Running Title: Electrical stimulation enhances cardiac phenotype

Mono and biphasic electrical stimulation induces a pre-cardiac differentiation in progenitor cells isolated from human heart

Stefano Pietronave[a*], Andrea Zamperone[a*], Francesca Oltolina[a], Donato Colangelo[a], Antonia Follenzi[ad], Eugenio Novelli[b], Marco Diena[b], Andrea Pavesi[c], Filippo Consolo[c], Gianfranco Beniamino Fiore[c], Monica Soncini[c] and Maria Prat[ad]

[a]Department of Health Sciences, Università del Piemonte Orientale "A. Avogadro" (Novara, Italy)

[b]Department of Cardiac Surgery, Clinica S. Gaudenzio (Novara, Italy)

[c]Dipartimento di Elettronica, Informazione e Bioingegneria, Politecnico di Milano (Milano, Italy)

[d]Centro di Biotecnologie per la Ricerca Medica Applicata (BRMA), Via Solaroli 17, 28100 Novara, Italy.

[*]These two authors equally contributed to the work

Correspondance: Maria Prat, Dipartimento di Scienze della Salute, Università del Piemonte Orientale "A. Avogadro", Via Solaroli 17, Novara, Italy; tel.: +39 0321 660662; fax:: +39 0321 620421; e-mail: mprat@med.unipmn.it

Keywords: human cardiac progenitor cells, stem cells, electrical stimulation, cardiac differentiation

ABSTRACT

Electrical stimulation (ES) of cells has been shown to induce a variety of responses, such as cytoskeleton rearrangements, migration, proliferation and differentiation. Herein we have investigated whether mono and biphasic pulsed ES could exert any effect on the proliferation and differentiation of cardiac progenitor cells (hCPCs) isolated from human heart fragments. Cells were cultured under continuous exposure to mono or biphasic ES with fixed cycles for 1 or 3 days. Results indicate that neither stimulation protocol affected cell viability, while cell shape became more elongated and re-oriented more perpendicular to the electric field direction. Moreover, the biphasic ES clearly induced the up-regulation of early cardiac transcription factors MEF2D, GATA-4 and Nkx2.5, as well as the *de novo* expression of the late cardiac sarcomeric proteins troponin T, cardiac alpha actinin and SERCA 2a. Both treatments increased the expression of connexin 43 and its relocation to the cell membrane; but biphasic ES was faster and more effective. Finally, when hCPCs were exposed to both mono and biphasic ES, they expressed *de novo* the mRNA of the voltage-dependent calcium channel Cav 3.1(α_{1G}) subunit, which is peculiar of the developing heart. Taken together, these results show that ES alone is able to set the conditions for early differentiation of adult hCPCs towards a cardiac phenotype.

INTRODUCTION

The discovery that adult myocardium has a low self-renewing ability responsible for the physiological replacement of cardiomyocytes throughout the heart life [1] has raised great expectations in the field of cardiac regenerative medicine. In this context bioengineered cell culture environments that combine microenvironmental control with tissue- or cell-specific signaling are critical for the expansion of the cellular component. While the above work has established with no doubts that there is a turnover of the myoblast-myocytic component of the heart, although at low level, and has quantified it, cardiac progenitor cells (CPCs), which could represent a less differentiated cell source able to replace the ones which are lost both physiologically and possibly pathologically, were already identified previously in different laboratories [2-5]. From a biochemical point of view CPCs are generally identified on the basis of membrane markers, such as c-kit, MDR, Sca-1 [2], whose expression, however, is not restricted to this cell population and in some cases was found to be somehow unstable [6]. The ideal candidate for myocardial cell-based therapy should meet the following criteria: *i)* cardiac/vascular commitment, *ii)* ability to integrate within the recipient tissue, thus developing connecting gap junctions with surrounding cells *iii)* resistance to apoptosis. Human cardiac progenitor cells (hCPCs) are endowed of most of these properties, thus representing a likely convenient cell source for tissue engineering applied to myocardium, when compared to other cell types of adult stem/progenitor cells, namely mesenchymal stem cells derived from bone marrow, cord blood, adipose tissue or skeletal muscle [7].

Indeed c-kit/Sca-1 hCPCs isolated from atrial biopsies which display stemness features [6-8] were used to fabricate scaffold-less patches, that were transplanted on the heart of mice and shown to engraft into the host tissue. The *in vivo* microenvironment provided important signals, since the transplanted cells acquired *de novo* expression of some cardiomyogenic markers [6]. Cardiac commitment/differentiation of hCPCs *in vitro* has been usually

approached by the application of exogenous biochemical factors [9-13]. Physical signals, such as electrical stimulation (ES) of stem/progenitor cells, are known to induce a variety of responses such as wound-healing and galvanotaxis [14], angiogenesis [15], neurogenesis, and myogenesis [16,17]. In particular, since the heart is composed of contractile cells driven by ion currents, the latter are recognized to play a key role in cardiomyogenesis *in vivo* [18]. *In vitro* ES can improve the functional assembly of neonatal mouse and rat cardiomyocytes into a contractile engineered cardiac tissues [19-21] and promote the differentiation toward the cardiac lineage of mouse embryonal stem cells [22]. However, few and recent studies have been undertaken to elucidate the possible effects of ES as cardiopoietic signals in adult human stem cells [23-26].

Herein hCPC were subjected to electric stimulation by means of an *ad hoc* designed bioreactor. In particular, the cardiomyogenic effectiveness of two protocols based on the application of pulsed monophasic and biphasic ES to induce pre-cardiac differentiation in c-kit/Sca-1 hCPCs was evaluated.

MATERIAL AND METHODS

Cell culture

Human cardiac progenitor cells were obtained from biopsies provided by the Department of Cardiac Surgery of the Clinica S. Gaudenzio, Novara (Italy) from patients undergoing cardiac surgery after signing a written informed consent according to a protocol approved by the Institutional Review Board (IRB) of Novara (Italy). Samples of right auricula were processed as described by Forte and colleagues [6]. Briefly, 1-3 mm^3 specimens were mechanically minced and enzymatically digested; partially digested tissue fragments were then plated on 0.02% gelatin-coated dishes in 1/3 Claycomb (Sigma Aldrich, St. Louis, MO, USA) and 2/3 F12K (Invitrogen Life Technologies Italia, Monza MB, Italy), 10% fetal bovine serum (FBS,

Lonza Biowittaker, Verviers, Belgium), 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma Aldrich) and cultured at 37°C in 5% CO_2, changing the medium every other day. After 7-14 days, the cells migrating from the fragments were harvested by trypsinization, expanded to obtain approximately 5 x 10^6/each biopsy and were sorted by magnetic immunobeads using anti-c-kit antibody (Miltenyi Biotec GmbH, Germany). Cells were used from the third until the seventh passage after immunselection.

The experiments reported in the present study were performed using five different preparations of hCPCs, AU110, AU572, AU1975, AU1778, AU 2115, which herein are named from 1 to 5 respectively.

Characterization of human cardiac progenitor cells

Cytofluorimetry. Cells from the third passage after immunoselection were detached with 5 mM ethylenediaminetetracetic acid (EDTA, Sigma-Aldrich), washed twice with phosphate buffered saline (PBS) and incubated 20 minutes with PE-labeled antibodies against CD117, CD90, CD34, CD45 (Biolegend, San Diego, USA), and Sca-1 (BD Bio-sciences, Buccinasco, Italy), FITC-labeled CD105, CD44 antibodies (Biolegend), following the manufacturer's instructions. Cells were then washed twice with ice cold PBS and fixed in buffered 1% paraformaldehyde, 2% FBS for 15 minutes at 4°C and analyzed with the FACScaliber flow cytometer (BD Bio-sciences) within 48 hours.

RT-PCR and quantitative real time PCR (qRT-PCR). Total RNA was extracted in Trizol® reagent (Invitrogen), followed by DNAse treatment (DNAse I, Fermentas, St. Leon-Rot, Germany). Then, 1 μg RNA was retrotranscribed in cDNA with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) using the oligo(dT) primers listed in Table 1. PCR reactions were performed using the PCR Master Mix 2x kit (Fermentas) in a final volume of 25 μl containing 50 ng cDNA and 200 nmol/l primers. The PCR conditions were:

94°C for 2 minutes, 35 cycles 94°C for 30 seconds, annealing temperature for 30 seconds (see Table 1), 72°C for 30 seconds and finally 72°C for 7 minutes. For β-actin, 25 cycles were performed. The amplified products were resolved in 2% agarose gel electrophoresis, stained with ethidium bromide and documented with GelDoc system (Biorad Laboratories, Milan, Italy).

