Published in final edited form as:

Exp Cell Res. 2009 December 10; 315(20): 3611–3619. doi:10.1016/j.yexcr.2009.08.015.

Electrical stimulation of human embryonic stem cells: Cardiac differentiation and the generation of reactive oxygen species

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Abstract

Exogenous electric fields have been implied in cardiac differentiation of mouse embryonic stem cells and the generation of reactive oxygen species (ROS). In this work, we explored the effects of electrical field stimulation on ROS generation and cardiogenesis in embryoid bodies (EBs) derived from human embryonic stem cells (hESC, line H13), using a custom-built electrical-stimulation bioreactor. Electrical properties of the bioreactor system were characterized by electrochemical impedance spectroscopy (EIS) and analysis of electrical currents. The effects of the electrode material (stainless steel, titanium-nitride coated titanium, titanium), length of stimulus (1 s and 90 s) and age of EBs at the onset of electrical stimulation (4 and 8 days) were investigated with respect to ROS generation. The amplitude of the applied electrical field was 1 V/mm. The highest rate of ROS generation was observed for stainless steel electrodes, for signal duration of 90 s, and 4-days old EBs. Notably, comparable ROS generation was achieved by incubation of EBs with 1 nM H₂O₂. Cardiac differentiation in these EBs was evidenced by spontaneous contractions, expression of Troponin T, and its sarcomeric organization. These results imply that electrical stimulation plays a role in cardiac differentiation of hESCs, through mechanisms associated with the intracellular generation of ROS.

Keywords

human embryonic stem cells; electrical stimulation; reactive oxygen species; cardiac differentiation

Introduction

Heart disease and stroke, the principal components of cardiovascular disease, are the first and the third leading cause of death in the U.S., accounting for nearly 40% of all deaths, more than all cancer combined [1]. Because cardiomyocytes are unable to regenerate following an injury

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in the adult heart, cell-based therapies provide a potential alternative approach to replace damaged myocardial tissue and restore cardiac function. A major limitation toward this goal is the lack of donor cells. Although human embryonic stem cells have enormous potential as a source of cardiovascular cells, the regulatory elements mediating their differentiation to cardiomyocytes are largely unknown [2]. The full therapeutic utility of human embryonic stem cells (hESC) will only be realized when they may be predictably differentiated into a range of cell lines [3-5].

Numerous studies have demonstrated the importance of chemical and physical stimuli in directing stem cell differentiation [6-9]. Endogenous electromagnetic fields (EF) are present in the developing and regenerating tissues, either in the extracellular space or in the cell cytoplasm, and they range in strength from a few mV/mm to several hundred of mV/mm [3, 4]. Combined with diffusible chemical gradients, electric fields lead to the polarization and the formation of spatial patterns in developing embryo [12-14] creating directional signals necessary for the proper placement of the components of the organism.

Exogenous electrical fields applied *in vitro* have been shown to influence cell behavior [5]. Several studies have reported galvanotaxis in a variety of cultured cells after stimulation with a constant direct current (DC) field comparable with those detected *in vivo* [6]. The cell responds to an externally applied electric field by the passive and active intracellular influx of ions, such as calcium and sodium [7,8], or the localization of lipid and epithelial growth factor (EGF) receptors in the membrane [9].

Exposure of mouse embryonic stem cells (mESCs) to electric fields has been found to promote cardiomyogenic [10] and angiogenic [21,22] differentiation. However, the course of action of electrical stimulation on the activation of cell differentiating pathways is poorly understood. One possible mechanism involves the generation of reactive oxygen species (ROS) within the cell [10,11]. Sauer and colleagues demonstrated that stimulation with an exogenous electric field increases intracellular ROS production in mouse EBs [10]. ROS are highly reactive molecules generated during the normal metabolism of oxygen by NADPH oxidases or as side products of several enzymatic systems (e.g., cyclooxygenases, nitric oxide synthases, mitochondrial cytochromes). Although excessive concentration of ROS, such as superoxide anions (O_2^-) and hydrogen peroxide (H_2O_2), is considered destructive and results in inhibition of gene expression [12,13], small amounts of ROS function as intracellular second messengers and activate signaling cascades involved in growth and differentiation of many cell types [26-29].

