Cardiovascular Research (2009) **82**, 411–420
doi:10.1093/cvr/cvp067

Differentiation of human adult cardiac stem cells exposed to extremely low-frequency electromagnetic fields

Roberto Gaetani[1†], Mario Ledda[2†], Lucio Barile[1], Isotta Chimenti[1], Flavia De Carlo[2], Elvira Forte[1], Vittoria Ionta[1], Livio Giuliani[3], Enrico D'Emilia[3], Giacomo Frati[1,4,5], Fabio Miraldi[6], Deleana Pozzi[1], Elisa Messina[1], Settimio Grimaldi[2], Alessandro Giacomello[1*], and Antonella Lisi[2]

[1]*Department of Experimental Medicine, Pasteur Institute, Cenci Bolognetti Foundation, 'Sapienza' University of Rome, viale Regina Elena 324, 00161 Rome, Italy;* [2]*Institute of Neurobiology and Molecular Medicine, CNR, via Fosso del Cavaliere 100 00133, Rome, Italy;* [3]*ISPESL-DIPIA, Rome, Italy;* [4]*Department of Cardiac Surgery, 'Sapienza' University of Rome, Polo Pontino-ICOT, Latina 04100, Italy;* [5]*I.R.C.C.S. Neuromed, 86077 Pozzilli, Italy; and* [6]*Department of Heart and Great Vessels 'Attilio Reale', 'Sapienza' University of Rome, viale Regina Elena 324, 00161 Rome, Italy*

Received 15 July 2008; revised 11 February 2009; accepted 17 February 2009; online publish-ahead-of-print 19 February 2009

Time for primary review: 23 days

KEYWORDS
Clinical translation protocols;
ELF-EMF;
Ca^{2+} ion cyclotron resonance;
Cardiac regenerative medicine

Aims Modulation of cardiac stem cell (CSC) differentiation with minimal manipulation is one of the main goals of clinical applicability of cell therapy for heart failure. CSCs, obtained from human myocardial bioptic specimens and grown as cardiospheres (CSps) and cardiosphere-derived cells (CDCs), can engraft and partially regenerate the infarcted myocardium, as previously described. In this paper we assessed the hypothesis that exposure of CSps and CDCs to extremely low-frequency electromagnetic fields (ELF-EMFs), tuned at Ca^{2+} ion cyclotron energy resonance (Ca^{2+}-ICR), may drive their differentiation towards a cardiac-specific phenotype.

Methods and results A significant increase in the expression of cardiac markers was observed after 5 days of exposure to Ca^{2+}-ICR in both human CSps and CDCs, as evidenced at transcriptional, translational, and phenotypical levels. Ca^{2+} mobilization among intracellular storages was observed and confirmed by compartmentalized analysis of Ca^{2+} fluorescent probes.

Conclusions These results suggest that ELF-EMFs tuned at Ca^{2+}-ICR could be used to drive cardiac-specific differentiation in adult cardiac progenitor cells without any pharmacological or genetic manipulation of the cells that will be used for therapeutic purposes.

1. Introduction

Cardiovascular diseases represent the leading cause for morbidity and mortality in the western world. Cell transplantation into the damaged myocardium (cell therapy) for heart regeneration has received extensive attention. The accumulated evidence from both pre-clinical and clinical studies suggests that cell therapy has the potential to restore heart function. Up to date, major limitations to the clinical applicability of adult stem/progenitor cells (aS/PCs) in heart failure settings are the low-specific cardiomyogenic potential of bone marrow-derived aS/PCs, and the arrhythmogenicity elicited by muscle-derived PCs.[1] The optimal cell type to be transplanted should be characterized by: (a) spontaneous disposition to integrate with the target tissue without induction of immune reaction; (b) cardiac commitment; (c) capacity to develop gap junctions with host cells; (d) preferably by some degree of resistance to ischaemia, in order to avoid massive apoptosis, which is currently observed during cell transfer.[2] In this context, cardiac stem cells (CSCs) seem to fulfil many of these characteristics. In fact, increasing attention has been focused on the finding that the heart contains a reservoir of S/PCs.[3–7] However, despite their cardiac origin, it is difficult to obtain terminally differentiated cells.[8] Therefore, many attempts have been made to increase their differentiative potential, mostly by pharmacological pretreatment (5-azacytidin,[6] trichostatin A, oxitocin[9]).

The possibility of using cardiac bioptic specimens as a source for *ex vivo* isolation and expansion of these cells, introduced for the first time by our group,[5,10] led to a new breakthrough in the field of cardiac cell therapy. In fact, CSCs obtained with this method promote cardiomyocyte regeneration and improvement in systolic function after their injection into the border zone of experimental infarcts

* Corresponding author. Tel: +39 064 461 481; fax: +39 064 461 481.
E-mail address: alessandro.giacomello@uniroma1.it
† These authors contributed equally to the study.

in SCID mice.[10] Expansion *ex vivo* over a period of weeks is necessary to obtain enough cells for experimental and clinical purposes. Their potential as a convenient source for autologous stem cell therapy has been considered as the basis for forthcoming clinical trials that will explore the use of CSCs for myocardial regeneration.

By using the method described previously,[5] CSCs are spontaneously shed from human surgical or bioptical specimens and murine heart samples in primary culture. The cells are self-renewing, clonogenic, and multipotent, giving rise spontaneously to cardiomyocytes, smooth muscle cells, and endothelial cells. In semi-suspension culture, they form multicellular clusters dubbed cardiospheres (CSps). The improved method, which allows to expand them manifold,[10] gives rise to a population of adherent growing cells (CSp-derived cells, CDCs), which is easier to use in experimental and clinical settings.

In order to increase the reliability and the clinical feasibility of CSC therapy, with particular attention to their cardiogenic potential, new methods are needed to allow modulation of the differentiation process with minimal cell manipulation and without any pharmacological treatment or gene modification.

Electromagnetic fields (EMFs) have been shown to interfere with cellular growth, proliferation[11–14] and differentiation, as recently demonstrated in murine and rat neuronal cells,[15,16] and to interfere in endorphinergic and cholinergic systems.[17,18] EMFs have also been reported to regulate Ca^{2+} homeostasis and fracture healing.[19] Studies by Albertini *et al.*[20] have suggested that EMFs can prevent or repair damages suffered following heart ischaemia–reperfusion injury. The authors found that continuous exposure to a 3 mT, 75 Hz-pulsed extremely low-frequency (ELF) EMFs decreased the amount of permanently injured myocardium after ligation of the left anterior descending coronary artery in rats. Moreover, results by Ventura *et al.*[21] and Lisi *et al.*[22] and our preliminary data have shown lineage-specific commitment in different cell types after exposure to ELF-EMFs.[21,22] However EMF-based technologies have not progressed to clinical translation because of skepticism due to differences in experimental exposure protocols and to static MF variation.

In the present study we introduced for the first time an exposure system inside an a-magnetic room in order to obtain totally controlled and reproducible conditions. We assessed the hypothesis that the exposure of human CSps and CDCs to define ELF-EMFs could interfere with their proliferation, and modulate the cardiac differentiation process without any other intervention. A combination of static and alternate EMFs, tuned to Ca^{2+} ion cyclotron energy resonance (Ca^{2+}-ICR), has been used to trigger human CSC-specific differentiation, verified by analysis of their immunophenotype, transcriptional activity, and by functional characterization.

2. Methods

2.1 Cell culture

Cardiospheres and cardiosphere-derived cells were obtained from surgical auricola samples, and cultured as described previously.[5,10] *Table 1* summarizes the features of the enrolled patients. Ethical approval was obtained from the Ethics Committee of the 'Sapienza' University of Rome. This investigation conforms with the principles outlined in the Declaration of Helsinki.