For qRT-PCR total RNA was extracted and retrotranscribed in cDNA as described above. Single gene expression was normalized to that of the ribosomal 18S rRNA housekeeping gene. Analyses were based on the methods previously described, adapted in our laboratory [8,27,28]. Briefly, assays were performed in triplicate for each treatment in a 20 µl reaction volume containing 1 µl of RT products, 10 µl EVA Green SMX (BioRad, USA), 300 nM each forward and reverse primers, as indicated in Table 1. An automated CFX96 thermocycler was used (BioRad, USA). The reaction conditions were 95°C for 3 minutes, followed by 35 cycles of data collection at 98°C for 5 seconds and an anneal–extend step for 5 seconds at 60 °C (Table 1), specific for each primers couple. At the end of these cycles, a melting curve (65°C to 95°C, with plate read every 0,5°C) was performed in order to assess the specificity of the amplification product by single peak verification (e.g. 18S rRNA melting temperature peak was 86°C, the other genes ranged between 82°C and 88°C). Results were analyzed with BioRad CFX Manager and exported to Excel (Microsoft, Redmond, WA) for calculation and statistical analysis. For each data point the Ct gene/Ct 18S rRNA value was calculated, where threshold cycle values Ct were determined from semilog amplification plots (log increase in fluorescence versus cycle number). The relative ratio of each target gene repeat copy number to the 18S rRNA copy number (ΔCt) for each time point was calculated as:

$[2^{Ct(target\ gene)} / 2^{Ct(18SrRNA)}]^{-1} = 2^{-\Delta Ct}$, and related to the reference samples, by using the formula: $2^{-(\Delta Ct\ treatment - \Delta Ct\ primary)} = 2^{-\Delta\Delta Ct}$ Samples with a $2^{-\Delta\Delta Ct}$ greater than or lower than 1, had an average expression level greater or lower than that of the reference samples, respectively.

Electrical stimulation

We developed a bioreactor able to apply highly controlled ES protocols to the cultured cells. It is composed of a multichamber culture system with a set of two chassis hosting 12 independent silicone culture chambers (Fig. 2a left, and connected to a custom made computer-based programmable electric stimulator for the pulse generation. Each chamber (30x15x12 mm), rectangularly–shaped, is made of polydimethylsiloxane (PDMS, Sylgard 184®, Dow Corning, USA). The production of the chambers was obtained by the mould casting technique. The bottom surface of the chambers and the covers are flat and transparent to allow optimal visualization of the cells. We included in each chamber a couple of parallel electrodes, fabricated with AISI 316 stainless steel rods (2.5-mm diameter) and placed 12 mm apart. Two spring connectors, mounted on the chassis, allow the electrical connection between the electrodes and the electric stimulator, which is able to generate mono and biphasic voltage stimulation with adjustable square waveforms (amplitude, pulse width and frequency). The electric stimulator is designed and integrated in the culture system based on a low-cost and open source I/O board (Arduino UNO, Smart Projects, Strambino, TO, Italy), coupled with a National Instrument data acquisition board (PCMCIA, NI DaqCard6024E, National Instrument, Austin, TX), the latter used to record the voltage signal delivered to the stimulating electrodes at a 25 kHz sampling rate. The whole system design was performed with advanced engineering techniques, including finite-element-method (FEM) simulations [29].

For ES, hCPCs were plated at a density of 5000 cells/cm^2 onto sterile, 0.1% gelatin-pre-coated glass slides (Marienfeld laboratory glassware, Lauda-Königshofen, Germany), which provided a square surface of 2 cm^2, and cultured for 24 hours in standard conditions, before slides were transferred into PDMS chambers and placed in the bioreactor. Either mono (2 ms, 1 Hz, 5V amplitude) or biphasic (2 ms, 1 Hz, ±2.5 V) square-wave pulses were applied to the cultured cells (Fig. 2b). Cells were stimulated for up to 3 days, the medium was changed daily.

Cell viability and proliferation assays

MTT assay. After ES for the different periods of time (1, 2 and 3 days), slides were transferred in conventional plates and cell viability was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (MTT, Sigma Aldrich). Briefly, 60 µl of MTT solution (5 mg/mL in PBS) were added to each sample, which contained 300 µl of culture medium. Samples were then incubated at 37ºC for 3 hours. Afterwards, medium was eliminated and 0.2 N HCl in isopropanol was added to dissolve formazan crystals. Optical density was measured in a multiwell reader (2030 Multilabel Reader Victor TM X4, Perkin Elmer, Monza (MI), Italy) at 570 nm. Three different experiments were performed analyzing 3 replicates for each sample. Results are expressed as the percentage of proliferation of ES cells relative to T0 (cell number before ES application). Each experimental condition was assessed in triplicate in three separate experiments.

LIVE/DEAD assay. Cell viability of stimulated and unstimulated hCPCs was evaluated with the live/dead staining kit (Biovision, Milpitas, California) after 3 days of ES. In this assay living cells are stained with the cell permeable green fluorescent dye and non-living cells are stained in red with propidium iodide (PI). Freshly stained cells were analyzed by laser scanning confocal microscope (Leica TCS SP2, Heerbrugg, Switzerland).

Morphometric analysis

Images taken at 20x magnification with microscope (Zeiss HB050 and Qimaging RETIGA 2000R camera) were analyzed by drawing cell outlines using ImageJ software. The length of the major axis of the cell was taken as cell length [26]. For each experimental time-point (1 and 3 days) 50 to 60 cells were considered in each of the ten images taken from three separate experiments. ImageJ software was used also to define the angle of the major axis of the cells. The direction parallel to the electric field was assumed as 0° (Fig. 3A, top left). The degree of alignment and orientation of hCPCs were represented as a percentage of the total number of cells referred to 10° angle intervals ranging from 0° to 90°.

Immunofluorescence analysis

Cells were fixed in 3% paraformaldehyde in PBS for 20 minutes, washed, and permeabilized with 0.2% Triton X100 in PBS for 10 minutes, washed and incubated with rabbit antibodies (Abcam, Cambridge, UK) against Cx43 (1/400), GATA-4 (1/400) in PBS, 1% bovine serum albumin (BSA), 4% goat serum for 2 hours at room temperature, followed by secondary Alexa Fluor® 488 and tetramethylrhodamine isothiocyanate (TRITC)-labeled goat-anti-rabbit-IgG antibodies (1:500, Invitrogen) for 45 minutes at room temperature. Cell nuclei were counter-stained with DAPI (1:200, Sigma-Aldrich). The expression of GATA-4 was quantified in control unstimulated, mono and biphasic stimulated cells by manual counting. Percentage of positive cells was calculated as number of nuclei stained for GATA-4 *versus* total nuclei stained with DAPI. For each experimental time-point (1 and 3 days) a number ranging between 50–200 nuclei were considered in each of the ten images taken from three separate experiments. Images were taken with fluorescence Leica DM5500B microscope.

Western blot analysis

Unstimulated and electrically stimulated hCPCs for 1 and 3 days were washed twice with cold PBS and lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM HEPES, 0.1% SDS, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 10% Glycerol and a cocktail of protease inhibitors (Sigma-Aldrich). Cell lysates were centrifuged at 13,000 rpm and 4°C for 15 min. Total cell extracts (20 μg of protein) were denatured by heating for 5 min at 95°C in reducing Laemmli buffer; proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in two identical gels (except that 1/10 of each extract was used for probing for actin), and then transferred onto PVDF filters. Filters were blocked with methanol for 5 min, rinsed in water, and probed either with monoclonal antibodies against Troponin T (1:1000, Abcam) or β-actin (1:2000, Thermo Scientific) diluted in Tris-buffered saline (TBS), 5% BSA, for 2 hours at room temperature. After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated secondary anti-mouse IgG antibodies followed by enhanced chemiluminescence (ECL kit, GE Healthcare Europe GmbH, Milano, Italy) and analyzed in a Versadoc instrument (Biorad, USA).

Statistical analysis

Quantitative analyses are presented as mean ± standard deviation (SD) and differences between samples were determined by Student's *t*-test. One-way ANOVA and Bonferroni post-test analyses on selected pairs of groups were also performed with Prism (GrapPad software Inc., USA, version 4.03). Values with a $p \leq 0.05$ or $p \leq 0.01$ were considered as statistically significant.