ROS have been implied in the regulation of cardiogenesis [14] via a variety of mechanisms. When cardiac cells are stimulated by cytokines [15], growth factors, hormones, or mechanical stress [16], they elicit a small oxidative burst and generate low concentrations of ROS. During myocardial infarction, cardiac cells generate large amounts of free radicals and ROS, which are involved in the signaling and activation of the intrinsic repair mechanisms of the damaged myocardium [17,18]. ROS activate the mitogen activated protein kinase (MAPK) pathways in mouse EBs [19], enhancing angiogenesis via activation of ERK1,2 and JNK and cardiomyogenesis via phosphorylation of ERK1,2, JNK and p38 [35-37]. An NADPH oxidase-like enzyme is involved in the ROS-related cardiogenesis during mESC differentiation [2].

In previous studies done in our lab, a biomimetic system designed to deliver electric signals mimicking those in native heart resulted in the progressive development of conductive and contractile properties characteristic of cardiac tissue, including cell alignment and coupling, increased amplitude of synchronous construct contractions, and a remarkable level of ultrastructural organization [38, 39]. In order to control cell behavior through the efficacious application of electric fields, we have also found it necessary to characterize the stimulation

and understand its mechanisms of action at the biological level [40-42]. Because electric charges are carried by *ions* in physiologic media and by *electrons* in electrodes and electric circuitry, at the electrode-medium interface, there is a transduction of charge carriers from electrons to ions.

Charge transfer can occur through three mechanisms: (i) non-Faradaic charging/discharging of the electrochemical double layer, (ii) reversible Faradaic reactions, and (iii) non-reversible Faradaic reactions. The relative presence of each mechanism can be determined by electrochemical impedance spectroscopy (EIS), from which an equivalent circuit of the stimulation system can be constructed. Equivalent circuit modeling can provide insight into the behavior of the system being analyzed (which can be highly non-linear, depending on geometry, electrolyte composition, electrode composition and surface properties). In order to control cell behavior through the application of electric fields, we have characterized various electrode materials.

Taken together, these previous studies imply that electrical stimulation could be utilized to mediate hESC differentiation towards cardiac lineages, and motivated the present study of the effects of electrical stimulation on the generation of ROS and cardiogenic differentiation of human embryonic stem cells. The use of a simple yet highly controllable bioreactor system enabled us to probe in a systematic way the effects of multiple experimental variables (electrode material, duration of electrical stimulation, developmental stage of the cells). The conditions of stimulation were determined based on our cardiac tissue engineering studies [30-32] and previously reported effects of electrical signals on stem cell differentiation [10,11]. The ROS generation and the expression of cardiac markers were correlated to each other and the experimental variables.

Material and Methods

Human Embryonic Stem Cell culture

hESCs (line H13, National Stem Cell Bank, Madison, WI) were grown on mouse embryonic fibroblasts (MEF, Chemicon) inactivated with mitomycin C (Sigma-Aldrich) in growth medium consisting of 80% KnockOut DMEM (Gibco), supplemented with 20% KnockOut Serum Replacement (Invitrogen Corporation, Carlsbad, CA), 4 ng/ml basic Fibroblast Growth Factor (Invitrogen Corporation, Carlsbad, CA), 1 mM L-glutamine (Gibco), 0.1 mM β -mercaptoethanol (Gibco), 1% non-essential amino acid stock (Invitrogen Corporation, Carlsbad, CA). hESCs were passaged to new feeder using 1mg/ml type IV collagenase (Invitrogen Corporation, Carlsbad, CA). To induce the formation of human embryoid bodies (EBs), undifferentiated hESCs were treated with 1 mg/mL collagenase, and then transferred to low attachment plates containing EB medium: 80% knockout- Dulbecco's modified Eagle medium (Gibco/Invitrogen Co.) supplemented with 20% defined fetal bovine serum (Hyclone), 1mM L-glutamine, and 1% nonessential amino acid stock (Gibco). Human EBs were cultured at 37 °C, and 5% CO₂ in a humidified incubator, with changes of media every 3 days. In order to check the influence of electrical stimulation in cardiac differentiation, 4- and 8-days old EBs were stimulated in the electrical stimulation system described below in presence of “pulsing buffer”.

