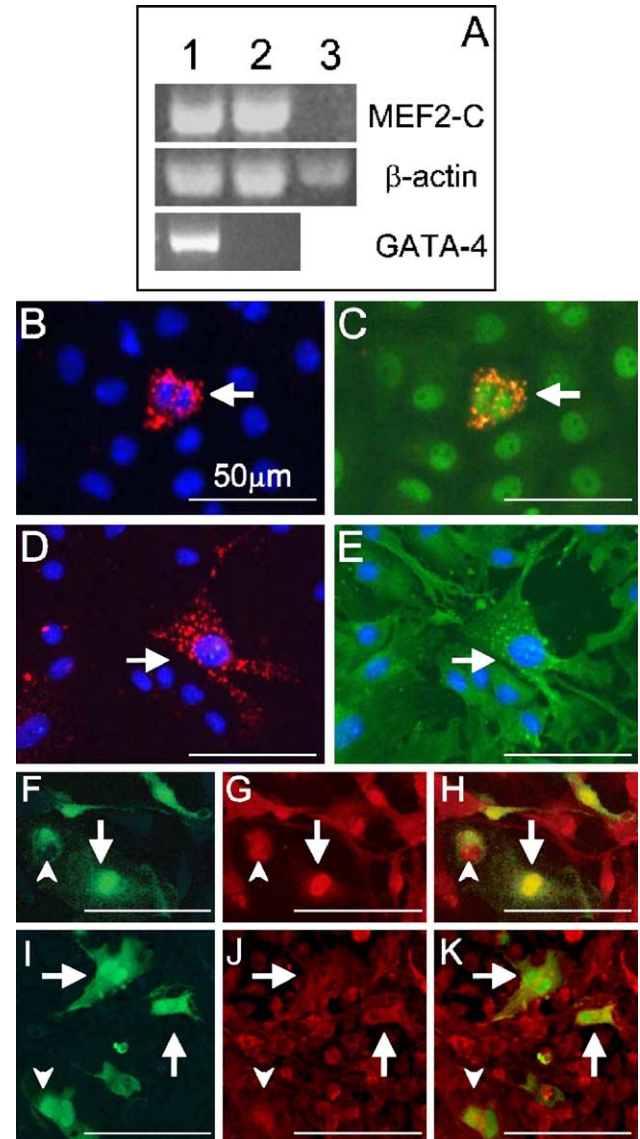


Fig. 1. Expression of cardiac markers in c-kit⁺ cells before and after co-culture onto cardiac myocytes. (A) RT-PCR on RNA extracted from neonatal cardiac myocytes (lane 1), c-kit⁺ (lane 2) and PB mononuclear cells (lane 3). (B and C) Expression of GATA-4 (C, nuclear green fluorescence) in DiI⁺ c-kit⁺ derived cells (B, cytoplasmic red fluorescence) co-cultured onto cardiac myocytes (arrows). (D–E) Expression of α -sarcomeric actin (E, green fluorescence) in DiI⁺ c-kit⁺ derived cells (D) co-cultured onto cardiac myocytes (arrows). (F–H) Confocal analysis of MEF-2C expression (G, red nuclear fluorescence) in GFP⁺ (F) c-kit⁺ derived cells onto cardiac myocytes. Panel H is the merger of panels F and G. Arrows indicate GFP⁺/MEF-2C⁺ cells. Arrowheads indicate GFP⁺/MEF2C⁻ cells. (I–K) Confocal analysis of α -sarcomeric actin (J, red fluorescence) in GFP⁺ (I) c-kit⁺ derived cells onto cardiac myocytes. Panel K shows the merger of panels I and J. Arrows indicate GFP⁺/ α -sarc⁺ cells. Arrowheads indicate GFP⁺/ α -sarc⁻ cells.

spontaneous electrical activity could be detected. To further characterize electrophysiologically undifferentiated HSCs, we performed voltage-clamp experiments. Membrane capacitance (C_m) was 3.6 ± 0.3 pF ($n=28$), while membrane resistance was 4.5 ± 0.7 G Ω ($n=28$). To assess whether undifferentiated HSCs express functional voltage-gated

channels, potential steps (50 ms duration) were applied to the patched cells held at -50 mV, testing potentials from -70 to $+50$ mV (Fig. 2A, top). In a fraction of the tested cells (11 out of 28, 11/28, 39%) an outward current like that shown in Fig. 2A (bottom) was observed. The mean density of the evoked current, measured at $+50$ mV, was 20.8 ± 4.5

pA/pF. The current was activated at potentials higher than -30 mV and increased linearly in the tested voltage range (Fig. 2B), with no inactivation, even when stimulated by voltage steps lasting up to 1 s. Bath application of TEA (20 mM), a blocker of several types of K^+ channels, reduced current amplitude by $73 \pm 6\%$ ($n=5$, see representative