Table 1 Features of the enrolled patients

	n	Percentage	Average age	Max	Min
Males	6	43	62	69	46
Females	8	57	72	77	67

2.2 Ca^{2+}-ion cyclotron energy resonance exposure parameters

The exposure parameters were calculated based on the following equation.

$$f = \frac{q \cdot \mathbf{B}_{DC}}{m \cdot 2\pi} \quad (1)$$

where q and m are charge and mass of an ion, respectively, $\mathbf{B}_{DC}$ is the flux density of the applied static MF, and f is the frequency of the superimposed EMF.

Since at resonance conditions the maximum possible extent of energy is believed to be transferred to the system, the intensity of the EMF applied was in the microtesla range. Under these conditions, the amount of heating due to Joule effect is negligible, and all the effects reported after cell exposure must be related to cyclotron exposure.

2.3 Ion cyclotron energy resonance generation system for chronic exposure

Cardiospheres and cardiosphere-derived cells were exposed for 3 or 5 days to a static MF (10 μT) and a sinusoidal ELF-EMF (2.5 μT RMS of intensity) at a 7 Hz frequency, close to the ICR frequency corresponding to the charge/mass ratio of the Ca^{2+} ion. All experiments were done under blind conditions.

The apparatus (*Figure 1A*) for EMF production (solenoid) is installed in an a-magnetic room. This equipment includes a cellular incubator made of an a-magnetic material, where temperature ($37 \pm 0.1^{\circ}C$), atmosphere composition (5% CO_2), and humidity regulation were provided and continuously controlled and recorded by a lab view program (control system). The main body of the solenoid is a cylinder in 5 mm-thick PVC, and it has a diameter of 33 cm and a height of 3 m. It is made of 3300 turns of 1 mm-thick copper wire. It is driven by three amplifiers and a signal generator, which create static and alternate currents for EMF production. This apparatus is able to produce a frequency from 0.01 Hz to 1 KHz, an EMF between 10 nT and 1 mT, and induction at 33 mV RMS. As a non-Ca^{2+}-ICR frequency control, CSps and CDCs were exposed for up to 5 days to a static MF (10 μT) and a sinusoidal EMF (2.5 μT RMS of intensity) at 18 Hz frequency, far from the ICR of the most important ions involved in cardiac metabolism, such as K^+, Na^+, Mg^{2+}, Cl^-. The technical details of the instrument are described in patent No. MI 2005A000693.

2.4 Acute ion cyclotron energy resonance exposure and direct fluorescence microscopy settings for Ca^{2+} imaging

The integrated EMF expositor system for acute exposure to Ca^{2+}-ICR is shown in *Figure 1B*. The EMF device consisted of a Helmholtz coil (b), shielded by μMetal (a), lowered into the incubation chamber surrounding the microscope objective. Since the equipment was not attached to the chamber, the chamber could be moved in the *x*- and *y*-axis to select the field of cells to be examined. The EMF, measured with a calibrated Hall probe and oscilloscope, showed a decrease of approximately 10% when the measuring probe was

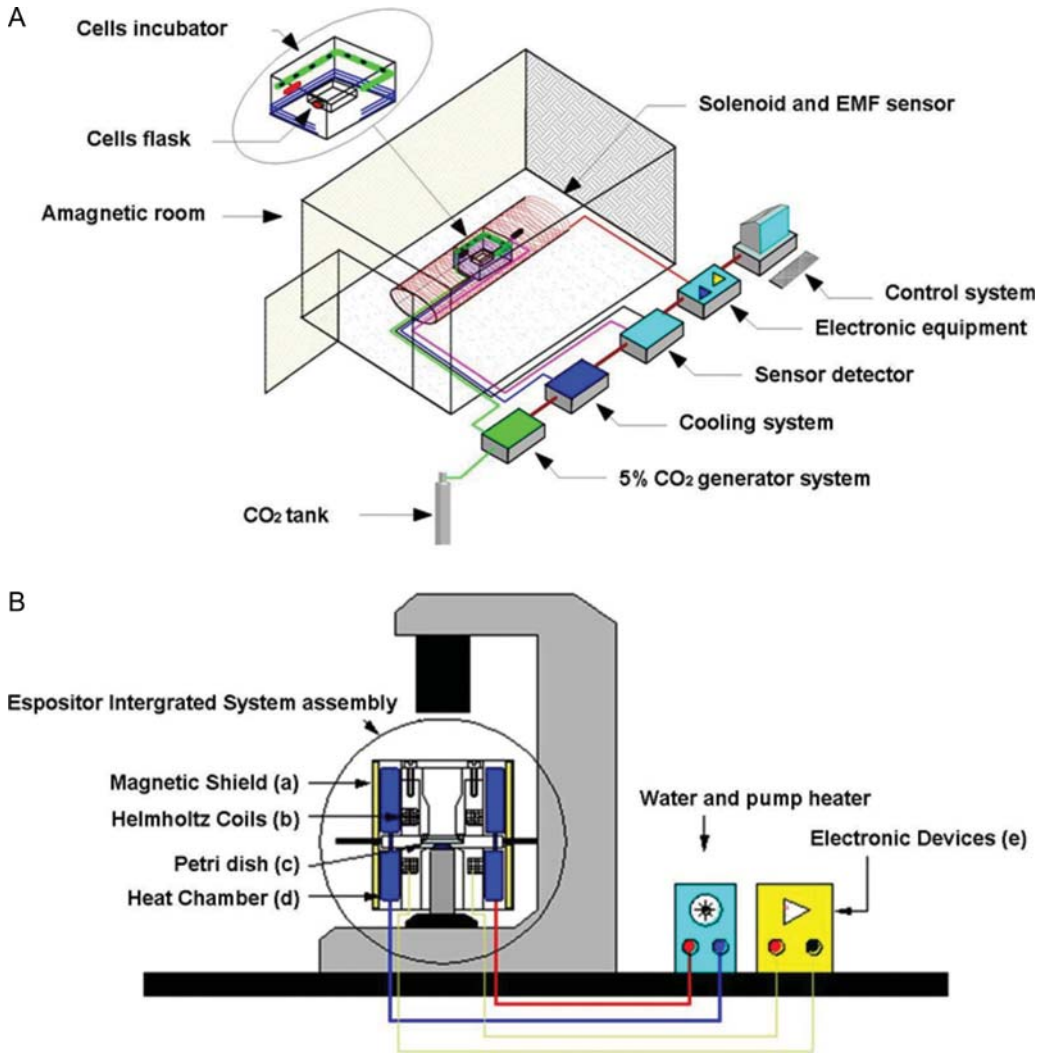

Figure 1 Extremely low-frequency electromagnetic field exposure systems. (*A*) Schematic representation of the apparatus for chronic extremely low-frequency electromagnetic field exposure. (*B*) Experimental setting for acute exposure to extremely low-frequency electromagnetic field and Ca^{2+} imaging.

displaced laterally within a 3 mm radius along the horizontal plane. The measured EMF change was approximately −15% for 1 mm steps along the microscope *z*-axis. Adherent cells on a glass coverslip were placed 2 mm below the centre of the coil. The presence of the microscope objective did not measurably alter the field. Cells seeded on a Petri dish (c) were maintained at 37°C by a heat chamber (d), and temperature was balanced by a water pump control system. Electronic devices (e) for appropriate field production were based on combined static and alternate MFs.