RESULTS

Characterization of cardiac progenitor cells (CPCs)

Cells migrated from digested fragments reached confluence after a few days, were expanded once and then enriched for the fraction expressing c-kit by an immunomagnetic procedure. The enriched population was expanded again and characterized for its immunophenotype in cytofluorimetry starting from passage 3. At this passage most of the cells remained positive for mesenchymal stemness markers Sca-1 and CD44, while the expression of the stemness marker c-kit was found to be decreased (Fig. 1a). Similar somehow unexpected data were already reported and explained by postulating that, rather than being different cell populations, c-kit(+)/Sca-1(+) cells and c-kit(-)/Sca-1(+) cells represent two phenotypic stages of the same original population. In such cells c-kit could mark the more immature cell pool, and Sca-1 could mark a more mature actively growing cell population, resembling more a transit-amplifying population [6]. Indeed both cell populations displayed similar multipotency [6]. Cells were also positive for the mesenchymal markers CD90 and CD105, while they were negative for the hematopoietic markers CD34 and CD45. The expression of such markers was maintained through all the used cell cultures.

The expression of early cardiac markers, as well as growth factors and their receptors (GF/GF-R), was evaluated in RT-PCR (Fig. 1b) Human heart tissues derived from the same patients were used as reference controls. The early cardiac markers GATA-4, MEF2C and TBX-5 were detectable in samples from all the patients, although a significant variability was found. Also growth factors were expressed in all samples, while in few cases the cognate receptors could not be detected.

Effects of ES on cell proliferation and viability

We first evaluated the effect of ES on hCPCs proliferation and viability. Pulse duration and rate were selected to provide the stimulated cells with a biomimetic-like electric stimulation

pattern: the selected values are indeed characteristic of electric activity of native myocardium of different animal species [30,31]. Additionally, the 2 ms pulse duration was adopted to dissipate the double-layer effect of the electrodes between subsequent pulses, as previously described in the literature [,32,33], Furthermore, based on preliminary stimulation experiments on hCPCs (not shown), a 5 mV pulse amplitude (which, according to our FEM simulations [29], produced a 3.4 V/cm pulse amplitude of the electric field in the cell-culture region) demonstrated to be the maximum safe stimulation voltage amplitude not inducing cell death/apoptosis and/or cell detachment from the glass substrate. Fig.2b, top depicts the voltage pulses recorded at the electrodes, whose shapes closely replicate pure square waves (superimposed noise <1% of the pulse amplitudes).

The proliferation rate of the cells exposed to both mono and biphasic ES, as determined in an MTT assay, was similar to that of the unstimulated cells in the first two days. In the third day, unstimulated and stimulated hCPS with monophasic ES slowed down their proliferation rate. By contrast, biphasic ES completely stopped cell growth (Fig. 2c). For both stimulation protocols (monophasic and biphasic pulses) cell viability, as determined by qualitative Live/Dead assay after 3 days of ES, was similar to that of unstimulated cells, with few nuclei positive for PI staining, thus suggesting that the ES protocols did not affect hCPCs viability (Fig. 2d).

Effects of ES on cell morphology and orientation

ES was responsible for evident modification of cell morphology that became spindle-shaped (Fig. 3a). Biphasic stimulation effects were significantly evident already after 1 day of treatment and increased after 3 days. In the case of monophasic ES, these changes were observed only after 3 days of stimulation. Furthermore, cells increased their length around 70% and 100% after 1 and 3 days of biphasic stimulation respectively (Fig. 3b). Monophasic stimulation affected cells length at a lesser extent, since they increased their length of 30%, but only after 3 days of stimulation (Fig. 3b). Moreover, the random spatial orientation of the unstimulated hCPCs was replaced by an evident alignment of about 30% of the cells with an angle of 30-40° relative to the direction of the electric field within the chamber after 1 day of biphasic ES (Fig. 3c). A higher percentage of oriented cells was observed after 3 days of ES, and, more interestingly, the angle interval increased up to 50-60°, with the final result that cells tend to align perpendicular to the direction of the electric field (Fig. 3d). A similar trend was observed also for hCPCs stimulated with monophasic pulses, although this effect was observed only after 3 days of ES and in a lower percentage of cells (oriented cells were around 22%) displaying an angle of only 30-40° (Fig. 3d).

ES induces the up-regulation of cardiac early commitment and delayed genes

Human cardiac progenitor cells were found to express the GATA-4, MEF2C and TBX5 markers typical of early cardiac commitment (Fig. 1b). To evaluate whether ES induced further a stronger commitment towards a more mature cardiac phenotype, qRT-PCR experiments were performed to investigate whether ES could induce the up-regulation of cardiac-related genes, such as MEF2D, GATA-4, Nkx2.5, Cx43, CAA (cardiac alpha-actinin), Troponin T, SERCA 2a and Cx43, which are known to be positively modulated during cardiac differentiation. The mRNA expression levels for the above-mentioned cardiac

markers was referred to those of hCPCs (Fig. 4a). Biphasic protocol had a clear effect in modulating the expression of these genes. Indeed, the three early cardiac markers MEF2D, GATA-4 and Nkx2.5 were up-regulated after three days of ES, and in the case of MEF2D and GATA-4, their levels were already increased after 1 day stimulation, although at a lower level than at 3 day treatment. The monophasic pulse was less efficient, since only a slight up-regulation could be detected, which was evident only after three days. Both stimulation protocols up-regulated the cardiac markers Cx43, Troponin T, CAA and SERCA 2a. In particular, Cx43 expression was already increased after 1 day stimulations, reaching its higher levels after 3 days biphasic stimulation, while the expression of the late markers CAA and SERCA 2a was up-regulated only after 3 days stimulation. The expression of the late marker Troponin T was highly upregulated already after 1 day biphasic stimulus, and, intriguingly, was down-regulated to basal levels after 3 days treatment. As for the other markers, monophasic stimulation induced the up-regulation of Troponin T gene expression only after 3 days treatments. Overall gene expression patterns time-course normalized *versus* the culture cells before ES (day 0) showed no significant differences of gene expression between the untreated cells (U) at day 1 and day 3 (data not shown).

It is thus clear that the biphasic stimulation is able to up-regulate all the genes investigated, while monophasic stimulus effect was always delayed and in many cases fainter.

ES induces the up-regulation of cardiac early and late differentiation proteins

The effect of ES on the expression of the early cardiac commitment transcription factor GATA-4 and of two proteins involved in the functional maturation of cardiomyocytes, e.g. Cx43 and Troponin T, was also evaluated by immunofluorescence or western blot. Unstimulated hCPCs expressed basal levels of GATA-4, that increased when cells were exposed to 1 day of mono or biphasic ES (Fig. 5a). This increased expression was even

stronger after 3 days of ES and biphasic stimulation was more efficient than monophasic stimulation (Fig. 5b).

qRT-PCR already indicated that ES up-regulated the expression of Cx43. In line with this finding this gap junction protein was detected also in immunofluorescence at higher levels upon both ES protocols carried on for 3 days. Moreover, while in unstimulated cells it was mainly detectable as a diffuse and faint signal within the cytoplasm, Cx43 was found to be partially relocated at the plasma membrane upon both treatments. As in the case of the other markers, the biphasic treatment was more effective (Fig. 5c).

Troponin T expression was evaluated after 3 day stimulation in western blot performed on total cell extracts. Indeed a faint band with the expected molecular weight of 40 kDa was detected in the case of cells stimulated with biphasic ES (Fig. 5d).

Finally, in view of the fact that other crucial effectors in the development of excitation-contraction machinery are the voltage-dependent calcium activated channels (VDCCs), the effect of ES on their expression was evaluated through the analysis of mRNAs expression by RT-PCR. We found that the cardiac specific Cav 1.2 (α_{1C}) subunit was constitutively expressed in human heart tissue (reference control), as well as in hCPCs, regardless of the applied ES. Interestingly, the mRNA levels of the Cav 3.1 (α_{1G}) subunit, that is usually highly expressed in developing heart, was induced in hCPCs exposed to both monophasic and biphasic ES, after both 1 and 3 days (Fig. 4b). The expression levels seem to be slightly higher in the case of biphasic ES rather than in the case of monophasic ES.

