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Current-Controlled Electrical Point-Source Stimulation of Embryonic Stem Cells

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Abstract

Stem cell therapy is emerging as a promising clinical approach for myocardial repair. However, the interactions between the graft and host, resulting in inconsistent levels of integration, remain largely unknown. In particular, the influence of electrical activity of the surrounding host tissue on graft differentiation and integration is poorly understood. In order to study this influence under controlled conditions, an *in vitro* system was developed. Electrical pacing of differentiating murine embryonic stem (ES) cells was performed at physiologically relevant levels through direct contact with microelectrodes, simulating the local activation resulting from contact with surrounding electroactive tissue. Cells stimulated with a charged balanced voltage-controlled current source for up to 4 days were analyzed for cardiac and ES cell gene expression using real-time PCR, immunofluorescent imaging, and genome microarray analysis. Results varied between ES cells from three progressive differentiation stages and stimulation amplitudes (nine conditions), indicating a high sensitivity to electrical pacing. Conditions that maximally encouraged cardiomyocyte differentiation were found with Day 7 EBs stimulated at 30 μ A. The resulting gene expression included a sixfold increase in troponin-T and a twofold increase in β -MHC without increasing ES cell proliferation marker Nanog. Subsequent genome microarray

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ELECTRONIC SUPPLEMENTARY MATERIAL

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analysis revealed broad transcriptome changes after pacing. Concurrent to upregulation of mature gene programs including cardiovascular, neurological, and musculoskeletal systems is the apparent downregulation of important self-renewal and pluripotency genes. Overall, a robust system capable of long-term stimulation of ES cells is demonstrated, and specific conditions are outlined that most encourage cardiomyocyte differentiation.

Keywords

Electrical stimulation; Embryonic stem cells; Cellular therapy; Cell differentiation; Cardiomyocytes

INTRODUCTION

The limited ability of the human heart to regenerate has made heart failure a devastating condition. Recently, an approach using pluripotent stem cells as a means to aid intrinsic repair mechanisms in damaged heart tissue is rapidly being explored. Some evidence suggests that it is possible for stem cell grafts to be both viable as well as physiologically functional.^{8,12} However, the lack of understanding of the differentiation and integration process, notably with respect to electrical signaling, significantly hampers the development of therapies. The mechanisms through which the graft contributes to increased heart function remain unclear, and may include differentiation into cardiomyocytes, angiogenesis, cell fusion, or paracrine effects. Poor control on the integration mechanisms and fate of implanted cells can also lead to serious complications such as teratoma formation^{4,23,26} or induced arrhythmia.⁵ The integration process is thus a very complicated one, where not only the graft influences the host, but the host influences the graft. This is particularly important for stem cell grafts, where many factors can influence their differentiation, one of which being the poorly understood concept of electrical stimulation that stem cell grafts are subjected to from endogenous *in vivo* pacing.

It is well known that during fetal development, electrical signals are present and may help guide stem cells toward a cardiomyocyte cell fate.²² Similarly, it is reasonable to hypothesize that comparable stimuli may play a role in the differentiation and integration of stem cells introduced into host cardiac tissue. In addition to providing further insight into the role of endogenous electric signals from native tissue, this may also provide a novel way to control the differentiation and integration of implanted cells.

Previous work has demonstrated the importance of electrical factors. It has been shown that electrical field stimulation over an eight-day period increased the amplitude of synchronous contractions in a tissue construct of cardiac cells.²⁰ Stimulation appears to be helpful in establishing physiological structure and function, as shown by the presence of striations, gap junctions, and cell coupling. With murine ES cells, the application of a DC electric field for 90 s over an embryoid body (EB) has in certain cases doubled the yield of beating EBs.²²

However, cells in the myocardium are depolarized by local currents, propagating in a wave-like pattern, and not by synchronous field stimulation. Such techniques would thus not adequately mimic the electrical micro-environment that stem cells may be subjected to in a graft. An alternative method is a local “point-source” stimulation approach using controlled current, as described in this study. By using a current source, stimulation thresholds stay relatively constant even with drifting electrode impedance.¹⁵ Voltage drop across the load can readily be measured to ensure that voltages stay within a safe margin, thus preventing electrode corrosion and water electrolysis.⁷ In addition, the waveform of the input current can be configured such that the first phase of a stimulus is entirely recovered during a

second phase with an opposite polarity, and hence “charge balanced.” It is understood that charge-balanced waveforms would be adequate for minimizing electrochemical reactions.⁷

In the case of this study, an active system was created that uses a voltage waveform and passes it through to a voltage-controlled current source. Creating a voltage waveform is typically not difficult with a waveform generator or a programmable microcontroller, but the current source is critical because non-linearities can lead to an imbalance in the waveform. Over time, a nonbalanced waveform can build up charges at the interface and push the electrode voltage outside the safe region of operation.