2.5 Cellular metabolic activity and proliferation

2.5.1 WST assay

The quantification of CSp and CDC metabolic activity, as an index of cellular proliferation, was performed by a colorimetric assay based on oxidation of tetrazolium salts (Cell Proliferation Reagent water-soluble tetrazolium salt (WST)-1; Roche Diagnostics, Basel, Switzerland). Exponentially growing CDCs and CSp-forming cells from seven different patients were seeded in 96-well plates at a density of 2000 or 3000 cells/well, respectively. Cells were cultured for up to 6 days in a normal humidified incubator (control) or in the presence of the Ca^{2+}–ICR EMFs (exposed), and they were analysed by means of the formazan dye every 24 h. WST reagent diluted to 1:10 was added in the wells at 4 h, 1, 2, 3 and 6 days after plating, and then incubated for 2 h in humidified atmosphere (37°C, 5% CO_2). Quantification of the formazan dye produced was performed by absorbance measurement at 450 nm with a scanning multiwell spectrophotometer (Biotrack II; Amersham Biosciences, Little Chalfont, UK).

2.5.2 Bromodeoxyuridine incorporation assay

Exponentially growing CDC- and CSp-forming cells from seven different patients were seeded 2000 or 3000 cells/well, respectively, in 96-well plates. Exposed and unexposed control cells were cultured for 4 days and tested every 24 h. Bromodeoxyuridine (BrdU) 10 μM was added to each well for the final 2 (CDCs) or 18 (CSps) hours in culture. Cells were then fixed and incubated for 30 min at 37°C with the anti-BrdU antibody (Cell Proliferation Kit; Roche Diagnostics). After incubation with 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) for 30 min, the absorbance of the samples was measured in an ELISA reader at 405 nm.

2.6 Immunofluorescence

CSps and CDCs were fixed in paraformaldehyde 4% (4°C, 10 min), washed twice in Ca^{2+}/Mg^{2+}-free PBS and permeabilized at room temperature (RT) for 15 min (0.1% Triton X-100, 1% BSA; Sigma-Aldrich, St Louis, MO, USA). Non-specific antibody binding sites were blocked with 10% goat serum (Sigma-Aldrich) prior to incubation with primary antibodies: cardiac troponin I (TnI; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), NK2 transcription factor related, locus 5 (Nkx2.5) and smooth muscle actin (SMA; Abcam, Cambridge, UK), vascular endothelial growth factor (VEGF), VEGF receptor [kinase insert

domain receptor (KDR)] and connexin-43 (Cx43; Chemicon, Millipore Corporation, Billerica, MA, USA), myosin heavy chain (MHC; clone MF-20, kindly provided by Maurizia Caruso). After washing in PBS containing 0.1% Triton X-100 and 1% BSA, slides were incubated with the nuclear dye TOPRO3 Iodide (Invitrogen, Carlsbad, CA, USA), anti-rabbit and/or anti-mouse secondary antibodies (Chemicon; Invitrogen), and mounted with Vectashield medium (Vector Laboratories, Burlingame, CA, USA).

Confocal microscopy was performed with a Leica TCS DMIRE 2 (LCS Lite Software; Leica, Wetzlar, Germany). No significant fluorescent signal was detectable with any of the secondary antibodies alone.

2.7 Real-time quantitative reverse transcriptase–polymerase chain reaction analysis

Total RNA was extracted after 5 days from control, Ca^{2+}-ICR exposed, non-Ca^{2+}-ICR exposed, and unexposed CSCs, deriving from seven different patients, using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. We obtained on average 5–10 mg total RNA per 70 cm^2 dish of cultured cells. We also isolated RNA from a whole cardiac biopsy as a positive control. One microgram of total RNA was used to synthesize first-strand cDNA with random primers, using 100 U of ImProm-II™ RT–PCR kit (Promega, Madison, WI, USA). The quantification of all gene transcripts was carried out by real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). Experiments were conducted to contrast relative levels of each transcript and endogenous control GAPDH in every sample. Gene expression was presented using the ($-2^{-\Delta\Delta Ct}$) method, described by Livak and Schmittgen,[23] where ΔCt = (average target Ct − average GAPDH Ct) and $\Delta\Delta Ct$ = (average ΔCt treated sample − average ΔCt untreated sample).

Table 2 Primers used for real-time reverse transcriptase–polymerase chain reaction

Target	Sequence	Annealing temperature (°C)
VEGF	5′-CTTGGGTGCATTGGAGCCT-3′ 5′-CTGCGCTGATAGACATCCAT-3′	60
KDR	5′-CAGCATCACCAGTAGCCAG-3′ 5′-TTATACAGATCTTCAGGAGCTT-3′	60
SMA	5′-ATGAAGATCCTGACTGAGCG-3′ 5′-GCAGTGGCCATCTCATTTTC-3′	60
Nkx2.5	5′-CAGCGACCCCGACCCAG-3′ 5′-GCTTCCTCCGCCGTCGC-3′	60
MHC	5′-CAGAAGAAGAAGATGGATGC-3′ 5′-CGCTGGTGTCCTGCTCCT-3′	60
TnI	5′-GGACAAGGTGGATGAAGAGA-3′ 5′-AGGGTGGGCCGCTTAAACT-3′	60
GAPDH	5′-CATCATCTCTGCCCCCTCT-3′ 5′-CAAAGTTGTCATGGATGACCT-3′	60

We performed a validation experiment to demonstrate that the amplification efficiency on target genes and reference GAPDH was equal. Real-time PCR was performed with Sybr Green I Mastermix, using an ABI PRISM™ 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each reaction was run in triplicate and contained 0.5–1 μL of cDNA template along with 250 nM primers in a final reaction volume of 25 μL. The sequences and annealing temperatures of the primers used are shown in *Table 2*. Cycling parameters were: 50°C for 2 min, 95°C for 10 min (to activate DNA polymerase), then 40–45 cycles at 95°C for 15 s and 60°C for 1 min. Melting curves were performed using Dissociation Curves software (Applied Biosystems) to ensure only a single product had been amplified. As negative controls, reactions were prepared in which RNA or reverse transcriptase had previously been omitted during reverse transcription.

2.8 Western blot analysis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli.[24] CSps and CDCs from six different patients were seeded on Petri dishes and cultured for 5 days in the absence (control) or in the presence (exposed) of Ca^{2+}-ICR EMF. Equal amounts of protein from control and exposed cells were loaded for each lane, after lysis in sample buffer