Bioreactor system for electrical stimulation

Bioreactor design—EBs were electrically stimulated within a bioreactor that was custom designed for this purpose and fabricated from poly(dimethylsiloxane) (PDMS). The bioreactor is formed as an array of 4 × 4 wells as shown in Figure 1. Electrodes (1.3 mm in diameter) are inserted at the two sides of each row of wells. Independent stimulation of each row was provided by insulating properties of PDMS. The bioreactor was fabricated by stereolithography, by

curing PDMS in a mold-fabricated by QuickParts, reproduced with a resolution of 50 μm . The 10:1 mixture of PDMS and initiator (Dow Corning) was poured into the mold where the electrodes were already inserted. The inlet and outlet holes for the positioning of electrodes were exactly matching the electrodes to provide tight seal. The PDMS bioreactor was attached to a 75 $\times$ 25 mm glass slide via plasma treatment (Plasma Cleaner PDC-002, Harrick Plasma). The optical transparency of the glass slide allowed observation of the EB during the stimulation. The electrodes were connected to electrical stimulator generating square-wave electric pulses (Astro-Med, Inc., West Warwick, RI).

Experimental Design—4- and 8-days old EBs were electrically stimulated as follows. Single EBs were suspended in a bioreactor well (positioned in the gray-shaded area in Figure 1B) in a low ionic content “pulsing buffer” (255 mM sucrose, 1 mM CaCl_2 , 1 mM MgCl_2 , 5 mM HEPES, all from Sigma-Aldrich, St. Louis, MO) that had pH = 7.2 and a conductivity of 500 $\mu\text{S}/\text{cm}$. A single electrical field pulse of 1 V/mm and duration of either 1 or 90 s was applied. Control (non-stimulated) EBs were subjected to the same procedure, except for electrical stimulation. The EBs were cultured freely suspended for three days and then transferred to adhesion culture until day 19. Media was replaced daily. Cell viability following electrical stimulation was verified after 2 hours from a single pulse and after 4 days of continuous stimulation (1 V/mm for 5 ms, at 1 Hz).

Stimulation with H_2O_2

In order to determine the amount of H_2O_2 necessary for intracellular ROS generation comparable to that resulting from electrical stimulation, 4- and 8-days old EBs were incubated for 15 minutes with 0.1, 1 and 10 nM H_2O_2 (Sigma Aldrich, St. Louis MO).

Characterization of the electrode-electrolyte system

The electrode/electrolyte interface was characterized as in our previous studies [26,30-32] through electrochemical impedance spectroscopy (EIS) and charge injection measurements for different electrode materials. Electrodes (10 cm length $\times$ 1.3 mm diameter) were fabricated from 304 stainless steel and titanium rods (McMaster-Carr, Atlanta, GA). Some of these titanium rods were sent to Eclat Industries Inc. (Levittown, NY) where they were coated with titanium nitride.

EIS measurements were performed with an electrochemical interface (Solartron 1287) and a frequency response analyzer (FRA, Solartron 1250) controlled by a computer with ZPlot software (Solartron Analytical, Oak Ridge, TN). EIS spectra were acquired for each electrode (in pulsing buffer) over a frequency range from 1×10^{-2} Hz to 1×10^6 , with perturbation amplitudes (input voltages) of 10, 100, and 1,000 mV. Collected data were evaluated in ZView 2.5b software to generate Nyquist and Bode plots, and from these, data were fit to a simple “Randles' cell” equivalent circuit (Figure 2A) using instant fit functions.

The current profile in the system during the 90 s stimulation was measured by the potential drop profile across a 330 ohm resistor placed in series in the stimulation loop. The current value was calculated by dividing the potential measured across the resistor (with an oscilloscope) by the value of the resistor. Since the current across the system was low enough to reach the 10 mV lower limit of the oscilloscope, at least 11 measurements were taken for each material.

Cell Viability

Cell viability in EBs was determined by a fluorescence based live/dead assay (Molecular Probes, Eugene), which detects live and dead cells using 3 μM calcein-AM and 3 μM ethidium homodimer-1 (EthD-1) dye. The calcein-AM is retained within live cells and converted enzymatically into green fluorescent calcein (excitation at ~ 495 nm, emission at ~ 515 nm).

EthD-1 enters all cells with damaged membranes where it binds to nucleic acids, thereby producing a bright red fluorescence in dead cells (excitation at ~495 nm, emission at ~635 nm). Before and after staining, the EBs were washed with DPBS, to minimize the esterase activity due to serum supplemented to growth medium, and to remove excess dyes. Cells were incubated in 250 μ L of the combined solution of calcein-AM and ethidium, for 45 minutes at room temperature. The labeled cells were analyzed using an inverted fluorescence microscope (Zeiss).