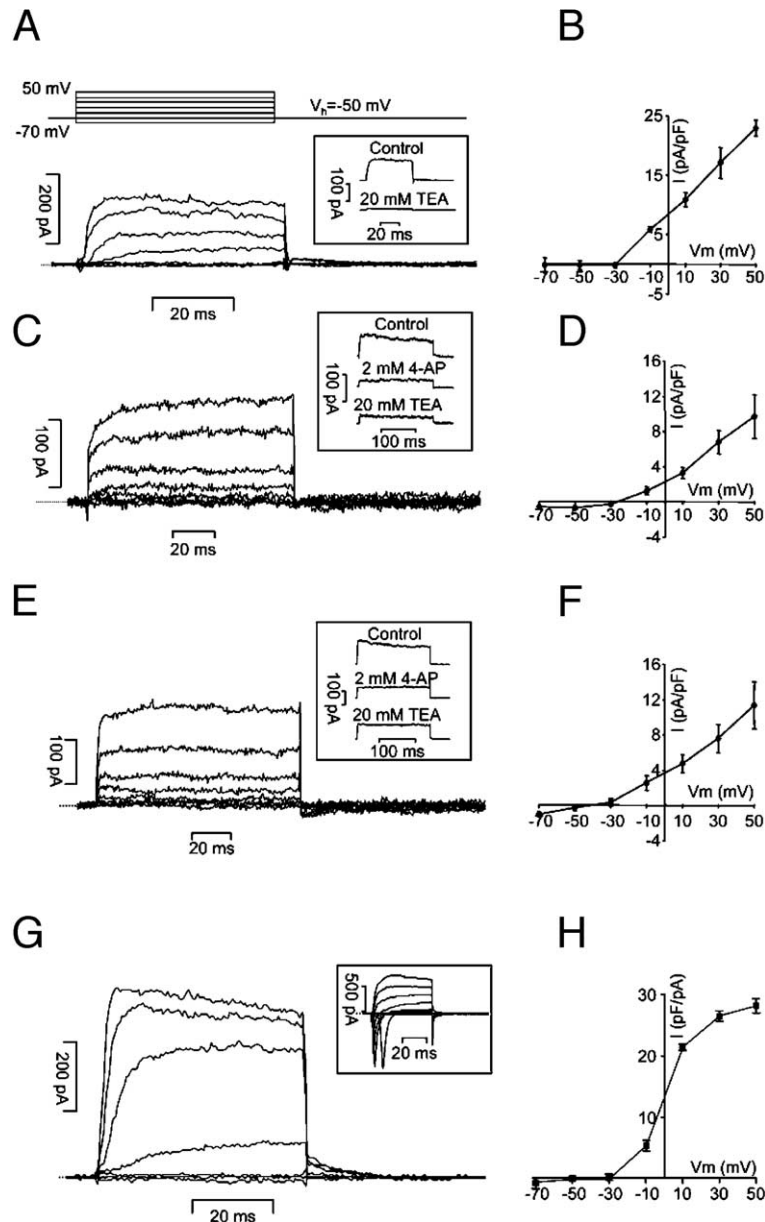


Fig. 2. Whole-cell recordings from $c\text{-kit}^+$ cells. (A) Current recordings evoked in undifferentiated $c\text{-kit}^+$ cells by the voltage protocol shown (upper traces). Dotted lines indicate 0 current level. Inset, extracellular application of TEA (20 mM, bottom) markedly reduces voltage-gated outward current (control). (B) Current/voltage (I/V) relationship for currents measured at the end of voltage steps. Each value is shown as mean \pm S.E. obtained from 6 different cells. (C) Representative voltage-gated currents from GFP^+ $c\text{-kit}^+$ -derived cell co-cultured onto neonatal cardiac myocytes. Inset: effect of extracellular 4-AP and TEA on voltage-gated currents in a single cell. (D) I/V relationship as a mean of 8 different cells. Note the significant reduction of current density in comparison to undifferentiated $c\text{-kit}^+$ cells (panel C) and linearity above -30 mV. (E) Currents from GFP^+ $c\text{-kit}^+$ -derived cell co-cultured onto cardiac myocytes in the presence of TGF- β and BMP-4. The current amplitude is not statistically different from co-cultured cells. Inset: effect of 4-AP and TEA on currents evoked in a treated cell. (F) I/V curve (mean from 5 recordings): note linear dependence on voltage. (G) Voltage-gated current of a GFP^+ $c\text{-kit}^+$ -derived cell co-cultured onto cardiac myocytes in the presence of TGF- β and BMP-4. Note the increased current amplitude in comparison with untreated cells. Inset: inward and outward currents evoked in a neonatal cardiac myocytes. (H) $I-V$ relationship of trace currents in panel G. Note the non-linear development of the voltage-dependence of the current.

example in Fig. 2A, inset), indicating that the outward current was mainly due to the activation of K^+ channels. No inward currents were observed using the KCl-standard intracellular solution, even when cells were held at more negative potentials (up to -90 mV), to rescue any functional channel from a possible steady-state inactivation. By contrast, inward currents (likely Na^+ -currents) were observed in cardiomyocytes under identical experimental conditions (see Fig. 2G, inset). As outward currents could mask the presence of inward currents, in particular Na^+ and Ca^{2+} currents, intracellular K^+ was substituted by Cs^+ , which generally has a limited permeability through K^+ channels. Under these conditions, neither inward nor outward currents were elicited by voltage steps to potentials ranging between -70 and $+50$ mV from a holding potential (V_h) of -90 mV (data not shown, $n=11$), indicating that undifferentiated c-kit⁺ stem cells are not endowed with functional Ca^{2+} and Na^+ channels, while their K^+ channels are impermeable to Cs^+ .

To investigate possible in vitro transdifferentiation, we seeded GFP-labeled c-kit⁺ cells onto neonatal cardiac myocytes. When co-cultured, a number of GFP⁺ cells lost their spherical shape and became larger and elongated (Fig. 1E,I). Since these morphological changes may be caused by a “response” of the stem cells to the host environment, electrophysiological investigations were performed on GFP⁺ elongated cells. Indeed, passive membrane properties were affected, as co-cultured cells exhibited a larger C_m compared to undifferentiated c-kit⁺ cells (12 ± 1.5 pF, $n=26$, $p < 0.0001$) and a more negative V_r (-55.2 ± 2.6 , $n=24$, $p < 0.0001$), while membrane resistance was 7.4 ± 1.4 G Ω ($n=23$). An outward current, similar to that observed in undifferentiated kit⁺ cells, was found in 15/32 cells (47%, Fig. 2C), with an activation threshold of -30 mV and a linear increase at more positive potentials (Fig. 2D). The current amplitude at $+50$ mV was 8.7 ± 1.2 pA/pF, significantly lower ($P < 0.00012$) than in undifferentiated cells. No significant difference in the passive membrane properties of excitable and non-excitable cells was observed ($P=0.5$), consistent with the idea that outward rectifying K^+ currents control cell excitability rather than its resting properties [17]. Application of 4-AP (2 mM), a potent blocker of several types of voltage-dependent K^+ channels or TEA (20 mM) blocked these currents by about 50% in all tested cells (3 for each blocker, Fig. 2C, inset). When a CsCl-based intracellular solution was used, no voltage-gated currents were detected, confirming that outward currents were indeed due to the activation of K^+ channels rather than to a specific outward conductance. Moreover, these experiments showed that co-cultured c-kit⁺ cells lacked Na^+ and Ca^{2+} currents, even when V_h was hyperpolarized to -90 mV, to rescue Na^+ channels from possible inactivation (data not shown, $n=19$). In some preparations (3/5), hyperpolarizing voltage steps up to -120 mV (from $V_h = -70$ mV) evoked inwardly rectifying currents in 6 out of the 17 GFP⁺ cells tested (35%, Fig. 3A) with an average current density of

-6.2 ± 2.4 pA/pF ($n=6$) at -120 mV. In these cells, membrane resting potential was markedly more negative than in the others, being -65.0 ± 5.6 mV ($n=6$), not far from the estimated reversal potential for K^+ . This, together with the prompt activation of the inward current suggests that these cells were endowed with inwardly rectifying K^+ currents (I_{Kir}), which are physiologically designed to set membrane resting potential [17]. The relationship between current density and test potentials (Fig. 3B) shows that rectification was incomplete, with a small current evoked at 0 mV, even in cells lacking an outward K^+ current. Cs^+ is a well known open-channel blocker of I_{Kir} , in several cell types, including cardiomyocytes [18,19]. As expected, in all tested cells (5/5, Fig. 3A) extracellular application of 1 mM Cs^+ resulted in a voltage-dependent block, with complete suppression of the responses evoked at negative potentials, but not at more positive potentials. This block was fully reversible upon Cs^+ wash-out (Fig. 3A). No depolarizing inward currents (Na^+ and Ca^{2+} currents) were detected ($n=19$), even when V_h was hyperpolarized to -90 mV, to rescue Na^+ channels from possible inactivation.