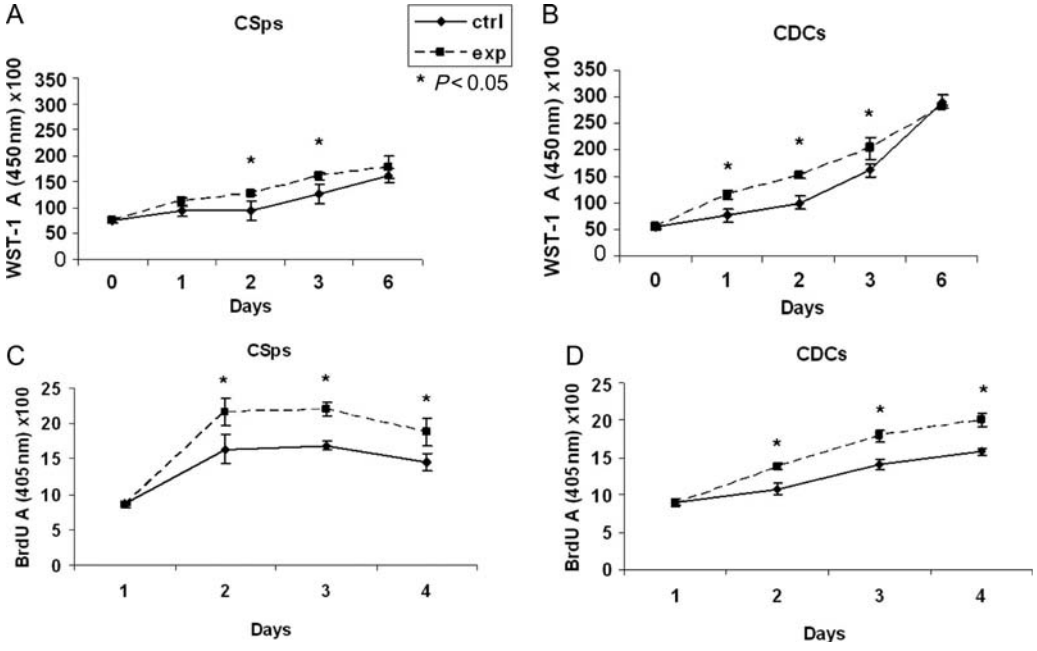

Figure 2 Metabolic activity and bromodeoxyuridine-incorporation of cardiac stem cells exposed to Ca^{2+}-ion cyclotron energy resonance. The WST assay on developing cardiospheres (*A*) and exponentially growing cardiosphere-derived cells (*B*) revealed a higher metabolic activity in exposed cells compared with unexposed controls. The BrdU pulse-labelling time-course on cardiospheres (*C*) and cardiosphere-derived cells (*D*) confirmed that the higher metabolic activity correlated with a higher proliferation rate. *$P < 0.05$; ctr, unexposed control; exp, exposed.

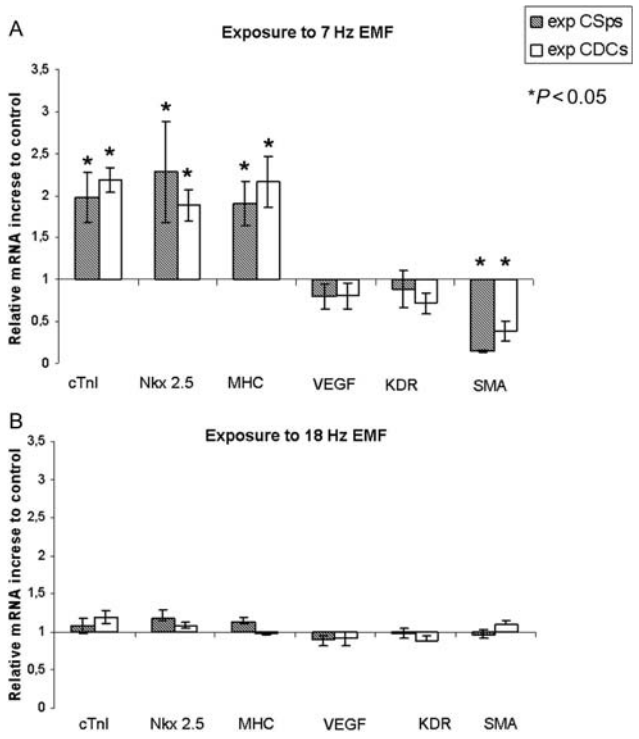

Figure 3 Quantitative real-time polymerase chain reaction for differentiation markers. (*A*) cardiospheres and cardiosphere-derived cells exposed to Ca^{2+}-ion cyclotron energy resonance for 5 days revealed a significant increase in relative TnI, Nkx2.5, and MHC mRNA levels (see Methods for details). No changes in VEGF and KDR mRNA levels were detected, but the decrease in SMA levels was statistically significant. (*B*) No statistically significant variation in any marker was detected after 5 days of exposure to the control non Ca^{2+}-ion cyclotron energy resonance frequency. $*P < 0.05$; exp, exposed.

(150 mM NaCl, 50 mM Tris/HCl, pH 8, 0.5 mM EDTA, 0.1 mM EGTA, 1% Triton X-100). Electrophoresis was performed on 7.5% SDS–polyacrylamide gels at 60V for 2 h. Transfer on nitrocellulose membranes (Biorad, Hercules, CA, USA) was then performed at 300 mA for 2 h. After blocking in 5% fat-free-milk for 1 h at RT, membranes were incubated with the following monoclonal antibodies: TnI (Santa Cruz), KDR and Cx43 (Chemicon), Nkx2.5 and SMA (Abcam), MHC (clone MF-20, kindly provided by Maurizia Caruso), β-Actin (Sigma-Aldrich), and then revealed by chemiluminescence system (Amersham Biosciences).

Densitometric analysis was performed with the Quantity One software (Biorad).

2.9 Ca^{2+}-fluorescence digital imaging

2.9.1 Rhod-2-AM and Oregon Green 488BAPTA-1 loading

Cardiosphere cells were plated on glass coverslips (7000 cells/cm^2) and chronically exposed to Ca^{2+}-ICR or to the control non-Ca^{2+}-ICR frequency for 3 or 5 days. After exposure, CSCs were loaded with 4.5 μM Rhod-2/AM (mytocondrial probe; Invitrogen) or 1 μM Oregon Green 488BAPTA-1 (Invitrogen) in presence of 0.005% Pluronics 127 (Sigma-Aldrich) for 30 min at RT or 45 min at 37°C, respectively, in HEPES buffer (156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose, and 7.5 mM HEPES at pH 7.35). Cells were washed thoroughly before fluorescence microscopy analysis. Exposed and unexposed cells, labelled with the probes, were then acutely re-exposed to Ca^{2+}-ICR in the miniaturized exposure system for direct visualization under a fluorescence microscope (*Figure 1B*).

2.9.2 Fluorescence acquisition and analysis

Fluorescence measurements were obtained using an inverted microscope (Olympus IX51, Tokyo, Japan) equipped with oil immersion 60× objective and with a cooled CCD camera (Spot RT Slider, acquisition rate five frames per second, full frame; Diagnostic Instruments, Sterling Heights, MI, USA). For the qualitative

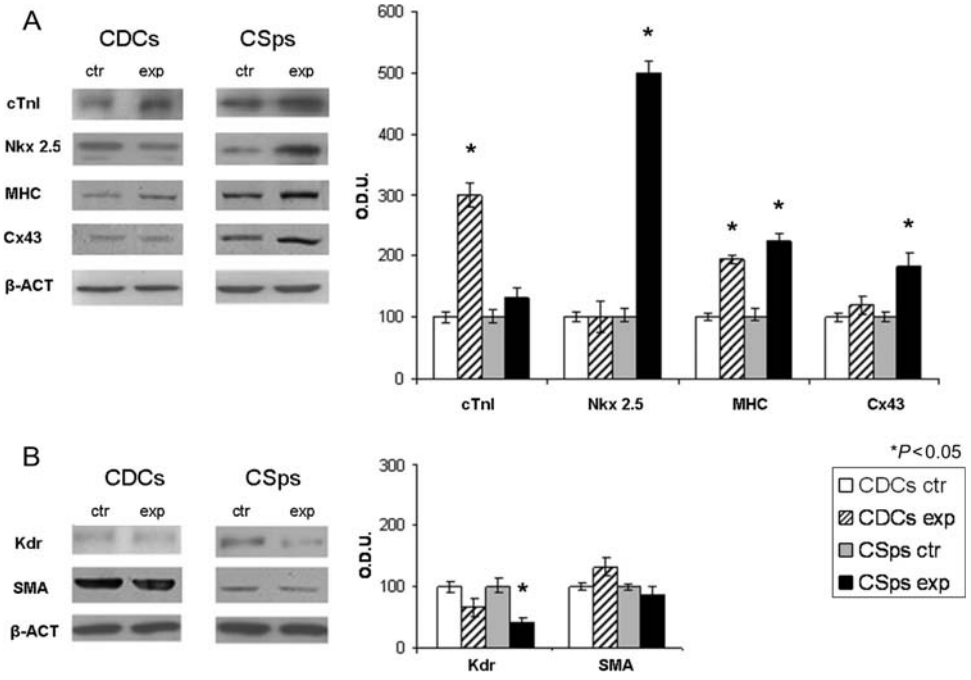

Figure 4 Representative western blots for differentiation proteins and corresponding densitograms. (*A*) After 5 days of exposure, cardiosphere-derived cells and cardiospheres showed a significant increase in the expression of cTnI and MHC, or in Nkx2.5, MHC and Cx43, respectively. (*B*) Protein levels of KDR and SMA were either reduced or unaffected by 5 day exposure to Ca^{2+}-ICR. $*P < 0.05$; ctr, unexposed control; exp, exposed.