Generation of Reactive Oxygen Species

Generation of intracellular reactive oxygen species (ROS) was measured using the fluorescent dye dichlorofluorescein diacetate (DCFH-DA) (Sigma Aldrich, St. Louis, MO). DCFH-DA is a nonpolar and nonfluorescent compound that can diffuse into the cell where it is deacetylated by cellular esterases into a nonfluorescent polar derivative 2',7'-dichlorofluorescein (DCFH) that is impermeable to cell membrane. DCFH is rapidly oxidized to the highly fluorescent dichlorofluorescein (DCF) in the presence of intracellular ROS.

Embryoid bodies were incubated for 30 min in knockout medium containing 20 μ M DCFH-DA, in dark, at room temperature. EBs were then washed twice with pulsing buffer and transferred into the bioreactor. Fluorescence images (corrected for background fluorescence) were taken with excitation wavelength of 488 nm and emission wavelength of 530 nm. For the validation of the ROS analysis, images were taken every 20 s for three minutes, and then every minute for the next 7 minutes.

The increase in fluorescence of stimulated and non-stimulated (control) EBs was calculated by the normalization of fluorescence intensity at each time point after stimulation (F) to the initial value (F_0). The increase in fluorescence due to electrical stimulation was calculated by subtracting the increase in fluorescence measured for a control EB $[(F/F_0)_K]$ from the increase in fluorescence measured for a stimulated EB $[(F/F_0)_{ES}]$. During the validation, we observed that the maximum in ROS generation was reached at 5 minutes. For this reason and with the aim of reducing the fluorescence due to DCF auto-oxidation, during data acquisition images were taken only at time 0 (F_0 , immediately before the onset of electrical stimulation) and after 5 minutes (F, maximum value). The fluorescence variation was calculated by normalization of fluorescence intensity at each time point (F/F_0). In the case of H_2O_2 stimulation, the fluorescence variation was calculated in exactly the same way as for electrical stimulation.

To validate the ROS acquisition methodology using an inverted microscope, we performed a set of experiments using a confocal microscope, using the exact same methodology of electrical stimulation, data acquisition and analysis. For this purpose, 4-days old EBs were stimulated using stainless steel electrodes. An inverted confocal laser scanning microscope (Leica) and a 20 $\times$ objective were used. Fluorescence excitation was performed using the 488-nm line of the argon ion laser. Full frame images (512 $\times$ 512 pixels) were taken at a 400 Hz frequency. All subsequent analysis was done as in the inverted microscope studies.

Expression of cardiac markers

EBs were cultured for 19 days and then fixed with 2% PFA for 7 minutes. Cardiac Troponin T primary antibody (mouse monoclonal, NeoMarkers, USA) was diluted 1 : 100 in PBS/3% BSA (Sigma-Aldrich, USA) and applied for 1 hour at 37°C. Secondary antibody, Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen, Italy) was diluted 1 : 250 in PBS/3% BSA and applied for 45 minutes at 37°C. Cells were counterstained with DAPI and mounted in fluorescent mounting medium (DakoCytomation, Italy).

Statistical Analysis

Significance of each data point was determined by ANOVA and the Tukey's post hoc test, with $p < 0.05$ considered statistically significant.

Results

Bioreactor characterization

Electrochemical impedance spectroscopy (EIS) data were used to construct an equivalent circuit of the electrical stimulation system, describing the electrical behavior, as the elements in the model have a basis in the physical electrochemistry. The bioreactor system was modeled as a “Randles cell” (Figure 2A), to calculate values for R_e , representing the resistance of the electrolyte solution, R_p representing the polarization resistance (or resistance to corrosion), and CPE (constant-phase element) corresponding to a non-ideal double layer-capacitance (with coefficient of ideality η ranging from 0 to 1, where 1 is considered ideal).

Because the electrical response of electrodes in electrolyte solutions can be highly non-linear (depending on geometry, electrolyte composition, electrode composition and surface properties), in EIS measurements, an AC potential is applied to the electrochemical system and its current is measured for a range of frequencies [20]. The amplitude (perturbation voltage) of this signal may also be varied to compare the electrode responses [21]. Figure 2B shows Nyquist plots (real versus imaginary parts of impedance) for stainless steel electrodes at increasing perturbation voltages. With increasing input voltage, the slope of the Nyquist plot decreases, suggesting lower resistance of the system to electrode corrosion. From Nyquist plots, electrical circuit parameters were determined for different electrode configurations. Table 1 lists the equivalent circuit parameters (corresponding to Figure 2A) of the electrode materials utilized in this study for a range of perturbation voltages.