DISCUSSION

Nowadays adult stem/progenitor cells are envisioned as potential tools for regenerative medicine. In view of their possible application, the ability to mimic as much as possible the cells they should replace *in vivo* should be mandatory when manipulating/expanding them *in*

vitro. In particular, they could be pushed towards a more mature phenotype, which, moreover, would eliminate the risk of tumorigenesis [34,35]. Most of the methods described for the effective differentiation of cardiac stem/progenitor cells rely on biochemical signals, such as the use of specific culture media, exogenously added growth factors or co-culture with neonatal cardiomyocytes [8,11,13,27,36], while very few adopt physical cues, such as electrical stimulation (ES). On the other side, direct currents induced through voltage difference are physiologically involved in cell migration during embryonic cardiac development, and pulsatile currents are mainly related to the functional cardiac syncytium development [30,37]. For the above reasons, pulsatile monophasic electrical stimulation has been used by some researchers to improve cardiomyocytes alignment, electrical coupling, and maintenance of a differentiated phenotype [31]. So far, however, only one study analyzed the effects of different voltage waveforms (mono or biphasic) [20]. This was done on rat neonatal cardiomyocytes and on organoids composed of these cells in combination with cardiac fibroblasts and endothelial cells.

For the first time, herein we have investigated whether mono and biphasic pulsed electrical stimuli may influence the cardiac commitment of c-kit/Sca-1 expressing progenitor cells isolated from human atrium and whether they can affect other parameters of the cell behavior, such as growth and survival, cell elongation and re-orientation. The cells used were initially enriched for expressing the c-kit stemness marker, as already described [6]; however, as already reported, the expression of this marker declined rapidly, while cells maintained the expression of the other stemness associated marker Sca-1. This cell population was already shown to be multipotent [6]. Cells were found to express different growth factors and cognate receptors, namely SDF-1, VEGF, and HGF, which have already been shown to play a role during heart development, both for the contractile and the vascular compartments, and also during heart responses after injury [38-40]. This study shows that ES induced cardiac

progenitor cells towards cardiac differentiation, since these cells acquired the morphology of myogenic cells, an increased expression of early cardiac-associated proteins, as well as the relocation of Cx43 to the cell membrane and, finally, the *de novo* expression of voltage-dependent calcium channel genes and of the sarcomeric proteins Troponin T, CAA and SERCA 2a.

In order to produce precise voltage waveforms, we used a computer assisted device. We designed a versatile and highly efficient apparatus as a cell culture platform, able to apply finely tuned and controlled ES to the cells. In particular, our system allowed to analyze the different cell responses to monophasic and biphasic ES. In line with other reports [20,21,31] we found that among the different pulse amplitude analyzed, 5V was the more appropriate, being compatible with cell survival and able to promote cell responses (not shown). We observed that both types of stimuli induced hCPCs to acquire a more spindled and elongated shape. Moreover, cells displayed the trend to orient perpendicularly to the direction of the applied electric field; this possibly represents the response of the cells trying to minimize the electric field gradient across them, as originally suggested by Tandon [26] and reported also for other cell types, such as mouse fibroblasts, neonatal cardiomyocytes [19], human adipose derived stromal cells [21], and human bone marrow-derived mesenchymal cells [25,41]. Noteworthy, in accord with Chiu, who analyzed the effects of mono- and biphasic electric stimulation of rat neonatal cardiomyocytes [20], we demonstrated that biphasic stimulation induced more effective responses and these were enhanced with increasing stimulation times.

When the modulation of different cardiac markers was analyzed, 3 days biphasic ES was found in general to be more effective than monophasic ES in inducing the up-regulation of the MEF2D, GATA-4 and Nkx2.5 early genes and of the delayed Cx43, Troponin T, and cav2.1 genes. Indeed at 1 day stimulation, only biphasic ES induced their up-regulation, while monophasic ES was uneffective and only after 3 day stimulation was this kind of ES

able to up-regulate Troponin T mRNA. Three days monophasic ES up-regulated also Cx43, CAA and SERCA 2a, other genes coding proteins associated with structural/functional features of cardiomyocytes.

At the same time point (3 days) hCPCs stimulated with biphasic ES expressed higher levels of the GATA-4 protein and the *de novo* Troponin T protein, as detectable in immunofluorescence or in western blot. Strikingly, at this time point, its corresponding mRNAs was down-regulated to basal levels. The same apparently contradictory results have already been reported in the case of cardiac differentiation induced in mesenchymal stem cells upon the combined treatment with carbon nanotubes and ES [25]. The explanation was that, once maximal effect was achieved, the corresponding genes were down-regulated.

GATA-4, MEF2C and Nkx2.5 are among the earliest transcription factors expressed in the developing heart [42] and may be considered "master" genes controlling the cardiac differentiation program by activating a cascade of downstream delayed genes, such as, for example, the Troponin T, CAA, SERCA 2a, Cx43 and calcium channels genes analysed herein. Among these genes, Cx43 and Troponin T are up-regulated precociously, while SERCA 2a and CAA are up-regulated only after 3 days.

Gap junctions allow the rapid electrical signaling between myocytes necessary for synchronous cardiac contraction. Cx43 is the predominant isoform of gap junction channel in ventricular myocytes. We showed that both mono and biphasic ES induces an enhanced expression of Cx43 and its re-localization to the cell-cell contact area, thus suggesting a possible formation of gap junctions between adjacent cells. This effect was observed for both ES protocols but it was stronger after 3 days of biphasic stimulation. Our data are in line with those of Chiu and colleagues, who reported that a pulsed electrical biphasic stimulus induced a significant increase in the expression of Cx43, and that this correlated with an increase in cell excitability in neonatal rat cardiomyocytes [20]. The acquisition of electrical competence

is also related with the expression of voltage-gated ion channels. In the heart, the low voltage activated calcium channel (L-type) represents the most abundant type and it is responsible for myocytes contraction induced by calcium currents [43,44]. Also the T-type Ca^{2+} low-voltage activated channels significantly contribute to many physiologic processes, namely cardiac automaticity, development and excitation–contraction coupling in normal cardiac myocytes [44]. Among the different L and T-type Ca^{2+} channels, we have found that the expression of the subunit Cav 1.2 (α_{1C}) (L-type channel), which is already basally expressed in adult heart [45], was not affected by the ES, in line with what reported [45]. Interestingly, for the first time we report that both mono and biphasic ES were able to induce the *de novo* expression of the subunit Cav3.1 (α_{1G}) (T-type). This observation is noteworthy since T-type Ca^{2+} channels play a pivotal role in cardiac development [44]. Indeed, T-type calcium currents (ICa,T) are regulated throughout the differentiation of mouse cardiomyocytes derived from embryonic stem cells, and Cav3.1 (α_{1G}) was found to be responsible for ICa,T in these cells. ICa,T was suggested to play a key role in contractile activity in the developing heart [47] and in the pacemaker apparatus [46,48]. The fact that the transcription factor Nkx2.5 is involved in the up-regulation of T-type Ca^{2+} channels in mouse cardiomyocytes [49] suggests that this pathway could be activated also in electrically stimulated hCPCs. The same holds also for the other genes involved in cardiac differentiation. In conclusion, we show that ES can be a tool for training hCPCs to become more differentiated towards the cardiomyocytic phenotype.

While both ES protocols were able to promote a differentiation program in hCPC cells, the biphasic stimulation resulted to be more effective, when the total duration of monophasic and biphasic pulses was set to be equal. Similar observations were reported also when ES was used to stimulate rat neonatal cardiomyocytes [20] and were explained assuming that the two phases of the biphasic pulse act synergistically [50,51]. The fact that the cellular responses triggered in hCPCs were induced solely by physical stimuli, that can readily be incorporated

and finely tuned into bioreactors to substitute for exogenously added expensive bioactive molecules, is particularly important in view of a possible translation to clinical applications.

AKNOWLEDGMENTS

The authors would like to thank Silvia Simonini (Clinica S. Gaudenzio - Novara, Italy) for her technical support in editing the manuscript. This study was supported by a grant from Fondazione Cariplo (grant No. 2008-2459). Stefano Pietronave is the recipient of a fellowship from Clinica San Gaudenzio - Novara (Gruppo Policlinico di Monza).

DISCLOSURES

The authors have no conflicts of interest nor financial interests.

REFERENCES

1. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabé-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H, Jovinge S, Frisén J. (2009). Evidence for cardiomyocyte renewal in humans. Science 324:98-102.
2. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114(6):763-76.
3. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, Pocius J, Michael LH, Behringer RR, Garry DJ, Entman ML, Schneider MD. (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 100(21):12313-8.
4. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, Lin LZ, Cai CL, Lu MM, Reth M, Platoshyn O, Yuan JX, Evans S, Chien KR. (2005) isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433(7026):647-53.
5. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, Salio M, Battaglia M, Latronico MV, Coletta M, Vivarelli E, Frati L, Cossu G, Giacomello A.