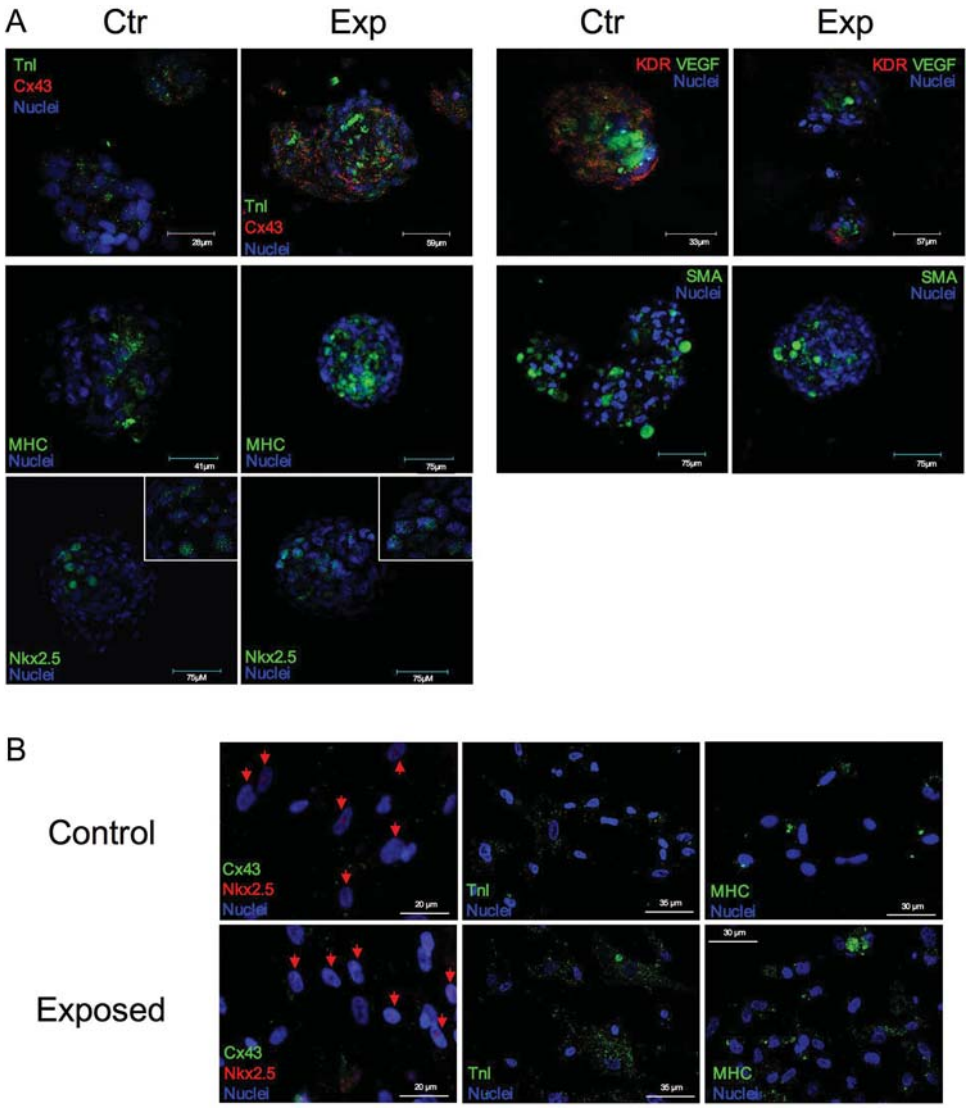

Figure 5 Immunofluorescence analysis for differentiation markers. Confocal images of exposed and unexposed cardiospheres (*A*) and cardiosphere-derived cells (*B*). In cardiospheres, cardiac markers, such as Cx43, TnI, MHC, and Nkx2.5 (higher magnification panels are shown in inserts), were up-regulated compared with controls; conversely expression of vascular markers, such as KDR, VEGF, and SMA, was slightly down-regulated or unaffected in exposed cardiospheres vs. controls. Consistently, in cardiosphere-derived cells TnI and MHC display increased expression. Red arrows: Nkx2.5-expressing cells; ctr, unexposed control; exp, exposed.

measurement of mytocondrial and cytosolic Ca^{2+}, Rhod-2 fluorescence was excited at 560 nm and collected through a 590 nm-long pass barrier filter. Oregon Green fluorescence was excited at 460 nm and collected through a 490 nm-pass barrier filter. High-resolution imaging enabled identification of individual mitochondria for analysis. All imaging data were acquired and analysed using Spot RT software 4.1 (Diagnostic Instruments). Since Rhod-2 and Oregon Green are single-wavelength fluorescent indicators, it was not possible to apply the ratiometric method for quantitative analysis. Therefore, the information derived from the measurements of Rhod-2 and Oregon Green probes were normalized as a function of the first image. Excitatory light was kept to a minimum with neutral density filters and a computer-controlled shutter to minimize photo-bleaching and photodynamic injury to cells. *In situ* calibration was performed by exposing loaded cells to the controlled Ca^{2+}-buffer in the presence of 10 μM ionomycin (Sigma-Aldrich).

Ca^{2+}-storage analysis with Oregon Green was performed on 15 sets of experiments. Statistical analysis for Rhod-2 was performed on five replicates for a total of 1000 single cells screened.

2.10 Statistical analysis

Statistical analysis of the data was performed using the Student's *t*-test, with $P = 0.05$ as the minimum level of significance. Data are shown as mean $\pm$ standard deviation.

3. Results

3.1 Extremely low-frequency electromagnetic field affects human cardiosphere and cardiosphere-derived cell metabolic activity and proliferation

Exponentially growing CSps and CDCs were plated in 96-well plates and exposed to Ca^{2+}-ICR frequency for up to 5 days. Exposed cells showed an increase in metabolic activity compared with the unexposed control, as assessed by the WST assay at different time points (*Figure 2A* and *B*). BrdU-pulse experiments on CSps and CDCs revealed that the higher

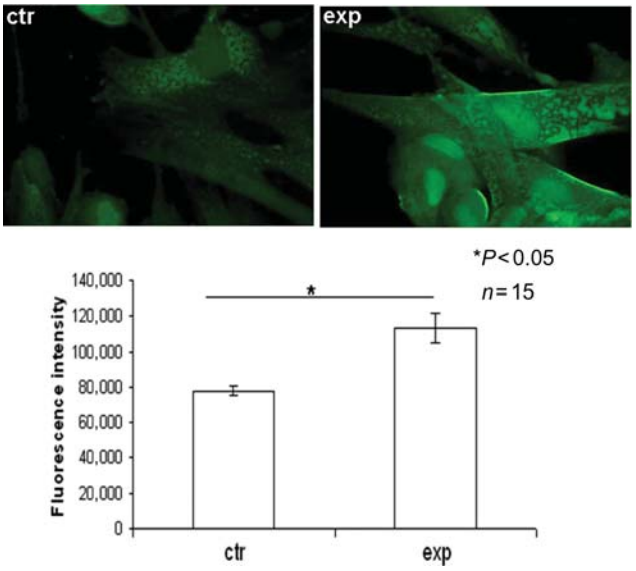

Figure 6 Ca^{2+} storage increase in chronically exposed cardiosphere-derived cells. Fluorescence images of Ca^{2+}-ion cyclotron energy resonance 5 day exposed cardiosphere-derived cells, loaded with Oregon Green, revealed higher intracellular Ca^{2+} storage compared with controls, as shown by quantification of green fluorescence intensity. $*P < 0.05$; ctr, unexposed control; exp, exposed.

metabolism corresponded to higher proliferation rates (*Figure 2C* and *D*). Data are representative of triplicate experiments on cells from three different patients.