Addition to the culture medium of a mixture of BMP-4 and TGF- β enhanced the functional differentiation degree of c-kit⁺ cells co-cultured onto cardiac myocytes (in 3/9 preparations). In fact, c-kit⁺ derived cells were further hyperpolarized, with a V_r of -62.2 ± 2.4 mV ($n=36$) that was significantly higher than in untreated co-cultured cells ($p=0.029$). However, neither C_m (13.5 ± 1.3 pF, $n=39$), nor membrane resistance (5.6 ± 0.8 G Ω , $n=36$) were affected as compared to untreated co-cultured cells ($P > 0.45$). As in untreated co-cultures, outward voltage-activated K^+ currents were observed in a subset of GFP⁺-cells (14/41, 34%) (Fig. 2E), and resting membrane potential was similar to that of the non-excitable cells ($P=0.8$). These currents had a mean current density of 10.4 ± 1.3 pA/pF at $+50$ mV, no inactivation and a linear voltage-dependence (Fig. 2F). Extracellular application of either TEA (20 mM) or 4-AP (2 mM) reduced current amplitude by about 50% (3 cells tested for each substance, Fig. 2E, inset). When 4-AP was increased to 20 mM, the block was total (not shown). Interestingly, $K_v1.4$ channels, present in cardiac cells, are blocked by high concentrations of 4-AP.

Hyperpolarizing voltage steps evoked inward currents in 7/15 cells (47%, Fig. 3C, control). The inward currents were reversibly blocked by extracellular Cs^+ (Fig. 3C, 1 mM Cs^+), and reduced by extracellular TEA (20 mM) by about 20% ($n=3$, not shown), indicating that they were I_{Kir} [18,20]. Mean current density at -120 mV was -11.8 ± 1.1 pA/pF ($n=4$), significantly larger than the value observed in untreated co-cultured cells ($P=0.037$). In good agreement with the increased inward K^+ permeability of these cells, their resting potential was -76.3 ± 3.1 mV, hyperpolarized both as compared to untreated co-cultured cells showing I_{Kir} ($P=0.042$) and to treated cells lacking this current ($P=0.03$). Na^+ and Ca^{2+} inward currents were invariably absent ($n=23$), even when cells were hyperpolarized to -90 mV, using Cs^+

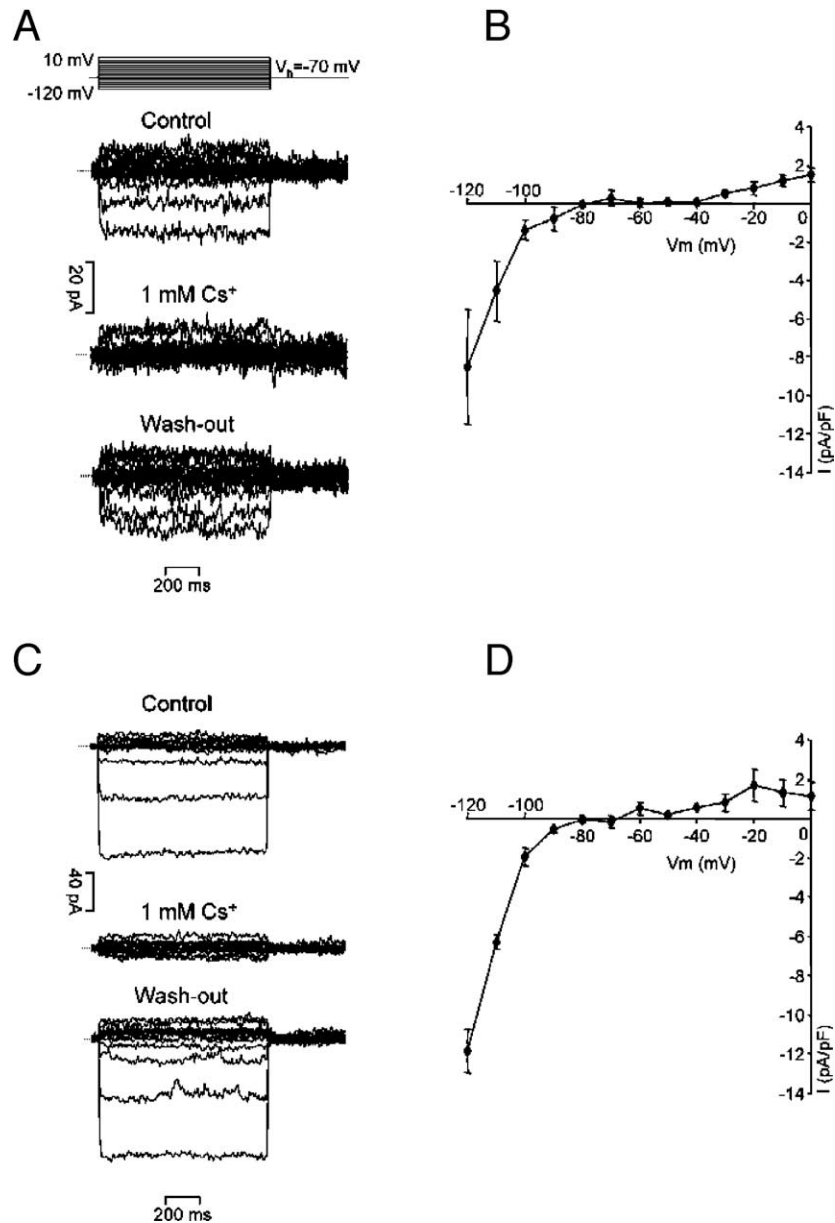


Fig. 3. Inward rectifier currents in c-kit⁺-derived cells onto cardiac myocytes in the absence or the presence of TGF-β and BMP-4. (A) Control: Inward rectifier currents were evoked by voltage steps ranging from -120 mV to +10 mV from -70 mV V_h (top). 1 mM Cs⁺: inward currents were blocked by extracellular perfusion of Cs⁺. The effect of Cs⁺ on inward currents was reversible (wash-out). All the recordings were obtained in the same cell. (B) I/V relationship of currents from 5 co-cultured cells (mean ± S.E.), showing inward rectification. (C) Control: Representative inward rectifier currents in TGF-β and BMP-4 treated GFP⁺ c-kit⁺-derived cells. 1 mM Cs⁺: block by extracellular Cs⁺. Wash-out: reversibility of Cs⁺-block of inward currents in the same cell. (D) I/V curve of inward rectifier currents from 4 treated cells.

in the patch pipette. The absence of depolarizing excitatory currents in c-kit⁺-derived cells was confirmed by the absence of spontaneous or evoked action potentials, while neonatal myocytes showed a spontaneous electrical activity, and action potentials were detected in both beating (Fig. 4F) and non-beating cells (Fig. 4E).