(2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95(9):911-21.

6. Forte G, Pietronave S, Nardone G, Zamperone A, Magnani E, Pagliari S, Pagliari F, Giacinti C, Nicoletti C, Musaró A, Rinaldi M, Ribezzo M, Comoglio C, Traversa E, Okano T, Minieri M, Prat M, Di Nardo P. (2011). Human cardiac progenitor cell graftsas unrestricted source of supernumerary cardiac cells in healthy murine hearts. Stem Cells 29(12):2051-2061.
7. Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. (2005) J Clin Invest 115(3):572-83.
8. Smits AM, van Vliet P, Metz CH, Korfage T, Sluijter JP, Doevendans PA, Goumans MJ. (2009). Human cardiomyocyte progenitor cells differentiate into functional mature cardiomyocytes: an in vitro model for studying human cardiac physiology and pathophysiology. Nat Protoc 4(2):232-43.
9. Duan Y, Liu Z, O'Neill J, Wan LQ, Freytes DO, Vunjak-Novakovic G. (2011). Hybrid gel composed of native heart matrix and collagen induces cardiac differentiation of human embryonic stem cells without supplemental growth factors. J Cardiovasc Transl Res 4(5):605-615.
10. Goumans MJ, de Boer TP, Smits AM, van Laake LW, van Vliet P, Metz CH, Korfage TH, Kats KP, Hochstenbach R, Pasterkamp G, Verhaar MC, van der Heyden MA, de Kleijn D, Mummery CL, van Veen TA, Sluijter JP, Doevendans PA. (2007). TGF-beta1 induces efficient differentiation of human cardiomyocyte progenitor cells into functional cardiomyocytes in vitro. Stem Cell Res 1(2): 138-149.
11. Lee SY, Ham O, Cha MJ, Song BW, Choi E, Kim IK, Chang W, Lim S, Lee CY, Park JH, Lee J, Bae Y, Seo HH, Choi E, Jang Y, Hwang KC. (2013). The promotion of cardiogenic differentiation of hMSCs by targeting epidermal growth factor receptor using microRNA-133a. Biomaterials 34(1):92-99.
12. Wen J, Zhang JQ, Huang W, Wang Y. (2012). SDF-1α and CXCR4 as therapeutic targets in cardiovascular disease. Am J Cardiovasc Dis 2(1):20-28.
13. Sadek H, Hannack B, Choe E, Wang J, Latif S, Garry MG, Garry DJ, Longgood J, Frantz DE, Olson EN, Hsieh J, Schneider JW. (2008). Cardiogenic small molecules that enhance myocardial repair by stem cells. Proc Natl Acad Sci U S A 22;105(16):6063-6068.
14. Rabaey K, Rozendal RA. (2010). Microbial electrosynthesis - revisiting the electrical route for microbial production. Nat Rev Microbiol 8(10):706–716.

15. Zhao M, Bai H, Wang E, Forrester JV, McCaig CD. (2004). Electrical stimulation directly induces pre-angiogenic responses in vascular endothelial cells by signalling through VEGF receptors. J Cell Sci 117:397-405.
16. Kern H, Salmons S, Mayr W, Rossini K, Carraro U. (2005). Recovery of long-term denervated human muscles induced by electrical stimulation. Muscle Nerve 31(1):98-101.
17. Mödlin M, Forstner C, Hofer C, Mayr W, Richter W, Carraro U, Protasi F, Kern H. (2005). Electrical stimulation of denervated muscles: first results of a clinical study. Artif Organs 29(3):203-206.
18. An RH, Davies MP, Doevendans PA, Kubalak SW, Bangalore R, Chien KR, Kass RS. (1996). Developmental changes in beta-adrenergic modulation of L-type Ca2+ channels in embryonic mouse heart. Circ Res 78(3):371-378.
19. Au HT, Cheng I, Chowdhury MF, Radisic M. (2007). Interactive effects of surface topography and pulsatile electrical field stimulation on orientation and elongation of fibroblasts and cardiomyocytes. Biomaterials 28:4277-4293.
20. Chiu LL, Iyer RK, King JP, Radisic M. (2011). Biphasic electrical field stimulation aids in tissue engineering of multicell-type cardiac organoids. Tissue Eng Part A 17(11-12):1465-1477.
21. Tandon N, Marsano A, Maidhof R, Wan L, Park H, Vunjak-Novakovic G. (2011). Optimization of electrical stimulation parameters for cardiac tissue engineering. J Tissue Eng Regen Med 5(6):115-125.
22. Serena E, Figallo E, Tandon N, Cannizzaro C, Gerecht S, Elvassore N, Vunjak-Novakovic G. (2009). Electrical stimulation of human embryonic stem cells: cardiac differentiation and the generation of reactive oxygen species. Exp Cell Res 315(20):3611-3619.
23. Castells-Sala C, Sanchez B, Recha-Sancho L, Puig V, Bragos R, Semino CE. (2012). Influence of electrical stimulation on 3D-cultures of Adipose Tissue derived progenitor cells (ATDPCs) behavior. Conf Proc IEEE Eng Med Biol Soc 2012:5658-61.
24. Llucià-Valldeperas A, Sanchez B, Soler-Botija C, Gálvez-Montón C, Prat-Vidal C, Roura S, Rosell-Ferrer J, Bragos R, Bayes-Genis A. (2013). Electrical stimulation of cardiac adipose tissue-derived progenitor cells modulates cell phenotype and genetic machinery. J Tissue Eng Regen Med doi: 10.1002/term.1710 [Epub ahead of print].

25. Mooney E, Dockery P, Greiser U, Murphy M, Barron V. (2008). Carbon nanotubes and mesenchymal stem cells: biocompatibility, proliferation and differentiation. Nano Lett 8(8):2137-2143.
26. Tandon N, Goh B, Marsano A, Chao PH, Montouri-Sorrentino C, Gimble J, Vunjak-Novakovic G. (2009). Alignment and elongation of human adipose-derived stem cellsin response to direct-current electrical stimulation. Conf Proc IEEE Eng Med Biol Soc 2009:6517-6521.
27. Wei HJ, Chen CH, Lee WY, Chiu I, Hwang SM, Lin WW, Huang CC, Yeh YC, Chang Y, Sung HW. (2008). Bioengineered cardiac patch constructed from multilayered mesenchymal stem cells for myocardial repair. Biomaterials 29(26):3547-3556.
28. Haferkamp A, Mundhenk J, Bastian PJ, Reitz A, Dörsam J, Pannek J, Schumacher S, Schurch B, Büttner R, Müller SC. (2004). Increased expression of connexin 43 in the overactive neurogenic detrusor. Eur Urol 46(6):799-805.
29. Pavesi A, M Soncini, A Redaelli, FM Montevecchi, GB Fiore. (2010). Computational characterization of electrical stimulation in a bioreactor for cardiac tissue engineering. In: *Proceedings of the 9th International Symposium on Computer Methods in Biomechanics and Biomedical Engineering*, J Middleman, SL Evans, C Holt, C Jacobs, C Atienza and B Walker, eds. ARUP, Solihull (UK), pp 963-968.
30. Nuccitelli R. (1992). Endogenous ionic currents and DC electric fields in multicellular animal tissues. Bioelectromagnetics Suppl 1:147-157.
31. Radisic M, Park H, Shing H, Consi T, Schoen FJ, Langer R, Freed LE, Vunjak-Novakovic G. (2004). Functional assembly of engineered myocardium by electrical stimulation of cardiac myocytes cultured on scaffolds. Proc Natl Acad Sci U S A 101(52):18129-18134.
32. Barr RC and Plonsey R. (2004). Field stimulation of 2-D sheets of excitable tissue. IEEE Trans Biomed Eng. 51(3):539-40.
33. Barash Y, Dvir T, Tandeitnik P, Ruvinov E, Guterman H, Cohen S. (2011). Electric field stimulation integrated into perfusion bioreactor for cardiac tissue engineering. Tissue Eng Part C Methods. 16(6):1417-26.
34. Li H, Fan X, Kovi RC, Jo Y, Moquin B, Konz R, Stoicov C, Kurt-Jones E, Grossman SR, Lyle S, Rogers AB, Montrose M, Houghton J. (2007) Spontaneous expression of embryonic factors and p53 point mutations in aged mesenchymal stem cells: a model of age-related tumorigenesis in mice. Cancer Res 67(22):10889-98.