All tested electrodes showed interfacial capacitance at the order of 10^{-6} F/cm², whereas the polarization resistance (R_p) for titanium and titanium nitride electrodes was an order of magnitude higher than that for stainless steel electrodes. For stainless steel, by increasing the amplitude of the stimulus up to 1 V, the capacitance increases whereas the resistance to the reactions becomes up to two orders of magnitude lower. These results are reflected with the changes of the Nyquist plot shape that becomes more curved (Figure 2B).

The current-time plots show an initial increase in the current (and thus the potential applied to the cells) followed by a rapid decrease due to the polarization of the medium and the shielding of charges on the electrode (Figure 2C). However the current reaches values close to zero only for titanium and titanium nitride electrodes, whereas for stainless steel electrodes the reactions that occur in the electrode-electrolyte interface keep the potential increasing to saturation by 20 s. Not surprisingly, stainless steel electrodes deliver much more total charge during the 90 s pulse than the other electrode materials: the amount of charge integrated over the 90 s stimulus was 35 mC for stainless steel, 2 mC for titanium nitride and 1 mC for titanium electrodes. As a consequence, only the stainless steel electrodes maintained constant electric field over the time of stimulation. Most of the comparative and verification data in this study are shown for stainless steel electrodes.

ROS expression

We first verified that the exogenous electrical stimulation does not compromise the viability of the EBs, by a live and dead assay performed 2 hours after a single pulse (Figure 3A), and then again 4 days after electrical stimulation (Figure 3C). The presence of dead cells was negligible in both stimulated and unstimulated EBs (Figure 3A-C).

Figures 3D and E show typical DCF fluorescence images of an EB before (Figure 3D) and after (Figure 3E) the application of exogenous electrical stimulation, for 90 s, using stainless steel electrodes. Figure 3F shows a typical time course of ROS evolution, measured as the difference in intensity of fluorescence between electrically stimulated and unstimulated EBs. The maximum difference was observed 5 minutes after the initiation of electrical stimulation.

The acquisition fluorescence needed to be optimized because, even though DCFH-DA has traditionally been used for detecting ROS, it is a highly photosensitive dye which tends to auto-oxidize and produce large background fluorescence [22]. For this reason, the measurements of fluorescence were systematically compared to unstimulated EBs. The DCF intensity analyses were performed at the onset of electrical stimulation (F_0) and 5 minutes later when a maximum was observed (F), and the data were expressed as the F/F_0 ratio. Figure 3G shows that the ROS generation was significantly higher in electrically stimulated 4- and 8-day-old EBs than in the corresponding unstimulated EBs.

These results were verified using a confocal microscope (Supplemental Fig. 1). A comparable increase in ROS generation (Supplemental Fig. 1D), due to electrical stimulation, was observed by confocal imaging, thus confirming the results obtained with the inverted microscope.

Significant changes in ROS expression were observed when the duration of electrical stimulation was increased from 1 s to 90 s (Figure 4A). All electrodes induced a significant increase in ROS production after electrical stimulation, if compared to unstimulated (control) EBs, at both stimulation regimes (1 and 90 s). Stainless steel electrodes (but not the titanium and titanium nitride electrodes) resulted in a statistically significant increase in ROS production when a stimulus of 90 s duration was applied as compared to the 1 s stimulus (Figure 4A). Also, the ratio F/F_0 significantly increased for each 1 s and 90 s stimulation from unstimulated control to titanium, titanium nitride and stainless steel electrode groups. This observation is consistent with the analysis of charge injection due to Faradaic reactions at the stainless steel electrodes, shown previously, which provide a constant electric field for all 90 s of stimulation (Figure 2C).

Since human embryonic stem cells have been shown to begin differentiation toward cardiac lineage 4 days after EBs formation [23] and their reactivity to exogenous electric fields can be different at different stages of development, it seemed interesting to analyze the ROS generation in EBs at various developmental stages (4 days and 8 days). As shown in Figure 4B, the ROS production increased after electrical stimulation for both groups of EBs, and all types of electrodes. The same trend was observed for 6-day old EBs (Figure 4A). At day 8, electrical stimulation generated a lower increase in ROS, when compared to 4-days old EBs, or 6-days old EBs in Figure 4A, suggesting that the effects of electrical stimulation are stronger at earlier developmental stages.