3.2 Extremely low-frequency electromagnetic field modulates cardiosphere and cardiosphere-derived cells differentiation

After 5 days of exposure to ELF-EMF, both human CSps and CDCs show a significant increase in the expression of cardiac markers, as evidenced by RT–PCR (*Figure 3*), western blotting (*Figure 4*), and immunofluorescent analysis (*Figure 5*).

Real-time RT–PCR revealed a significant increase in relative TnI, MHC, and Nkx2.5 mRNA levels (*Figure 3A*) after Ca^{2+}-ICR exposure. In contrast, vascular markers either did not show any change at the mRNA level, as for VEGF and KDR, or showed a statistically significant decrease, as for SMA (*Figure 3A*). Exposure to the control non-Ca^{2+}-ICR frequency (18 Hz) did not affect the expression of any marker (*Figure 3B*).

We also normalized the mRNA levels of cardiac markers (TnI, Nkx2.5 and MHC) of exposed and unexposed cells to those of adult heart tissue from a whole biopsy (see Supplementary material online, *Figure S1*). From this graph it is evident that the ratio between cardiac markers' mRNA levels and the positive control is higher in CSps than in CDCs. This difference further increases in exposed compared with unexposed samples, confirming the differentiation effect of Ca^{2+}-ICR exposure.

Results of western blot analysis are shown in *Figure 4*: an increase in band thickness, relative to Nkx2.5, MHC, and Cx43, was detected in exposed CSps compared with those unexposed (*Figure 4A*). Moreover, exposed CDCs showed a statistically significant increase in the expression of TnI and MHC (*Figure 4A*), while in normal conditions CDCs are usually characterized by weak expression of terminal cardiac-specific markers. The opposite effect was observed for the VEGF receptor, KDR, which displayed a statistically significant decrease at the protein level, while SMA was not affected (*Figure 4B*).

Confocal analysis of CSps (*Figure 5A*) showed a brighter positive signal for cardiac Cx43, TnI, MHC and Nkx2.5 in the 5-day exposed CSps compared with the unexposed control. In normal conditions, these cardiac proteins are spontaneously expressed mostly in the external layers of the CSp, as previously described.[5] A reverse pattern of expression was observed in CSps for KDR and VEGF staining, while changes in SMA levels and distribution were undetectable. Immunofluorescence for cardiac markers on CDCs (*Figure 5B*) showed a slight increase in TnI and MHC signals, while Cx43 and Nkx2.5 staining did not reveal a noticeable modulation.

3.3 Extremely low-frequency electromagnetic field effect on intracellular Ca^{2+} in human cardiosphere-derived cells

Pulse-labelling experiments with the Ca^{2+} probe Oregon Green showed a marked increase in cytoplasmic fluorescence intensity in Ca^{2+}-ICR exposed cells vs. the unexposed (*Figure 6*), suggesting intracellular Ca^{2+} accumulation. During acute exposure of CDCs to ELF-EMF, realized in the miniaturized a-magnetic chamber adjusted for microscope analysis, Ca^{2+} mobilization among cellular compartments was observed (*Figure 7A–E*). This acute effect was more pronounced when CDCs had been previously exposed for 5 days to the Ca^{2+}-ICR frequency (*Figure 7A–C*), compared with acute exposure alone (*Figure 7D* and *E*). Compartmentalized analysis of fluorescence intensity confirmed Ca^{2+} mobilization from storage compartments to the cytosol, and viceversa (*Figure 7A–C*; see Supplementary material online *Figure S2* and *Movies 1* and *2*). Chronically exposed cells to the non-Ca^{2+}-ICR frequency and unexposed cells did not display any detectable Ca^{2+} flux (*Figure 7F* and *G*; see Supplementary material online, *Figure S3*, respectively).

4. Discussion

The question whether ELF-EMFs can affect biological systems has attracted the attention of many research groups for quite some time. Nowadays, the theoretical possibility of such an interaction is still debated and the hypothetical mechanisms are unclear. There is substantial evidence indicating that moderate-intensity static MFs are capable of influencing a number of biological processes, particularly those whose function is closely related to the properties of membrane channels. Most of the reported effects may be explained on the basis of alterations in membrane Ca^{2+} flux.[16] The model that has been suggested is based on the diamagnetic anisotropic properties of membrane phospholipids. It is proposed that reorientation of these molecules during exposure to MFs would result in the deformation of imbedded ion channels, thereby altering their dynamics.[25]

In the present report, we studied the effect of combined static and alternate EMFs, tuned at Ca^{2+}-ICR, on a biological system consisting of human CSCs. We suggest that suitable combinations of EMFs may affect intracellular Ca^{2+} levels,