Finally, in 2/9 preparations, 40% (6/15) of BMP-4/TGF-β treated cells showed inactivating outward currents (Fig. 2G), that were never observed using intracellular Cs⁺. Current density at +50 mV was 28 ± 2.6 pA/pF, much greater than that measured in untreated cells ($P < 0.0002$).

The current density–potential curve (Fig. 2H) revealed that the current was activated at potentials more positive than -30 mV, and showed an I–V relation typical of an outward rectifier K⁺ currents [21].

The development of voltage-dependent ionic currents is linked to the expression of functional voltage-gated channels. Thus, we investigated by confocal microscopy whether c-kit⁺ cells co-cultured onto neonatal cardiac myocytes expressed voltage-gated Na⁺ and Ca²⁺ channels (Fig. 5). Immunoreactivity for Na_v channels was undetectable in progenitor-derived cells co-cultured onto cardiomyocytes

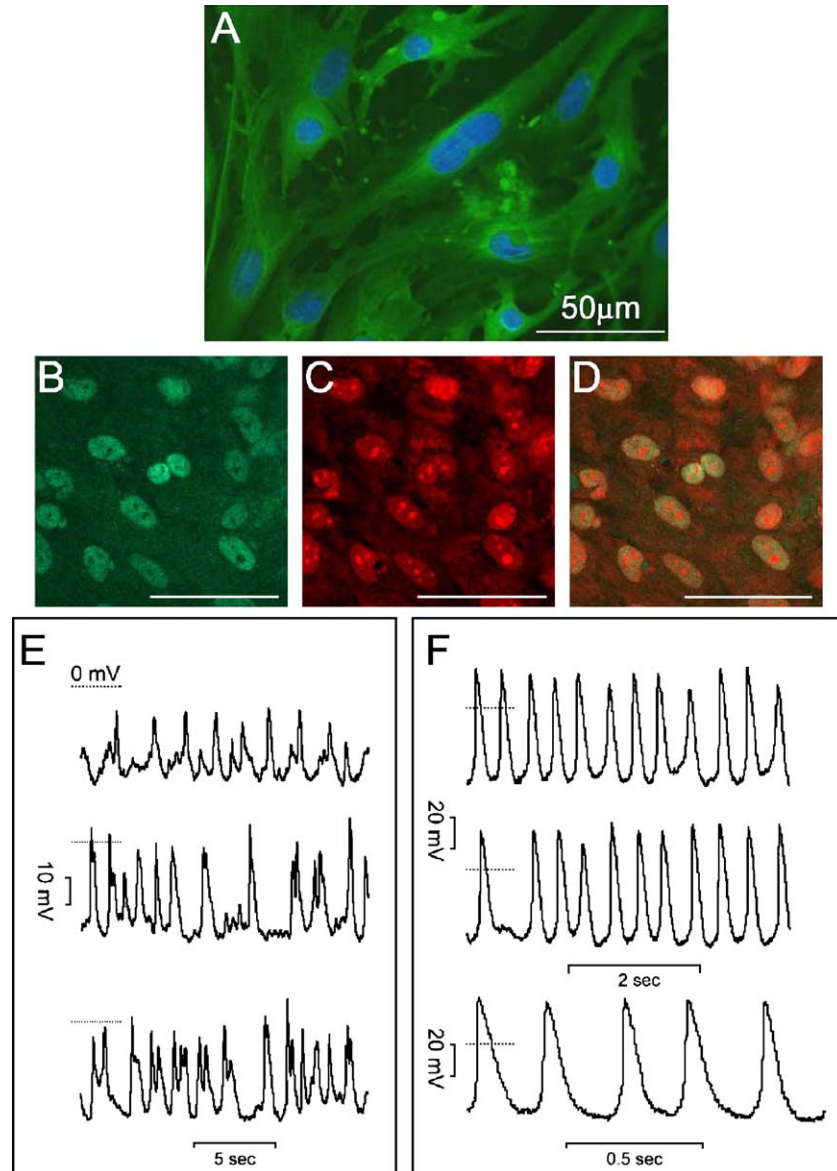


Fig. 4. Assessment of purity and function of neonatal cardiac myocytes. (A) Immunohistochemistry for Hoechst (blue staining) and α -sarcomeric actin (green staining) in beating neonatal cardiac myocytes. (B–D) Confocal analysis of GATA-4 (B, nuclear green fluorescence) and PI (C, nuclear red fluorescence) staining in beating cardiac myocytes. Panel D shows a merge of the green (GATA-4) and red (PI) channels. (E) Spontaneous electrical activity in a non-beating neonatal cardiomyocyte. Note the irregularity of amplitude and frequency of transients. Dotted lines indicate the 0 mV level. (F) Action potentials recorded in a beating neonatal cardiomyocyte. Representative consecutive recordings from the same cells (top and middle traces); bottom: action potentials showed on an expanded time scale. Dotted lines indicate the 0 mV level.

alone (Fig. 5A–C), while in a scant subpopulation of these cells, expression of little levels of these channels was observed (Fig. 5D–E) in the presence of BMP-4 and TGF- β . Voltage-gated Ca^{2+} channels were, instead, expressed in most of the $c\text{-kit}^+$ -derived cells in co-culture, independent of BMP-4 and TGF- β treatment (Fig. 5). It is important to note that, although specific, as confirmed by negative controls (not shown), most of the Na_v and Ca_v channel immunoreactivity was cytoplasmic, while the channels are mostly clustered at the cell membrane in mature cardiac myocytes [22]. It is likely that these channels are not mature and/or not functional (see Discussion).