The interface of the system relies on the use of planar microelectrode array technology (MEA) with integrated recording and stimulation electrodes.²⁵ The stimulation electrodes provide localized current injection into the cell culture, and more closely reproduces the boundary conditions of the graft, where only the peripheral cells in contact with parts of the healthy myocardium would be subjected to local currents and their resulting fields. In addition, electrical stimulation over a limited area helps determine the importance of being physically coupled to an electrically active substrate in the differentiation process. The recording electrodes allow the detection of nascent depolarization in differentiating cells and conduction properties of the differentiated tissue.

This study demonstrates the use of this platform to examine the role of localized current injection, providing insight into the response of ES cells to electrical stimulation. In simulating the electrical environment that implanted stem cells are exposed to, the extent of electrically driven cardiac differentiation was analyzed through real time PCR, immunofluorescence, and genome microarray analysis. A range of responses based on the differentiation stage and stimulus amplitude were found, which demonstrated the sensitivity of ES cells to electrical stimulation, but also revealed certain conditions that optimally increased cardiac differentiation without increasing ES cell proliferation.

MATERIALS AND METHODS

Electrical Stimulation

The MEAs used for this study contain stimulation electrodes symmetrically arranged across the surface with varying surface geometries (see Fig. 1), and have been previously described by Whittington *et al.*²⁵ All of the outer stimulation electrodes were connected in parallel and then used to stimulate the cells with the same signal. An MSP430 microcontroller (EZ430-T2012; Texas Instruments, Dallas, TX) was used to control a 16-bit bipolar digital-to-analog converter (DAC), which drove a modified Howland voltage-controlled current source (see Fig. 2).

The desired waveform, rate, duration, and pulse amplitude were programmed for continuous pacing.⁶ The type of stimulation applied was an anodic-first biphasic waveform with 10 ms duration, and with the load actively grounded in between pulses to clear away residual charges. The amplitude of the applied current was varied depending on the experiment between 10, 30, and 60 μA (8.3, 24.9, and 49.8 $\mu\text{A}/\text{mm}^2$, respectively). These values were chosen to be of a similar order of magnitude to transmembrane currents estimated through a cell during a physiological action potential.¹⁴ A high-side current sense was also placed over the load to verify appropriate current waveforms and amplitudes. The pulse frequency was found to play no significant influence on the gene expression when applied at 1, 2, or 4 Hz (60, 120, and 360 pulses per minute, respectively), and the system was set to 1 Hz for all presented data.

In order to prevent hydrolysis at the electrodes, the maximum total current across the electrodes was kept under 65 μ A, corresponding to a voltage under 0.9 V.²¹ This was monitored with a high-side current sense circuit to ensure compliance. In each culture, a platinum wire was placed in the medium bath to serve as a return path for the injected current. Control samples were placed under the same conditions, but with no electrical stimulation applied.

Cell Culture

Murine ES-D3 cell line (CRL-1934) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). The ES cells were cultured to keep them in an undifferentiated, pluripotent state using 1000 IU leukemia inhibitory factor (LIF; Millipore, Billerica, MA), and grown over a layer of murine embryonic fibroblast feeder cells. The fibroblasts were inactivated using 10 μ g/mL mitomycin C (Sigma–Aldrich, St. Louis, MO). The surfaces of the culture dishes were coated with 0.1% gelatin. Cells were cultured in ES medium containing Dulbecco's Modified Eagle Medium supplemented with 15% fetal calf serum, 0.1 mmol/L β -mercaptoethanol, 2 mmol/L glutamine, and 0.1 mmol/L nonessential amino acids as described previously.² The culture medium for the ES cells was changed on a daily basis, and cultures were passaged every one to two days. To develop the ES cells into embryoid bodies, the “hanging drop” method was used as described previously.¹⁷ LIF was withdrawn from the medium, and a cultivation of about 400 cells was suspended in 18 μ L hanging drops to form an aggregate of cells termed embryoid bodies (EB). At this point, the differentiation stage of the EB was noted as Day 0. After 2 days, each EB was transferred into its own well in an ultra-low attachment 96-well plate (Corning Life Sciences, Lowell, MA) for 2 days, after which they were further seeded onto 48-well plates. At the desired differentiation stage, whole EBs were dissociated with collagenase B (Worthington, Lakewood, NJ) and cells were plated onto the MEA surface at a density of 32,000 cells/cm² (80 K total cells). EBs were dissociated before plating for two reasons. First, it ensured that an evenly distributed layer of cells were attached to the electrode surface prior to the experiment, which was especially helpful in controlling variability between samples as well. Second, the process of dissociating EBs was done to mimic the approach used in *in vivo* transplantation, as described by Cao *et al.*⁴ At least 2 h prior to plating, the MEA surface was coated with hESC-qualified matrix Matrigel™ (BD, Franklin Lakes, NJ). The cells were allowed to settle between 20 and 30 h before electrical stimulation was applied for 4 days. The medium was replaced every 2 days. Stimulation was then applied at 10, 30, and 60 μ A for 4 days in order that the end of the stimulation period corresponded to three differentiation stages, described as Early (Days 3–9), Intermediate (Days 9–17), and Terminal (Days 17 and on). During the Early Stage, undifferentiated ES cells are destabilized and begin differentiating. The Intermediate Stage sees a modulation of cell fate direction, and Terminal Stage cells have differentiated to a mature cell state.^{2,27}