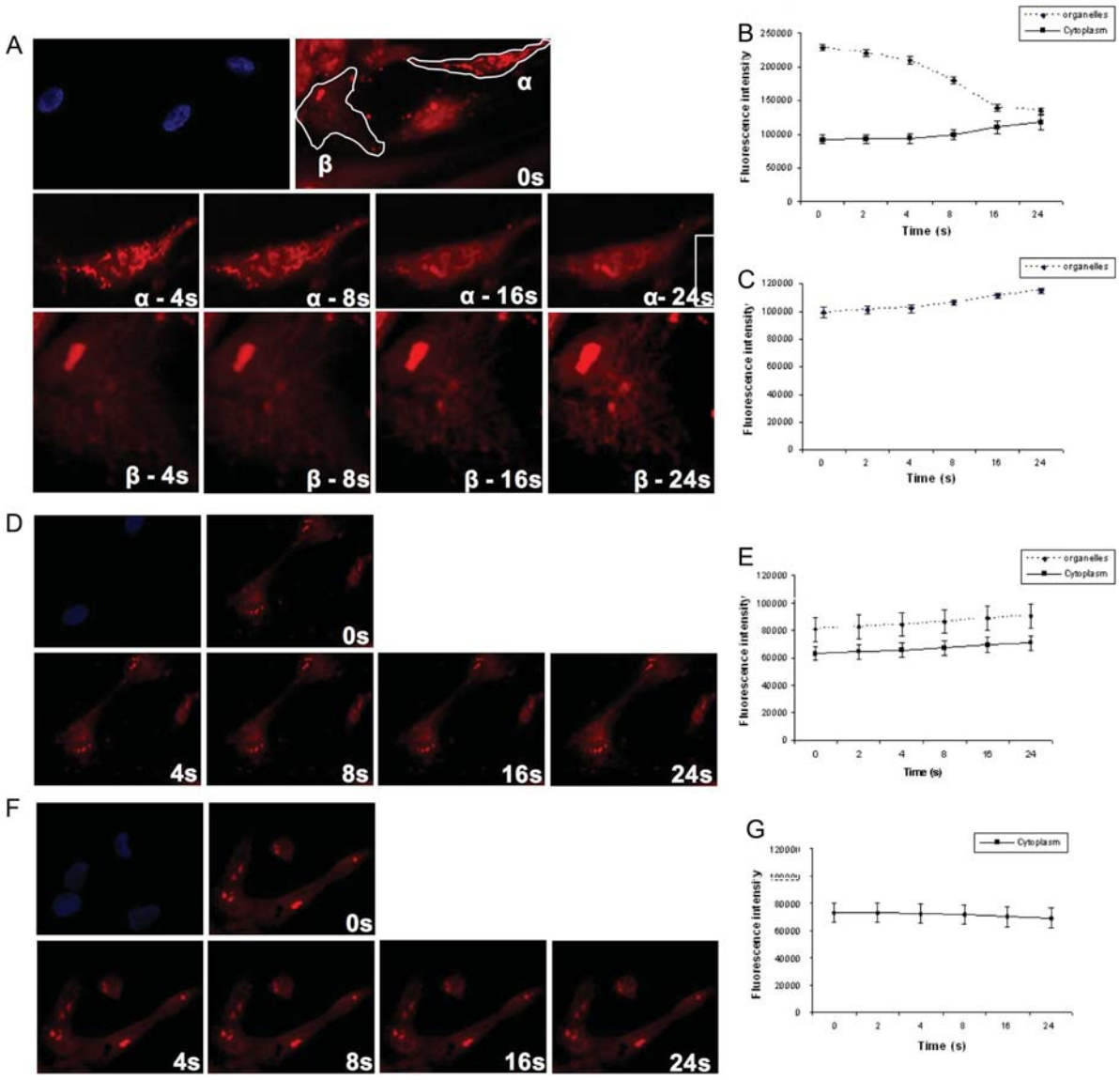

Figure 7 Qualitative measurements of mitochondrial and cytosolic Ca^{2+} mobilization by Rhod-2 fluorescence. (*A*) Chronically exposed cardiosphere-derived cells showed Ca^{2+} mobilization from storage compartments to the cytosol (α), and viceversa (β), as shown by compartmentalized analysis of fluorescence intensity (*B* and *C*, respectively). Cardiosphere-derived cells acutely exposed to Ca^{2+}-ion cyclotron energy resonance displayed slight Ca^{2+} mobilization (*D*), as confirmed by compartmentalized fluorescence analysis (*E*). After chronic exposure for 5 days to the non Ca^{2+}-ion cyclotron energy resonance frequency, cardiosphere-derived cells did not show any Ca^{2+} flux among cytoplasmic compartments (*F*), as confirmed by fluorescence intensity analysis (*G*). s, seconds.

triggering PCs proliferation, and differentiation. A number of mechanisms have been postulated to explain the observed effects of combined MFs and EMFs. Among them, based on Eq. (1), ICR occurs for predictable combinations of static MFs and EMFs. Liboff *et al*.[26] suggested that EMFs can interact in a resonant manner with endogenous alternate current electric fields in biological systems. Lednev[25] elaborated a theory to explain ICR at a biological level. He considered an ion in its protein-binding site as a dipole; when the ion is exposed at its ICR, energy is transferred to the dipole and, as a consequence, the ion is released in solution.

Ca^{2+} ions are essential regulatory components of all organisms. Being a second messenger, Ca^{2+} is involved in regulation at all stages of cellular growth and development, including proliferation, differentiation, assembling and disassembling of cytoskeleton elements.[27–31]

In the present study, CSps and CDCs were exposed for up to 5 days to ELF-EMFs close to the ICR frequency corresponding to the charge/mass ratio of the Ca^{2+} ion, on the basis of our previous results obtained with other cellular models.[15,32] Exposure to Ca^{2+}-ICR frequency produced several effects in both CSps and CDCs. *Figure 2* shows that both CSps and CDCs exposed to ELF-EMF have a higher metabolic activity compared with unexposed cells. This can be related to an increase in cell proliferation, as evidenced by the BrdU incorporation curves (*Figure 2C* and *D*). The trend is reduced after 3 days of exposure, perhaps due to both contact inhibition and/or the beginning of the differentiation process, well documented after 5 days in both CSps and CDCs at transcriptional and translational level (see Results). Usually proliferation and differentiation are considered mutually exclusive paths, but since both CSps and CDCs represent heterogeneous populations of progenitor cells at various stages of commitment, one could expect slightly different responses to proliferative and differentiative stimuli at each intermediate stage. To a certain

extent, these responses are possibly overlapping in the progressive maturation process of the whole progenitor population.

The increase in mRNA levels of cardiac-specific markers, demonstrated by real-time PCR, was associated to an increase in the corresponding protein expression, as evidenced in *Figures 3A* and *4A*. Although CSps spontaneously differentiate towards the cardiogenic phenotype, this process was improved by ELF-EMF exposure. The improvement in the differentiation process was cardiac specific (see Results), although not terminal, and more relevant in CDCs, in which spontaneous differentiation does not normally reach an advanced stage. After Ca^{2+}-ICR exposure, cardiac markers such as TnI, MHC, Cx43, and Nkx2.5 were up-regulated, while vascular markers, such as KDR and SMA, were either unaffected or reduced (*Figures 3A* and 4). Cardiac-specific differentiation was further evidenced when mRNA levels of cardiac markers (TnI, Nkx2.5, and MHC) of exposed and unexposed cells were compared with those of adult heart tissue from a whole biopsy (see Supplementary material online, *Figure S1*). The upregulation of cardiac markers, normalized to heart tissue, in Ca^{2+}-ICR exposed compared with unexposed samples represents an effective plotting option to evidence the progress of cardiac differentiation.

Confocal analysis (*Figure 5*) confirmed an increase in the expression of cardiac markers, as indicated by higher fluorescence intensity for TnI, Cx43, MHC, and Nkx2.5, although with some variability between CSps and CDCs. Altogether these results suggest that, in our experimental conditions, a lineage-specific differentiation is driven by consequence of the exposure to Ca^{2+}-ICR. As we previously described, the CSp structure allows more advanced differentiative features, compared with CDCs. Therefore, different expression levels and balances of early (e.g. Nkx2.5) vs. late (e.g. TnI) cardiac markers are not inconsistent.

The same experiments repeated at a frequency not matching ICRs of biologically relevant ions did not show any significant effect at transcriptional level (*Figure 3B*), endorsing the hypothesis of a Ca^{2+}-mediated result.

The role of cytosolic Ca^{2+} has long been recognized in the regulation of cellular and molecular interactions. Signal transduction related to Ca^{2+} oscillations can provide molecular cues for cell functions such as differentiation[16] and proliferation.[33,34] Although Ca^{2+} dynamics are versatile and likely to depend on cell type, their role in human CSCs differentiation is yet to be fully elucidated.

In the present work, although we did not investigate the involved mechanisms, we unequivocally demonstrated increased intracellular calcium accumulation in CDCs after chronic exposure to Ca^{2+}-ICR (*Figure 6*). Furthermore, by compartmentalized fluorescence analysis through the Ca^{2+} probe Rhod-2, we detected that chronic and acute exposure to Ca^{2+}-ICR correlates to Ca^{2+} mobilization among cellular compartments (*Figure 7A–E*; see Supplementary material online, *Figure S2*, Movies 1 and 2). Since Rhod-2 is a mitochondria-specific probe, the mobilization is most likely to be between mitochondria and the cytosol.