3.2. Absence of gap junctions and RyR-receptors in $c\text{-kit}^+$ cells

The presence of gap junctions between human peripheral blood-derived EPCs and the surrounding cardiac myocytes has been already shown [6]. We thus injected GFP $^+$ cells with the red-fluorescent tracer Alexa Fluor 594, a gap junction permeable dye. In none of the 5 patched cells a dye transfer was observed (Fig. 6A), indicating that functional gap junctions between stem cells and the neighboring cells were absent. As a positive control, we injected the dye into neonatal cardiac myocytes, whence dye diffusion was

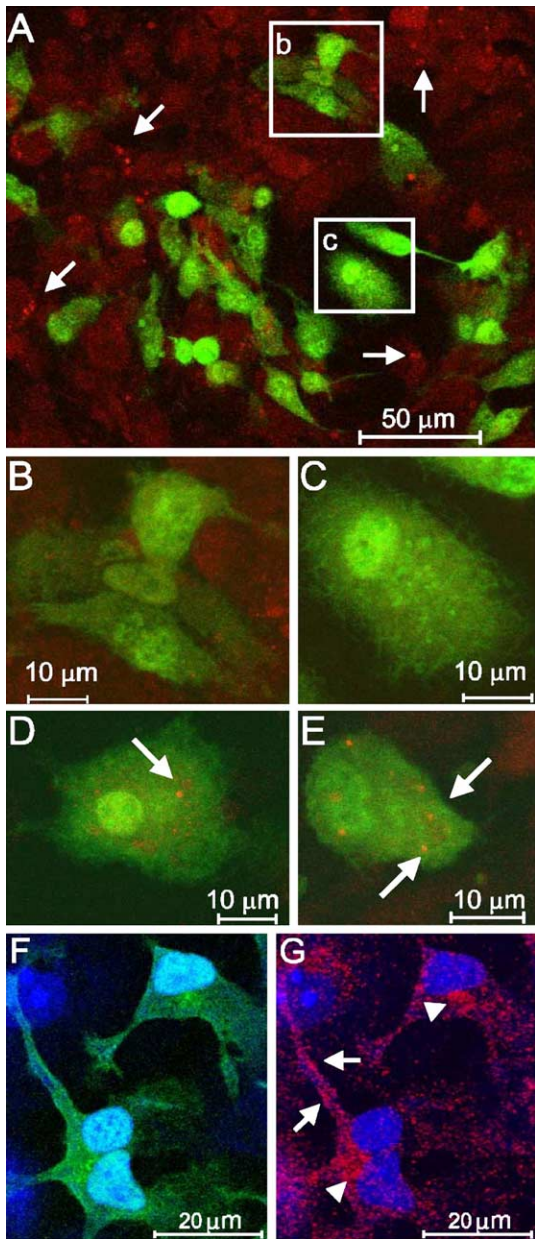


Fig. 5. Confocal analysis of voltage-gated Na^+ α subunits and Ca^{2+} channel expression in cardiomyocytes and $\text{GFP}^+/\text{c-kit}^+$ -derived cells in culture. (A) α subunits of Na_v channels were expressed at the junctions between adjacent cardiomyocytes (arrows) but not in GFP^+ cells. For example, b and c areas indicate cells that are shown at higher magnification in panels B and C, respectively, and which do not express Na_v channels. (B–C) Higher magnification of GFP^+ cells indicated in insets in panel A. Note that no expression of Na_v channels was observed in these cells. (D–E) Expression of α subunits of Na_v channels in $\text{GFP}^+/\text{c-kit}^+$ -derived cells upon treatment by $\text{TGF-}\beta$ and BMP-4 . The expression of few dots (arrows) suggests relatively little expression levels, while cytoplasmic localization indicates non correct membrane clustering. (F–G) Confocal analysis of $\text{Ca}_v1.2\alpha$ L-type Ca^{2+} channel subunit expression in $\text{GFP}^+/\text{c-kit}^+$ -derived cells onto cardiomyocytes. Panel F shows nuclear (PI, blue fluorescence) and GFP staining (green fluorescence) in two adjacent cells, while panel G shows relatively high expression of $\text{Ca}_v1.2\alpha$ channel subunits (red dots) in co-cultured cells. Most of the fluorescence was confined in the cytoplasm (arrowheads), except for few membrane clusters (arrows on the left cell).

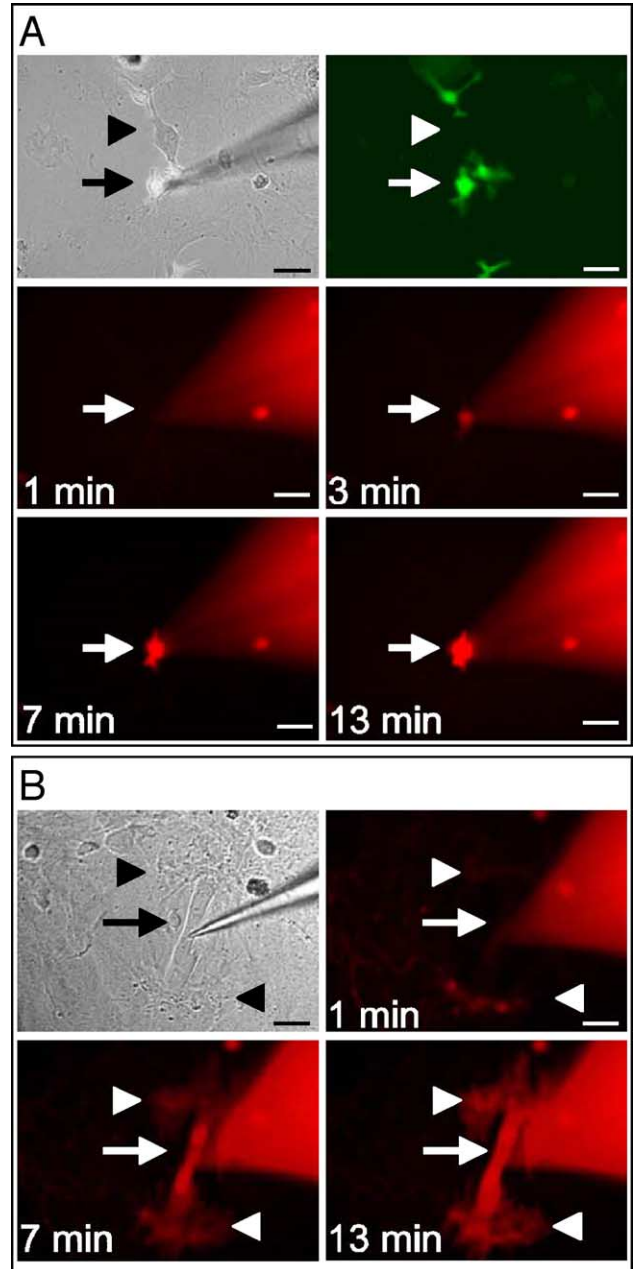


Fig. 6. Coupling between cardiac myocytes in culture by gap-junctions. Cardiomyocytes or $\text{GFP}^+/\text{c-kit}^+$ -derived cells were injected with Alexa Fluor 594. Diffusion of this gap junction permeable dye was followed up to 13 min after injection. (A) No diffusion was observed when the dye was injected into $\text{GFP}^+/\text{c-kit}^+$ cells (arrow) co-cultured onto cardiomyocytes (arrowhead). (B) The presence of gap junctions in cultured cardiomyocytes is confirmed by diffusion of the dye from the injected cell (arrow) into its neighbors (arrowheads).

invariably observed ($n=3$, see example in Fig. 7B). The absence of gap-junctions in $\text{GFP}^+/\text{c-kit}^+$ co-cultured cells was further confirmed by immunohistochemistry, revealing no expression of Connexin-43 protein (not shown).