35. Knoepfler PS. (2009) Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine. Stem Cells. 27(5):1050-6.
36. Pagliari S, Vilela-Silva AC, Forte G, Pagliari F, Mandoli C, Vozzi G, Pietronave S, Prat M, Licoccia S, Ahluwalia A, Traversa E, Minieri M, Di Nardo P. (2011). Cooperation of biological and mechanical signals in cardiac progenitor cell differentiation. Adv Mater 23(4):514-518.
37. Berger HJ, Prasad SK, Davidoff AJ, Pimental D, Ellingsen O, Marsh JD, Smith TW, Kelly RA. (1994). Continual electric field stimulation preserves contractile function of adult ventricular myocytes in primary culture. Am J Physiol 266:H341-H349.
38. Rappolee DA, Iyer A, Patel Y. Hepatocyte growth factor and its receptor are expressed in cardiac myocytes during early cardiogenesis. (1996) Circ Res 78(6):1028-36.
39. Nakamura T, Mizuno S, Matsumoto K, Sawa Y, Matsuda H, Nakamura T. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. (2000) J Clin Invest 106(12):1511-9.
40. Liu SQ, Tefft BJ, Zhang D, Roberts D, Schuster DJ, Wu A. Cardioprotective mechanisms activated in response to myocardial ischemia. (2011) Mol Cell Biomech 8(4):319-38.
41. Mooney E, Mackle JN, Blond DJ, O'Cearbhaill E, Shaw G, Blau WJ, Barry FP, Barron V, Murphy JM. (2012). The electrical stimulation of carbon nanotubes to provide a cardiomimetic cue to MSCs. Biomaterials 33(26):6132-6139.
42. Vincentz JW, Barnes RM, Firulli BA, Conway SJ, Firulli AB. (2008). Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. Dev Dyn 237(12):3809-3819.
43. de Boer TP, van Veen TA, Jonsson MK, Kok BG, Metz CH, Sluijter JP, Doevendans PA, de Bakker JM, Goumans MJ, van der Heyden MA. (2010). Human cardiomyocyte progenitor cell-derived cardiomyocytes display a maturated electrical phenotype. J Mol Cell Cardiol 48(1):254-260.
44. Ono K, Iijima T. (2010). Cardiac T-type Ca(2+) channels in the heart. J Mol Cell Cardiol 48(1):65-70.
45. Welling A. (2009). Voltage-dependent calcium channels. Biotrend Reviews 4:1-11.

46. Zhang YM, Shang L, Hartzell C, Narlow M, Cribbs L, Dudley SC Jr. (2003). Characterization and regulation of T-type Ca2+ channels in embryonic stem cell-derived cardiomyocytes. Am J Physiol Heart Circ Physiol 285(6):H2770-H2779.
47. Irisawa H, Brown HF, Giles W. (1993). Cardiac pacemaking in the sinoatrial node. Physiol Rev 73(1):197-227.
48. Hüser J, Blatter LA, Lipsius SL. (2000). Intracellular Ca2+ release contributes to automaticity in cat atrial pacemaker cells. J Physiol 524:415-422.
49. Wang Y, Morishima M, Zheng M, Uchino T, Mannen K, Takahashi A, Nakaya Y, Komuro I, Ono K. (2007). Transcription factors Csx/Nkx2.5 and GATA4 distinctly regulate expression of Ca2+ channels in neonatal rat heart. J Mol Cell Cardiol 42(6):1045-1053.
50. Jones JL, Jones RE, Balasky G. (1987). Improved cardiac cell excitation with symmetrical biphasic defibrillator waveforms. Am J Physiol 253:H1418-1424.
51. Tung L, Borderies JR. (1992). Analysis of electric field stimulation of single cardiac muscle cells. Biophys J 63(2):371-386.

TABLES

Table I. Primers and annealing temperatures used for RT-PCR and q-RT-PCR.

Primers used for RT-PCR			
Gene	*Forward primers*	*Reverse Primers*	*tm**
β-actin	5'-acttcgagcaagagatggcc	5'-cacatctgctggaaggtgg	56 °C
GATA-4	5'-agacatcgcactgactgagaac	5'-gacgggtcactatctgtgcaac	55°C
MEF2C	5'-gccctgagtctgaggacaag	5'-agtgagctgacagggttgct	55°C
TBX5	5'-aaatgaaacccagcataggagctggc	5'-acactcagcctcacatcttaccct	55°C
SDF-1-R	5'-ttctaccccaatgacttgtg	5'-atgtag taaggcagccaaca	55°C
SDF-1	5'-ctgggcaaagcctagtga	5'-gtcctgagagtccttttgcg	60°C
VEGF-R_2	5'-catgtggtctctctggttgtg	5'-tccctggaagtcctccacact	60°C
VEGF	5'-ggcagcttgagttaaacgaac	5'-atggatccgtatcagtctttcctgg	55°C
HGF-R	5'-tggtgtcccggatatcagc	5'-gagcaaagaatatcgatggcc	55°C
HGF	5'-aggagaaggctacaggggcac	5'-tttttgccattcccacgataa	60°C
Cav 1.2 (α_{1C})	5'-aatgcctacctccgcaacggctg	5'-tgatgccgtgcttgggaccatcc	60 °C
Cav 3.1(α_{1G})	5'-acc cctggtttgagcgcatcagc	5'-agcaggacgttgcccagcatgg	60 °C
Primers used for quantitative real time qPCR			
MEF2D	5'-cccctgctggaggacaagta	5'-tgcatggagctctgattgga	
GATA-4	5'-gacaatctggttaggggaagc	5'-accagcagcagcgaggagat	
Nkx-2.5	5'-cgccgctccagttcatag	5'-ggtggagctggagaagacaga	
Cx43	5'-ggaatgcaagagaggttgaaag	5'-ggcatttggagaaactggtaga	
TropT	5'-gtgggaagaggcagactgag	5'-atagatgctctgccacagc	
CAA	5'-gctcctgtggtgtcagagaa	5'-cagcttcccgtagtcaatca	
SERCA2a	5'-catggatgagacgctcaagt	5'-acatcagtcatgcacagggt	
18SrRNA	5'-gtggagcgatttgtctggtt	5'-acgctgagccagtcagtgta	