The effects of electrical stimulation were compared to the effects of a molecular agent, H_2O_2 , in an attempt to establish correlations between the two stimuli of ROS production. EBs at two different developmental stages (4 days and 8 days) were stimulated with a range of concentrations of H_2O_2 . These results are reported in Figure 5, as differences in F/F_0 between the stimulated and unstimulated EBs for electrically stimulated EBs (stainless steel electrodes, 90 s signal duration), and EBs stimulated by H_2O_2 (concentrations of 0.1 nM, 1 nM, 10 nM; incubation time: 15 min). Figure 5 shows that the production of ROS increased with an increase in H_2O_2 concentration, and that the increase in F/F_0 in response to 90 s of electrical stimulation was equivalent to that in response to 15 min of incubation with 1 nM H_2O_2 . Interestingly, the highest rates of ROS generation, observed following incubation in 10 nM H_2O_2 , were also different for 4-days old EBs and 8-days old EBs. This result, is consistent with that observed

for electrically stimulated EBs (Figure 4) and suggests the role of the developmental stage of EBs in the magnitude of their responses to the induction of ROS production.

Cardiac differentiation

To evaluate whether electrical field and intracellular ROS expression affect cardiac differentiation of hESC, EBs of two different developmental stages (4 days and 8 days) were analyzed for up to 19 days of culture for spontaneous contractions and cardiac Troponin T expression. After 3 days in culture, embryoid bodies started to beat spontaneously in the untreated samples (4-days old: 14%; 8-days old: 6%) and electrically stimulated EB (TiN, 4-days old: 11%; TiN, 8-days old: 13%; SS, 4-days old: 3%; SS, 8-days old: 17%). The expression of cardiac Troponin T was observed in beating EBs (Fig. 6 A-C). The sarcomeric organization of the cells, an ultrastructural marker of cardiac differentiation, was also verified (Fig. 6C).

Discussion

We show that the applied electric field leads to generation of intracellular endogenous ROS in EBs derived from human embryonic stem cells, a result consistent with the report of Sauer for murine embryonic stem cells [10]. The application of an exogenous electrical field on hESC-derived EBs resulted in a significant increase in intracellular generation of ROS. The highest production was obtained for the stimulus of 90 s duration (as compared to 1 s duration stimulus), stainless steel electrodes (as compared to titanium and titanium nitride electrodes) and 4-day old EBs. ROS production under these conditions was comparable to that observed after 15 min incubation of EBs in 1 nM H₂O₂. Cardiac differentiation of human EBs was verified by the detection of cardiac Troponin T, the presence of sarcomeric structure and spontaneous contractions.

Strong influence of electrode material was observed in the generation of intracellular ROS. To rationalize this effect, the electrochemical properties of the system were studied in detail, to properly relate the biological outcome to exogenous electrical stimulation. Because the charge-transfer at the electrode-electrolyte interface occurs through three mechanisms: (i) non-Faradaic charging/discharging of the electrochemical double layer, (ii) reversible Faradaic reactions, and (iii) non-reversible Faradaic reactions [25,26], we used electrochemical impedance spectroscopy (EIS) to determine the relative presence of each mechanism. The values of capacitance and resistance to reactions were measured for each type of electrode - stainless steel, titanium and titanium nitride. The polarization resistance (R_p), which corresponds to the resistance of the electrode to chemical reaction, was lowest for stainless steel, consistent with a steady-state current of 0.4 mA after ~20 ms maintained by galvanic corrosion. For titanium and titanium nitride electrodes, an initial increase of the current was followed by a rapid decrease due to the polarization of the medium and the shielding of charges on the electrode. Taken together, the analysis of the electrochemical properties of the three electrode systems confirms the importance of the charge transfer mechanism between the electrode and the solution on the biological response of electrical stimulation. The EIS results were useful to correlate the duration of electrical stimulation with ROS expression on human EBs. The analysis showed that titanium or titanium nitride electrodes do not maintain an electrical stimulus of 90 s duration, due to their high resistance to Faradaic reactions (galvanic corrosion).