Since stimulated Ca^{2+} transients are reported in human EPCs co-cultured onto cardiac myocytes [6], we performed Ca^{2+} imaging recordings by loading co-cultured c-kit^+ cells with the Ca^{2+} sensitive dye X-rhod-1 AM. Following

depolarization of cells by a KCl-enriched extracellular solution, no variations of intracellular free Ca^{2+} concentration were observed in GFP^+ cells. A possible explanation for the absence of Ca^{2+} transients may be that c-kit^+ -derived cells do not have intracellular Ca^{2+} stores or that they do not express endoplasmic reticulum (ER) receptors that are able to trigger Ca^{2+} -induced Ca^{2+} release. Therefore, we stimulated GFP^+ co-cultured cells with caffeine, an agonist of ryanodine (RyR) receptor. Only a subset of c-kit^+ cells became loaded with X-Rhod1, possibly because of active dye extrusion by stem cells. However, when GFP^+ cells were exposed to 5 mM caffeine for 10 s, Ca^{2+} release was never observed ($n=6/6$) while neonatal cardiomyocytes were responsive ($n=13/15$), as expected (not shown). Finally, to assess whether the lack of intracellular Ca^{2+} release depends on the absence of intracellular Ca^{2+} stores, we stimulated undifferentiated c-kit^+ cells using cyclopiazonic acid (CPA, Fig. 7A), known to induce Ca^{2+} release from intracellular stores by blocking Ca^{2+} -ATPase [23]. A high percentage of c-kit^+ cells (37/39, Fig. 7B) was responsive to CPA, showing CPA-dependent fluorescence increase ($0.34 \pm 0.04 \Delta R$, Fig. 7B), suggesting the presence of intracellular Ca^{2+} stores. We also stimulated undifferentiated c-kit^+ cells using SDF-1, a chemokine which has been shown to be important for release of intracellular Ca^{2+} stores in HSCs [24] and differentiation of these cells into endothelial progenitor cells [7]. When stimulated by SDF-1, at a concentration of 0.1 $\mu\text{g/ml}$ (Fig. 8A), about 26% (31/120, Fig. 7B) of the c-kit^+ cells showed intracellular Ca^{2+} increase ($\Delta R=0.46 \pm 0.07$, Fig. 7B, bottom). Taken together, these data demonstrate that c-kit^+ cells possess caffeine-insensitive intracellular Ca^{2+} stores, indicating the absence of cardiac-like Ca^{2+}

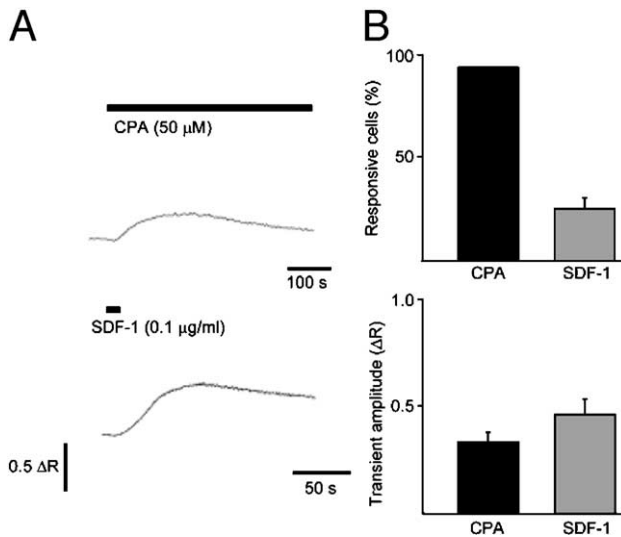


Fig. 7. Lack of elicited Ca^{2+} transients was not due to absence of Ca^{2+} intracellular stores. In fact, stimulation with CPA and chemokine SDF-1 induced Ca^{2+} release from c-kit^+ cells intracellular stores as shown by increase in fluorescence (ΔR). (A) Time course of fluorescence increase in c-kit^+ cells during application of CPA (50 μM , top) and SDF-1 (0.1 $\mu\text{g/ml}$, bottom). (B) Top: percentage of both CPA- and SDF-1-responsive cells; bottom: response amplitudes in CPA-SDF-1-stimulated c-kit^+ cells.

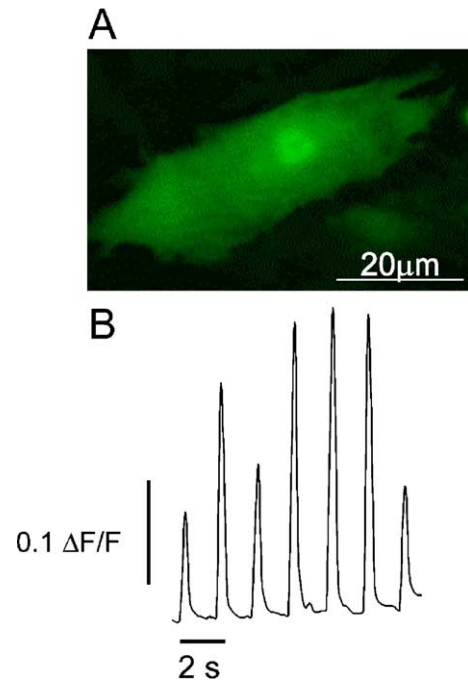


Fig. 8. Ca^{2+} transient in UCB, GFP labeled, CD34^+ cells [9] co-cultured onto cardiomyocytes. (A) GFP fluorescence of CD34^+ cells. (B) Ca^{2+} imaging recording ($\Delta F/F$ as a function of time) of one GFP^+ UCB CD34^+ -derived cell onto cardiac myocytes and loaded with X-rhod-1 showing spontaneous Ca^{2+} transient.

transients. As these results are in contradiction with those reported by [6], we asked if this discrepancy was due to our experimental and/or co-culturing conditions. To resolve this issue, we co-cultured CD34^+ stem cells extracted from human cord blood onto neonatal cardiomyocytes. CD34^+ stem cells, stained with GFP, showed spontaneous Ca^{2+} cyclic variations typical of cardiomyocytes [6] (Fig. 8) that were synchronous with beating neonatal cardiac myocytes.