FIGURES

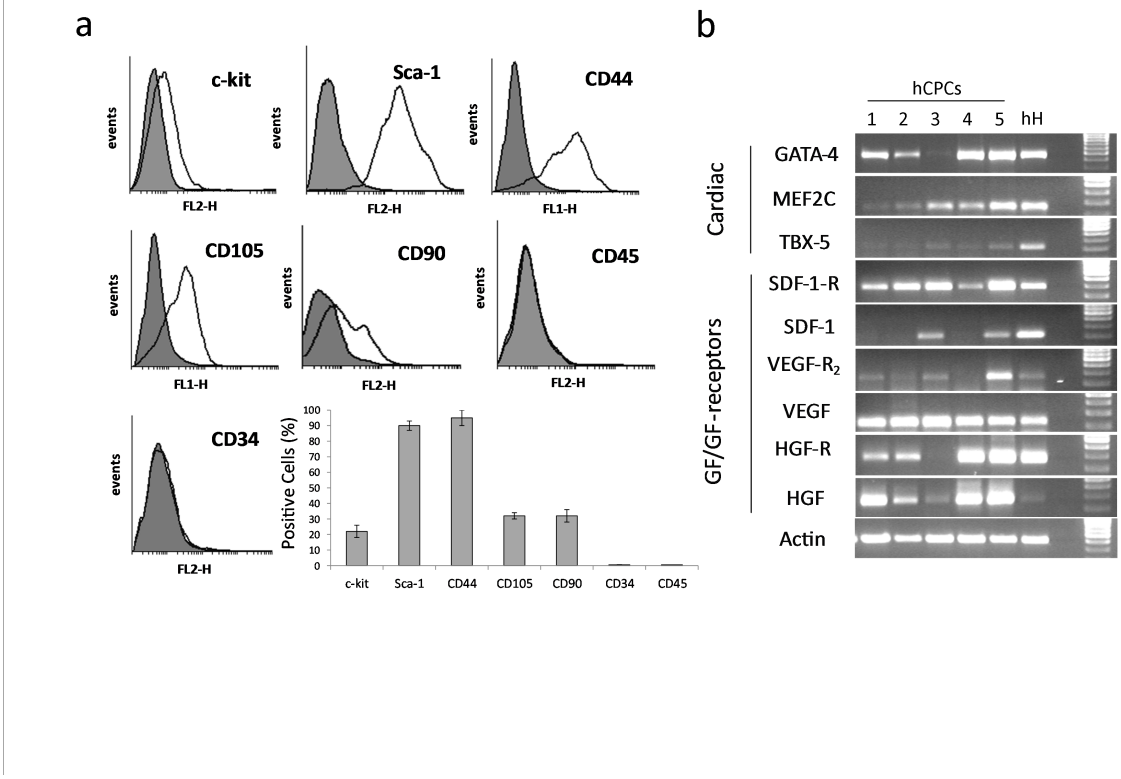

Figure 1. Characterization of hCPCs. Phenotype of hCPCs at passage 3 after immunomagnetic selection for c-kit. **(a)** At this passage cells stably expressed the mesenchymal stem cells markers Sca-1 and CD44, while the expression of c-kit was already declined (see the Results section for more details). These cells expressed also CD90 and CD105. Representative cytograms and a graph relative to the analysis of the 5 hCPC preparations, each one analyzed in two independent experiments ± SD are reported. **(b)** In RT-PCR analysis the early cardiac markers GATA-4, MEF2C and TBX-5 were detectable in samples from all the patients, although at different levels. A similar variability was observed also when growth factors expression was analyzed. In few cases the cognate receptors could not be detected. One representative experiment series out of the three performed with similar results are reported. hH, human heart tissue used as control.

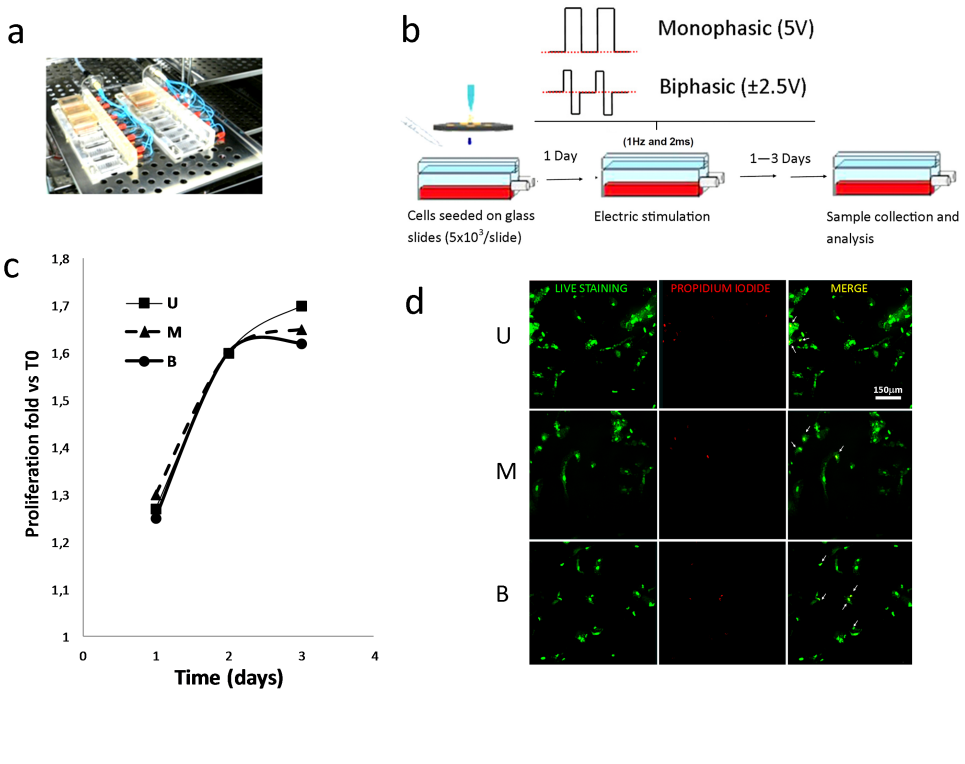

Figure 2. Electrical stimulation cell culture system and its validation in proliferation and viability tests. (a) The multichamber culture system with the two chassis housing the 12 independent silicon culture chambers. **(b)** Scheme of the experiments of electrical stimulation (ES) and time tracings of the voltage applied at the stimulating electrodes, as measured during the tests (top monophasic; bottom: biphasic). The noise superimposed to the pure square wave pulses was <1% of the signal amplitudes. **(c)** Effects of ES on hCPC proliferation, as evaluated in an MTT assay. Results are expressed as the percentage of proliferation of ES cells relative to cell number before ES (Day 0). Values are the mean of triplicates ± SD and representative of three independent experiments. Student's *t*-test was performed to determine significant variation induced by monophasic (M) and biphasic (B) protocols *versus* unstimulated (U) cells at each time point. No significant differences were found ($p \geq 0.05$). **(d)** Effects of ES on hCPC cell viability, as qualitatively evaluated by LIVE/DEAD staining after 3 days of ES. Images are representative of three separate experiments. Dead cells are red stained, while viable cells are green stained. Scale bar is equivalent to 150 µm.

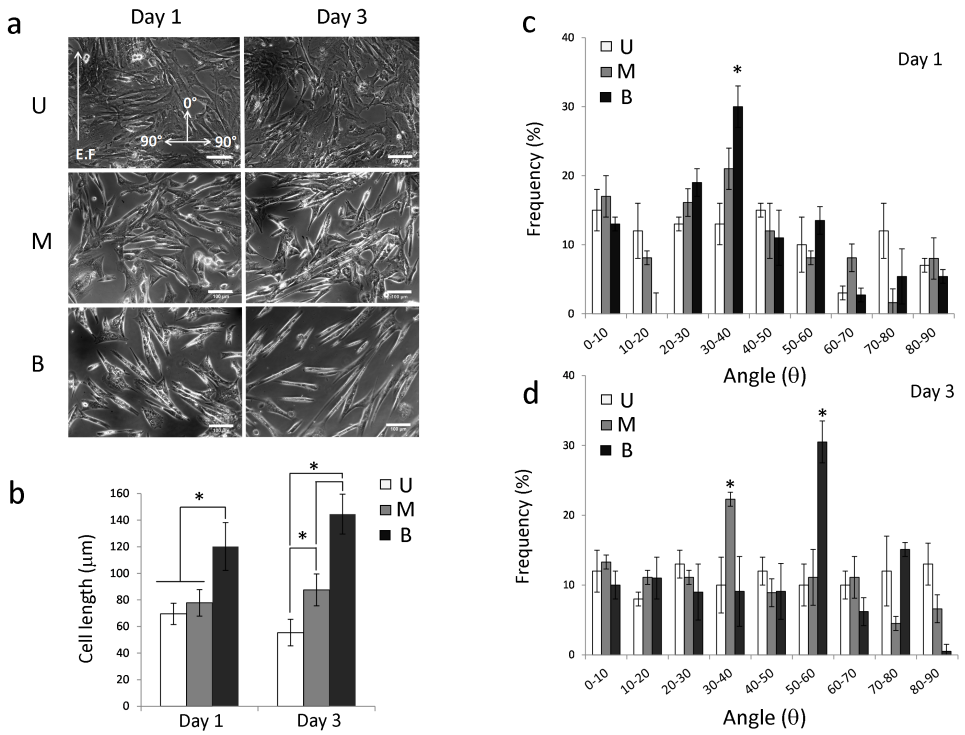

Figure 3. Effects of ES on hCPC elongation and alignment. (a) hCPCs morphology, as shown in bright field images. Cells plated at day 0 adhered to the substrate and within 4 h they displayed the same morphology (not shown) recorded for unstimulated cells at day 1. **(b)** The major axis of the cell was used to measure cell length with the ImageJ software. Student's *t* tests were performed to determine significant variation of the angle induced by each ES protocol for each time point vs unstimulated controls (* $p \leq 0.05$). Groups were compared with one-way ANOVA and Bonferroni post-test (M *versus* B or U, and B *versus* U). The frequency of orientation at a determined angle range was determined in each field by ImageJ analysis for hCPC exposed for 1 day **(c)** and 3 days **(d)** to mono and biphasic ES. The direction parallel to the electric field (EF) was assumed to represent an angle of 0°. Values represent the mean counts ± SD obtained from 10 microscope fields (50-60 cells each) in three independent experiments. U, unstimulated cells; M, cells stimulated with monophasic ES; B, cells stimulated with biphasic ES. * $p \leq 0.05$.