In principle, Faradic reactions at the electrodes could result in the production of electrochemical species, such as H₂O₂, that can diffuse to the EB through the pulsing buffer. This effects is however not consistent with our experimental conditions. In particular, the characteristic time, τ , of H₂O₂ diffusion from the electrode to the EB ($\tau = L^2/D$, where $L = 2 \times 10^{-3}$ m is the electrode-EB distance and $D = 1.3 \times 10^{-5}$ m²/s is the H₂O₂ diffusion coefficient, [27]) is estimated to 10³ s. This time is two orders of magnitude larger than the duration of ROS measurements, as

compared to data in Figure 3F, where ROS generation starts only few seconds after the application of electrical stimulation. These considerations were confirmed by experimental data for 4-days old EBs stimulated by adding a pulsing buffer solution that was previously exposed to the electrical stimulation (stainless steel, 1 V/mm, 90 s). ROS detection did not show any increase in fluorescence for 10 minutes (data not shown). These results confirm that the ROS signal is due to the electrical stimulation and it is not caused by electrochemical species generated at electrodes.

Relatively short pulses (1 ms range) are more commonly used, in particular in membrane depolarization studies, and during that same time frame most common electrodes are subject to polarization. Because polarization “filters out” the exogenous electrical fields, the EBs would normally not experience electrical stimulation beyond this initial period of time. However, the polarization of the stainless steel electrodes remains incomplete, as clearly shown in electrode characterization experiments. When SS electrodes are used, an electrical field is constantly generated throughout the 90 seconds of electrical stimulation, which was the reason for investigating these long periods of stimulation (in addition to 1 s). Previous work [10] also demonstrated beneficial effects of stimulation of mESCs, during the 90 s time period.

Overall, EIS measurements show that stainless steel (a material with the lowest corrosion resistance) was the only electrode material providing electrical stimuli longer than ~1 second. Given the ability of stainless steel to provide steady-state current, we were not surprised to observe that lengthening the stimulation duration to 90 s enhanced the intracellular ROS expression only in EB stimulated with stainless steel electrodes.

Because Sauer and colleagues demonstrated that the endogenous generation of ROS in mESC-derived EB reached a maximum 2-3 days after EB generation and observed a significant decay in ROS production with increased cultivation time [19], we investigated the response of human embryonic stem cells at different time points. When we stimulated human EBs of different stages of development (4- and 8-days old), we observed a maximum expression of ROS in 4-days old EBs. The decay of ROS generation may be correlated to the down-regulation of NADPH oxidase activity during the development, as previously shown for mESC [19]. The active role of NADPH oxidase in differentiation, shown for several cell types [28], suggests higher predisposition of the hESC to cardiac differentiation and a more efficacious electrical stimulation at earlier stage of development.

Increased ROS production after electrical stimulation at 1 V/mm for 90 s was comparable to that following chemical stimulation with 1nM of H₂O₂. These results suggest a possible downstream effect of electrical stimulation resulting in the production of intracellular endogenous ROS and the activation of ROS-activated signaling pathway, relevant for cardiac differentiation of hESC-derived EB [29]. It is plausible that the application of electrical stimulation can enhance the number of cardiac-differentiated human embryonic stem cells through mechanisms involving ROS generation.

Recent studies suggest that control of ROS may be essential for regulation of stem cell, and that ROS signaling participates in regulation of cell proliferation, DNA damage, and cell aging. However, the mechanisms of ROS generation (either by molecular or electrical factors) are not well defined and the underlying signaling pathways remain to be mapped.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge funding by NIH (P41-EB002520 to GVN), Regione Veneto, “Azione Biotech II” and Programma Operativo F.S.E. Asse “Capitale Umano”, and Italian Ministry of University and Research (MIUR). We thank Keith Yeager (Columbia U) for his expert help with bioreactor design and Andrea Legati (Padova U) for help with experimental work.

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Figure 1. Bioreactor for electrical stimulation

Schematic representation of the mold (A) and the PDMS bioreactor (B); C Image of the bioreactor; D Schematic representation of a single chamber of stimulation (25 mm²). The electrodes are 5 mm spaced and the EBs are placed in the gray area (1 mm²).

Figure 2. Electrical properties of the electrode-electrolyte system

A Equivalent circuit of the electrode/electrolyte systems, where R_e is the resistance of the electrolyte. **B** Nyquist plot of stainless steel electrodes at increasing perturbation voltage. **C** Effect of electrode material on effectiveness of electrical stimulation (1 V/mm, 90 s). Current vs time profile in the system during stimulation with stainless steel, titanium nitride and titanium electrodes.