4. Discussion

In this report, we investigated the plasticity of BM c-kit^+ cells to become cardiac myocytes from a functional point of view. To this aim, we cultured c-kit^+ cells onto layers of beating cardiac myocytes that were shown to promote cardiac myocyte differentiation of human peripheral blood stem cells [6]. C-kit^+ hematopoietic stem cells in co-culture were investigated by expression of cardiac markers and voltage-gated depolarizing Ca^{2+} and Na^+ channels, dye-transfer through gap-junction, whole-cell recordings and intracellular Ca^{2+} imaging.

Immunofluorescence studies revealed the expression of markers typical of cardiac myocytes. However, physiological experiments demonstrated preliminary signs of differentiation. In fact, we observed that co-cultured cells were larger and hyperpolarized as compared to c-kit^+ cells before culturing. The hyperpolarization was enhanced in co-cultures exposed to BMP-4 and TGF- β . Co-culture of c -

kit⁺-derived cells with cardiomyocytes also induced the appearance, in a subset of cells which were particularly hyperpolarized, of an inward rectifying current, known to be involved in setting up the resting potential in cardiomyocytes [17,25]. In a different subset of cells, outward rectifying currents were also observed. Our data show that the resting potential changes are not related to voltage-gated currents, except for the contribution of $I_{K_{ir}}$. They might be caused by modifications in the expression of ion pumps, or other voltage-independent conductances. For instance, the Na/Ca²⁺ exchanger, which affects membrane potential, is developmentally regulated in the mouse heart [26]. Given the high membrane resistance of the co-cultured c-kit⁺ cells, even minor changes in current fluxes would yield large potential changes. The contribution of $I_{K_{ir}}$ is proportional to its weight relative to these other conductances, and increases in BMP-4/TGF- β -treated cells, as its density is larger. A progressive hyperpolarization of differentiating stem cells has been reported for mesoangioblasts differentiating into skeletal muscle cells [27] and in muscle-resident stem cells, the satellite cells [28]. The beginning of differentiation is marked by the appearance of an outward K⁺ current, together with a mild hyperpolarization of the cells. At the final stages of differentiation, the expression of an inward rectifying K⁺ current shifts membrane resting potential towards K⁺ equilibrium potential. It is remarkable that a comparable sequence of events takes place in our c-kit⁺ cells.

Experiments performed by Badorff and colleagues [6] suggested that human circulating progenitor cells acquired physiological features of cardiomyocytes, when co-cultured onto cells isolated from neonatal heart. In line with these and other evidences [13], we found that human cord blood CD34⁺ stem cells have the ability to physiologically differentiate into cardiac-like cells showing Ca²⁺ transients and electro-mechanical coupling to surrounding cardiomyocytes (Fig. 8). Differences in source of stem cells (BM vs. PB), in the species of stem cell (mouse vs. human) and labeling techniques (GFP vs. Ac-LDL-DiI) to recognize co-cultured cells may explain the discrepancy of results obtained using mouse BM c-kit⁺ cells. However, these results are in line with recent findings by Balsam and colleagues [29] and Murry and colleagues [30] showing no differentiation of mouse BM c-kit⁺ cells in infarcted hearts. Thus, at least under these experimental settings, mouse BM hematopoietic stem cells exhibited a limited plasticity to transdifferentiate into cardiac myocytes.

In the mammalian, chick and *Xenopus* embryo, pre-cardiac mesoderm differentiation is subject to inductive signals by pathways involving TGF- β [31], Wnt and BMP [32,33] molecules. Additionally, HSCs are affected in differentiation and proliferation/self-renewal pathways by treatment with TGF- β and BMP-4 in culture [4,5]. Adding TGF- β and BMP-4 to culture medium, c-kit⁺ cells were hyperpolarized compared to co-cultured untreated cells, showed markedly increased inward rectifier current density, acquired immunoreactivity for voltage-dependent Na⁺

channels (Fig. 5), although they did not acquire an excitable/contractile phenotype like cardiac cells. Expression of Na⁺ and Ca²⁺ channels was quite surprising, because in none of the tested co-cultured cells alone or in the presence of BMP-4 and TGF- β depolarizing inward currents could be recorded. One possibility to explain this finding is that, although being expressed, assembly of Na_v and Ca_v channels protein complex is not optimal or is immature in c-kit⁺-derived cells. This interpretation is sustained by findings reporting that interactions of Na_v channels with ankyrin are likely involved in their correct exposure at the cell membrane of cardiomyocytes [34], or that interaction of different subunits is necessary for tethering the functional channel to the cell membrane [35,36]. Interestingly, our confocal microscope observations showed that most of the immunoreactivity for Na_v or Ca_v was localized in the cytoplasm rather than at the cell membrane (Fig. 5), suggesting that most of the α subunits of Na_v and Ca_v channels were not correctly exposed at the cell surface either due to the lack of functional interaction with cytoskeleton or the expression of other channel subunits.

In this study, we observed for the first time that expression of cardiac lineage markers do not correspond to functional differentiation of c-kit⁺-derived cells, thus calling for caution about possible therapeutic use of these cells for reconstructing infarcted hearts. Future studies performed by prolonging culture period, genetically manipulating these cells or embedding them in a three dimensional, *in vitro* reconstituted, heart tissue will allow to assess whether what we report here consists in an initial maturation of BM HSCs toward a fully functional cardiomyocyte phenotype, or whether it reflects indeed lack of cardiac plasticity of blood borne stem cells.

Acknowledgements

This work has been supported by: (1) EU FP6, contract no. LSHB-CT-2004-5029288, “Application and process optimization of stem cell for myocardial repair” (SC and CR) and (2) Italian Ministry of Health, contract no. 186, and contract no. 164, issued to MP and MCC.

References

- [1] He JQ, Ma Y, Lee Y, Thomson JA, Kamp TJ. Human embryonic stem cells develop into multiple types of cardiac myocytes: action potential characterization. *Circ Res* 2003;93:32–9 [Electronic publication 2003 Jun 5].
- [2] Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. *Circulation* 2003;107:2733–40 [Electronic publication 2003 May 12].
- [3] Behfar A, Zingman LV, Hodgson DM, Rauzier JM, Kane GC, Terzic A, et al. Stem cell differentiation requires a paracrine pathway in the heart. *FASEB J* 2002;16:1558–66.