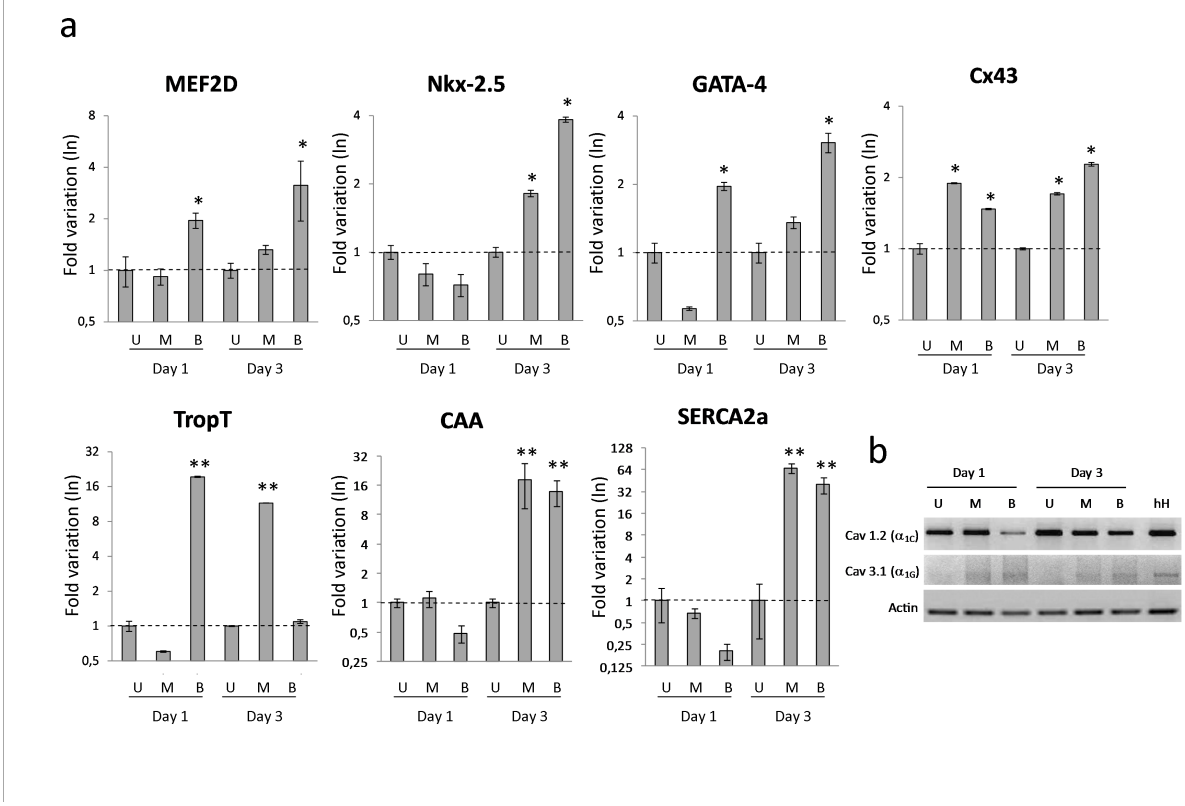

Figure 4. Effects of ES on gene expression. (a) qRT-PCRs for markers associated to cardiac differentiation, after 1 and 3 days of mono (M) and biphasic (B) ES. Data are normalized relative to the unstimulated (U) cells cultured in the same conditions and time points. Values represent the mean ± SD gene expression measured from six independent experiments of ES (one-way ANOVA and Bonferroni post-tests vs control U; * $p \leq 0.05$, ** $p \leq 0.01$). **(b)** Expression of mRNA for Ca^{2+} low voltage activated channels in unstimulated (U) and in mono (M) and biphasic (B) stimulated cells at Day 1 and Day3. Human atrial biopsy (hH) was used as control. Image shows a typical experiment out of the three performed relative to a donor patient. In general early genes are up-regulated more efficiently with bi-phasic stimulation, while structural/functional genes are up-regulated more efficiently after three days, both upon mono and biphasic electric stimulation.

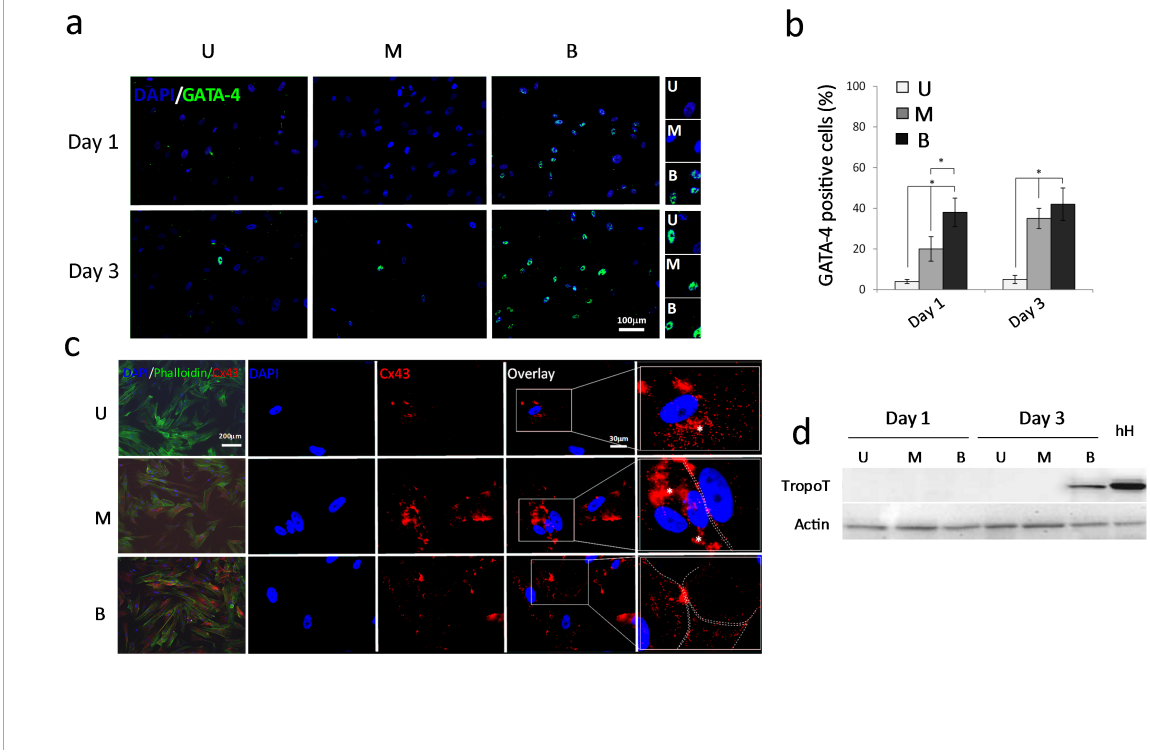

Figure 5. Expression of cardiac markers (proteins) in electrically stimulated hCPCs. (a) GATA-4 was evaluated by immunofluorescence in unstimulated hCPCs (U) and at Day1 and Day 3 after mono (M) or biphasic (B) ES. The labeled nuclei are shown in detail on the right side of the picture. **(b)** The percentage of GATA-4 positive cells was calculated over the total number of nuclei. The quantification was performed by manual counting of 8 images (average 50-200 nuclei each) in 3 independent experiments. Results are presented as the mean ± SD. Student's *t*-test was performed to determine significant variation of ES protocols. Groups were compared with one-way ANOVA and Bonferroni post-test (M *versus* B or U, and B *versus* U; * $p \leq 0.05$). **(c)** Cx43 detection in immunofluorescence. Cx43 is present in scanty amounts within the cytoplasm (white * symbol) in unstimulated (U) control cells; upon ES (M, monophasic; B, biphasic) the protein is expressed at higher levels and it is also partially relocated at membrane boundaries between adjacent cells (white dotted line). **(d)** Troponin T expression was determined by western blot in one donor preparation in unstimulated (U) cells or in cells undergoing mono (M) or biphasic (B) ES for 1 and 3 days, Troponin T expression was only detectable upon 3 days of biphasic ES. Human atrial biopsy (hH) was used as reference control. The level of actin expression in the same extracts was used as internal control to normalize for the amount of total proteins. Only one tenth of the amount of cell extract used for Troponin T detection was used. Image shows one experiment out of the three performed relative to a donor patient.