Figure 3. Effect of electrical stimulation on cell viability and reactive oxygen species (ROS) generation

A-C: Live/Dead assay of EB. **A** EB in suspension culture 2 hours after a single pulse of electrical stimulation; **B** non stimulated adherent EBs; **C** adherent EBs after 4 days of continuous stimulation. **D-G:** Analysis of intracellular ROS generation through DCFH-DA. **D-E:** Fluorescence image of EB loaded with dichlorofluorescein (DCF) before (**D**) and 20 minutes after (**E**) electrical stimulations (90 s, SS). **F** An example of time course of ROS generation (DCF intensity). The graph reports the point difference of normalized fluorescence between a stimulated EB (ES) and a control EB (Ctrl). The electric field (EF) was applied for the first 90 seconds. **G** ROS generation on control (Ctrl) EB and electrically stimulated EB (ES) (90 s, SS), 4- and 8-days old. The fluorescence intensity 5 minutes after electrical stimulation (F) was normalized by the intensity at time 0 (F_0).

Figure 4. Generation of ROS in EB after electrical stimulation with different electrode materials
A Effect of duration of electrical stimulation on ROS production on 6-daysold EB; **B** Effect of EBs age on ROS generation. About 10 EBs in each of three independent experiments were used for the determination of each data point. * indicates $p<0.05$; ** indicates $p<0.01$; the difference between groups of same electrode material and different EB age is significant for all the groups. No significant differences are observed in EB at the same developmental stage (4- or 8-days old) when stimulated with different electrodes material; while EB at different developmental stage stimulated with the same material are significantly different, with $p<0.05$.

Figure 5. ROS generation in EBs in response to electrical stimulation (ES) and treatment with peroxide (H₂O₂)

Data are shown for 4-days old EBs (open bars) and 8-days old EBs (filled bars) after electrical field (ES) and H₂O₂ treatment. H₂O₂ EBs were incubated for 15 min with increasing concentrations of H₂O₂: 0.1 nM, 1 nM and 10 nM. About 4 EBs in each of three independent experiments were used for the determination of each data point. ** indicates $p < 0.01$.

Figure 6. Effect of electrical stimulation on cardiomyocyte differentiation

Cardiac Troponin T immunostaining of electrical field-treated representative EB. Nuclei were counterstained with DAPI. Scale bars: 75 μm in **A** and **B**, 30 μm in **C**.

Table 1
Electrical properties of the electrode/electrolyte system

Constant phase element (CPE), polarization resistance (Rp) and capacitance dispersion (η) calculated fitting the equivalent circuit of the electrode/electrolyte systems (please also see Fig 2.A).

Material	Perturbation Voltage	CPE, F/cm ² (% error)	Rp, Ω /cm ² (% error)	η (% error)
Stainless Steel	10mV	3.17E-06 $\pm$ 8.88E-09	1.68E+06 $\pm$ 4.20E+04	0.71 $\pm$ 5.04E-03
	100mV	6.28E-06 $\pm$ 5.08E-08	8.76E+05 $\pm$ 8.76E+04	0.66 $\pm$ 2.38E-03
	1000mV	3.02E-05 $\pm$ 1.39E-06	1.87E+04 $\pm$ 1.92E+03	0.53 $\pm$ 1.78E-02
Titanium nitride	10mV	3.27E-06 $\pm$ 2.06E-08	6.86E+06 $\pm$ 6.86E+05	0.77 $\pm$ 1.43E-02
	100mV	4.51E-06 $\pm$ 2.89E-08	3.18E+06 $\pm$ 5.84E+05	0.76 $\pm$ 2.20E-03
	1000mV	1.23E-05 $\pm$ 1.28E-07	1.78E+05 $\pm$ 9.66E+03	0.73 $\pm$ 4.16E-03
Titanium	10mV	1.61E-06 $\pm$ 8.51E-09	4.69E+06 $\pm$ 3.41E+05	0.80 $\pm$ 5.21E-03
	100mV	1.60E-06 $\pm$ 1.06E-08	4.05E+06 $\pm$ 2.95E+05	0.79 $\pm$ 2.29E-03
	1000mV	3.02E-05 $\pm$ 2.82E-08	1.08E+06 $\pm$ 7.69E+04	0.53 $\pm$ 2.26E-03