In conclusion, in the present experimental strategy, the modulation of both proliferation and cardiac differentiation observed in Ca^{2+}-ICR exposed cells correlates to induced changes in intracellular Ca^{2+} accumulation and mobilization, potentially modulating signal cascade pathways.[16,31] Independently of the involved mechanisms, the induced differentiation towards the cardiac phenotype has relevant implications for the use of CSCs in cell therapy and tissue engineering. The modulation of proliferation and specific differentiation elicited by our system through ELF-EMFs could represent an effective, minimally manipulating, and safe biotechnological tool to improve their cardiac regenerative potential.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

We would like to aknowledge: Emanuela Rosola, Francesca Bertani, Francesco Angelini and Alberto Di Consiglio for their technical support; Maurizia Caruso (INMM, CNR, Rome, Italy) for kindly providing the anti-MHC antibody.

Conflict of interest: none declared.

Funding

This work was supported by 'Istituto Pasteur—Fondazione Cenci Bolognetti' and by a grant ISPESL-B1 4/DIPIA/2005.

References

1. Abraham MR, Henrikson CA, Tung L, Chang MG, Aon M, Xue T *et al*. Antiarrhythmic engineering of skeletal myoblasts for cardiac transplantation. *Circ Res* 2005;**97**:159–167.
2. Cho HJ, Lee N, Lee JY, Choi YJ, Ii M, Wecker A *et al*. Role of host tissues for sustained humoral effects after endothelial progenitor cell transplantation into the ischemic heart. *J Exp Med* 2007;**204**:3257–3269.
3. 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**:763–776.
4. Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S *et al*. Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. *Nature* 2005;**433**:647–653.
5. Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F *et al*. Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 2004;**95**:911–921.
6. Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y *et al*. Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci USA* 2003;**100**:12313–12318.
7. Ott HC, Matthiesen TS, Brechtken J, Grindle S, Goh SK, Nelson W *et al*. The adult human heart as a source for stem cells: repair strategies with embryonic-like progenitor cells. *Nat Clin Pract Cardiovasc Med* 2007;**4**(Suppl. 1):S27–S39.
8. van Vliet P, Roccio M, Smits AM, van Oorschot AA, Metz CH, van Veen TA *et al*. Progenitor cells isolated from the human heart: a potential cell source for regenerative therapy. *Neth Heart J* 2008;**16**:163–169.
9. Oyama T, Nagai T, Wada H, Naito AT, Matsuura K, Iwanaga K *et al*. Cardiac side population cells have a potential to migrate and differentiate into cardiomyocytes *in vitro* and *in vivo*. *J Cell Biol* 2007;**176**:329–341.
10. Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E *et al*. Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. *Circulation* 2007;**115**:896–908.
11. Cossarizza A, Monti D, Bersani F, Scarfi MR, Zanotti M, Cadossi R *et al*. Exposure to low-frequency pulsed electromagnetic fields increases mitogen-induced lymphocyte proliferation in Down's syndrome. *Aging (Milano)* 1991;**3**:241–246.
12. Cossarizza A, Monti D, Bersani F, Cantini M, Cadossi R, Sacchi A *et al*. Extremely low frequency pulsed electromagnetic fields increase cell proliferation in lymphocytes from young and aged subjects. *Biochem Biophys Res Commun* 1989;**160**:692–698.

13. Cadossi R, Bersani F, Cossarizza A, Zucchini P, Emilia G, Torelli G *et al*. Lymphocytes and low-frequency electromagnetic fields. *FASEB J* 1992; **6**:2667–2674.
14. Cossarizza A, Angioni S, Petraglia F, Genazzani AR, Monti D, Capri M *et al*. Exposure to low frequency pulsed electromagnetic fields increases interleukin-1 and interleukin-6 production by human peripheral blood mononuclear cells. *Exp Cell Res* 1993;**204**:385–387.
15. Lisi A, Ciotti MT, Ledda M, Pieri M, Zona C, Mercanti D *et al*. Exposure to 50 Hz electromagnetic radiation promote early maturation and differentiation in newborn rat cerebellar granule neurons. *J Cell Physiol* 2005; **204**:532–538.
16. Lisi A, Ledda M, Rosola E, Pozzi D, D'Emilia E, Giuliani L *et al*. Extremely low frequency electromagnetic field exposure promotes differentiation of pituitary corticotrope-derived AtT20 D16V cells. *Bioelectromagnetics* 2006;**27**:641–651.
17. Thomas AW, Kavaliers M, Prato FS, Ossenkopp KP. Pulsed magnetic field induced 'analgesia' in the land snail, *Cepaea nemoralis*, and the effects of mu, delta, and kappa opioid receptor agonists/antagonists. *Peptides* 1997;**18**:703–709.
18. Vorobyov VV, Sosunov EA, Kukushkin NI, Lednev VV. Weak combined magnetic field affects basic and morphine-induced rat's EEG. *Brain Res* 1998; **781**:182–187.
19. Ibiwoye MO, Powell KA, Grabiner MD, Patterson TE, Sakai Y, Zborowski M *et al*. Bone mass is preserved in a critical-sized osteotomy by low energy pulsed electromagnetic fields as quantitated by in vivo micro-computed tomography. *J Orthop Res* 2004;**22**:1086–1093.
20. Albertini A, Zucchini P, Noera G, Cadossi R, Napoleone CP, Pierangeli A. Protective effect of low frequency low energy pulsing electromagnetic fields on acute experimental myocardial infarcts in rats. *Bioelectromagnetics* 1999;**20**:372–377.
21. Ventura C, Maioli M, Asara Y, Santoni D, Mesirca P, Remondini D *et al*. Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. *FASEB J* 2005;**19**:155–157.
22. Lisi A, Ledda M, de Carlo F, Pozzi D, Messina E, Gaetani R *et al*. Ion cyclotron resonance as a tool in regenerative medicine. *Electromagn Biol Med* 2008;**27**:127–133.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;**25**:402–408.
24. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
25. Lednev VV. Possible mechanism for the influence of weak magnetic fields on biological systems. *Bioelectromagnetics* 1991;**12**:71–75.
26. Liboff AR. Electric-field ion cyclotron resonance. *Bioelectromagnetics* 1997;**18**:85–87.
27. Nakanishi S, Okazawa M. Membrane potential-regulated Ca^{2+} signalling in development and maturation of mammalian cerebellar granule cells. *J Physiol* 2006;**575**:389–395.
28. Cai H, Liu D, Garcia JG. CaM kinase II-dependent pathophysiological signalling in endothelial cells. *Cardiovasc Res* 2008;**77**:30–34.
29. Means AR. Calcium, calmodulin and cell cycle regulation. *FEBS Lett* 1994; **347**:1–4.
30. Takuwa N, Zhou W, Takuwa Y. Calcium, calmodulin and cell cycle progression. *Cell Signal* 1995;**7**:93–104.
31. Whitfield JF, Boynton AL, MacManus JP, Sikorska M, Tsang BK. The regulation of cell proliferation by calcium and cyclic AMP. *Mol Cell Biochem* 1979;**27**:155–179.
32. Manni V, Lisi A, Pozzi D, Rieti S, Serafino A, Giuliani L *et al*. Effects of extremely low frequency (50 Hz) magnetic field on morphological and biochemical properties of human keratinocytes. *Bioelectromagnetics* 2002;**23**:298–305.
33. Walleczek J, Budinger TF. Pulsed magnetic field effects on calcium signaling in lymphocytes: dependence on cell status and field intensity. *FEBS Lett* 1992;**314**:351–355.
34. Manni V, Lisi A, Rieti S, Serafino A, Ledda M, Giuliani L *et al*. Low electromagnetic field (50 Hz) induces differentiation on primary human oral keratinocytes (HOK). *Bioelectromagnetics* 2004;**25**:118–126.