- [4] Marone M, Scambia G, Bonanno G, Rutella S, de Ritis D, Guidi F, et al. Transforming growth factor-beta1 transcriptionally activates CD34 and prevents induced differentiation of TF-1 cells in the absence of any cell-cycle effects. *Leukemia* 2002;16:94–105.
- [5] Chadwick K, Wang L, Li L, Menendez P, Murdoch B, Rouleau A, et al. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood* 2003;102:906–15.
- [6] Badorff C, Brandes RP, Popp R, Rupp S, Urbich C, Aicher A, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation* 2003;107:1024–32.
- [7] De Falco E, Porcelli D, Torella AR, Straino S, Iachininoto MG, Orlandi A, et al. SDF-1 involvement in endothelial phenotype and ischemia-induced recruitment of bone marrow progenitor cells. *Blood* 2004;29:29.
- [8] Fucile S, Palma E, Mileo AM, Miledi R, Eusebi F. Human neuronal threonine-for-leucine-248 alpha 7 mutant nicotinic acetylcholine receptors are highly Ca²⁺ permeable. *Proc Natl Acad Sci U S A* 2000;97:3643–8.
- [9] Pesce M, Orlandi A, Iachininoto MG, Straino S, Torella AR, Rizzuti V, et al. Myoendothelial differentiation of human umbilical cord blood-derived stem cells in ischemic limb tissues. *Circ Res* 2003;14:14.
- [10] Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. *FEBS Lett* 1997;407:313–9.
- [11] Iijima Y, Nagai T, Mizukami M, Matsuura K, Ogura T, Wada H, et al. Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. *FASEB J* 2003;17:1361–3.
- [12] Condorelli G, Borello U, De Angelis L, Latronico M, Sirabella D, Coletta M, et al. Cardiomyocytes induce endothelial cells to transdifferentiate into cardiac muscle: implications for myocardium regeneration. *Proc Natl Acad Sci U S A* 2001;98:10733–8 [Electronic publication 2001 Sep 4].
- [13] Botta R, Gao E, Stassi G, Bonci D, Pelosi E, Zwas D, et al. Heart infarct in NOD-SCID mice: therapeutic vasculogenesis by transplantation of human CD34+ cells and low dose CD34+KDR+ cells. *FASEB J* 2004;18:1392–4 [Electronic publication 2004 Jul 01].
- [14] Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* 2003;114:763–76.
- [15] Torrente Y, Tremblay JP, Pisati F, Belicchi M, Rossi B, Sironi M, et al. Intraarterial injection of muscle-derived CD34(+)/Sca-1(+) stem cells restores dystrophin in mdx mice. *J Cell Biol* 2001;152:335–48.
- [16] Suslov ON, Kukekov VG, Ignatova TN, Steindler DA. Neural stem cell heterogeneity demonstrated by molecular phenotyping of clonal neurospheres. *Proc Natl Acad Sci U S A* 2002;99:14506–11 [Electronic publication 2002 Oct 15].
- [17] Tomaselli GF, Marban E. Electrophysiological remodeling in hypertrophy and heart failure. *Cardiovasc Res* 1999;42:270–83.
- [18] Nagashima M, Ishii K, Tohse N, Taira N, Yabu H. Unitary current through the inward rectifier K⁺ channel cloned from rabbit heart—comparison with the native K⁺ channel. *J Mol Cell Cardiol* 1996;28:957–65.
- [19] Picones A, Keung E, Timpe LC. Unitary conductance variation in Kir2.1 and in cardiac inward rectifier potassium channels. *Biophys J* 2001;81:2035–49.
- [20] Brouillette J, Clark RB, Giles WR, Fiset C. Functional properties of K⁺ currents in adult mouse ventricular myocytes. *J Physiol* 2004;559:777–98 [Electronic publication 2004 Jul 22].
- [21] Honore E, Barhanin J, Attali B, Lesage F, Lazdunski M. External blockade of the major cardiac delayed-rectifier K⁺ channel (Kv1.5) by polyunsaturated fatty acids. *Proc Natl Acad Sci U S A* 1994;91:1937–41.
- [22] Maier SK, Westenbroek RE, McCormick KA, Curtis R, Scheuer T, Catterall WA. Distinct subcellular localization of different sodium channel alpha and beta subunits in single ventricular myocytes from mouse heart. *Circulation* 2004;109:1421–7 [Electronic publication 2004 Mar 8].
- [23] Seidler NW, Jona I, Vegh M, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca²⁺-ATPase of sarcoplasmic reticulum. *J Biol Chem* 1989;264:17816–23.
- [24] Shen H, Cheng T, Olszak I, Garcia-Zepeda E, Lu Z, Herrmann S, et al. CXCR-4 desensitization is associated with tissue localization of hemopoietic progenitor cells. *J Immunol* 2001;166:5027–33.
- [25] Lopatin AN, Nichols CG. Inward rectifiers in the heart: an update on I(K1). *J Mol Cell Cardiol* 2001;33:625–38.
- [26] Liu W, Yasui K, Ophof T, Ishiki R, Lee JK, Kamiya K, et al. Developmental changes of Ca(2+) handling in mouse ventricular cells from early embryo to adulthood. *Life Sci* 2002;71:1279–92.
- [27] Grassi F, Pagani F, Spinelli G, De Angelis L, Cossu G, Eusebi F. Fusion-independent expression of functional ACh receptors in mouse mesoangioblast stem cells contacting muscle cells. *J Physiol* 2004;560:479–89 [Electronic publication 2004 Aug 19].
- [28] Liu JH, Bijlenga P, Fischer-Lougheed J, Occhiodoro T, Kaelin A, Bader CR, et al. Role of an inward rectifier K⁺ current and of hyperpolarization in human myoblast fusion. *J Physiol* 1998;510:467–76.
- [29] Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 2004;428:668–73.
- [30] Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature* 2004;428:664–8.
- [31] MacLellan WR, Brand T, Schneider MD. Transforming growth factor-beta in cardiac ontogeny and adaptation. *Circ Res* 1993;73:783–91.
- [32] Olson EN. Development. The path to the heart and the road not taken. *Science* 2001;291:2327–8.
- [33] Czyz J, Wobus A. Embryonic stem cell differentiation: the role of extracellular factors. *Differentiation* 2001;68:167–74.
- [34] Mohler PJ, Schott JJ, Gramolini AO, Dilly KW, Guatimosim S, duBell WH, et al. Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. *Nature* 2003;421:634–9.
- [35] Chen C, Bharucha V, Chen Y, Westenbroek RE, Brown A, Malhotra JD, et al. Reduced sodium channel density, altered voltage dependence of inactivation, and increased susceptibility to seizures in mice lacking sodium channel beta 2-subunits. *Proc Natl Acad Sci U S A* 2002;99:17072–7 [Electronic publication 2002 Dec 12].
- [36] Qu Y, Isom LL, Westenbroek RE, Rogers JC, Tanada TN, McCormick KA, et al. Modulation of cardiac Na⁺ channel expression in *Xenopus* oocytes by beta 1 subunits. *J Biol Chem* 1995;270:25696–701.