Real-Time PCR

Gene expression analysis was performed at the end of the stimulation period for cardiac markers (β -MHC and troponin-T)² and embryonic stem cell markers (nanog).¹ After 4 days of continuous stimulation, cells were harvested from the MEA using trypsin–EDTA (Invitrogen, Carlsbad, CA). The RNA was then extracted and prepared for reverse-transcriptase (RT) PCR. Gene expression of the stimulated stem cells were quantitatively measured using real-time PCR (7900 HT; Applied Biosystems, Foster City, CA). The four probes of interest were the cardiac cell markers β -MHC and troponin-T, the ES cell marker nanog, and gapdh, a housekeeping gene for control (Applied Biosystems, Foster City, CA.). β -MHC, a ventricular specific protein, is typically regarded as an early stage marker while Troponin-T is essential for cardiac contraction and is regarded as a late stage marker.² The

numbers reported for the real-time PCR are the ratio of the relative expression of the gene of interest divided by the expression of *gapdh*, and then normalized to the control samples.

Immunofluorescence

Cardiomyocyte differentiation was verified using immunofluorescence. Cells on MEA surfaces were fixed with 4% paraformaldehyde, permeabilized by 0.1% Triton X-100, and stored at 4 °C with primary antibodies overnight followed by 30 min with secondary antibody incubation. The primary antibodies selected in this study were goat anti-Troponin-T. Secondary antibodies were FITC conjugated rabbit antigoat IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Nuclei were stained by propidium iodide or DAPI (Sigma–Aldrich Inc, Milwaukee, WI).

Genome Microarray: Labeling Reaction, Hybridization, and Data Acquisition

All sample processing for microarray analysis was performed at the same time to negate potential technical variability. Using Low RNA Input Fluorescent Linear Amplification Kits (Agilent Technologies, Santa Clara, CA), cDNA was reverse transcribed from each RNA sample ($N = 8$ per group), and cRNA was then transcribed and fluorescently labeled from each cDNA sample. cRNA samples derived from four biological replicates of a pooled collection of electrically stimulated ES cells, as well as a pooled reference of RNA taken from control (non-stimulated) ES cells, were labeled with Cy5 and Cy3, respectively. The resulting cRNA was purified using an RNeasy kit (Qiagen, Valencia, CA) followed by quantification of the cRNA by spectroscopy using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). A mixture of 825 ng of Cy3- and Cy5-labeled and amplified cRNA was fragmented according to the Agilent technology protocol. cRNA was hybridized to 4×44K whole mouse genome microarray slides from Agilent according to the manufacturer's instructions. The hybridization was carried out in a rotating hybridization chamber in the dark at 65 °C for 17 h. The array was scanned using an Agilent G2505B DNA microarray scanner. The image files were extracted using Agilent Feature Extraction software version 9.5.1 applying LOWESS background subtraction and dye normalization.

Genome Microarray: Data Analysis

Microarray data analysis was performed using GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA). Genes were considered significant if the fold change was greater or less than 1.4 and had a P -value of less than 0.05. Because of the small changes in overall transcription between the two groups, multiple testing corrections to remove false positives in the resulting data were not possible. Further analysis was performed using GeneSpring's Gene Ontology browser and Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

Statistics

All data is presented as mean $\pm$ standard error of the mean (SEM). For the gene expression studies, each experiment consisted of six to eight stimulated samples, and six to eight control samples of a particular differentiation stage, and at a certain amplitude (nine total experiments, with an equal number of samples per group each time). After each experiment, the gene expression for the three genes was examined as a differential measurement between stimulated and non-stimulated samples. A Student's t -test was performed between these two groups, where significance was defined at $P < 0.05$.

RESULTS

Current-Controlled Electrical Stimulation

The two operational amplifier configuration used for the voltage-controller current source (see Fig. 2) fixes a user-defined voltage across a resistor in series with the load, ensuring precise and accurate current control. The configuration as well as choice of components allowed a slew rate of $2.96 \mu\text{A}/\mu\text{s}$, and an SNR of 59.4 dB with a $60 \mu\text{A}$ sine wave output over a $100 \text{ k}\Omega$ load.

When used for electrical point-source stimulation through a parallel set of microelectrodes, analysis of the individual electrode impedance values indicated that the current density through each electrode did not vary by more than about $2.6 \mu\text{A}/\text{mm}^2$. The system was capable of successfully pacing ES cells over long periods of time without increased cell death. After 4 days of continuous pacing, the number of cells in both stimulated and non-stimulated samples increased by about a factor of five, and appeared healthy under a microscope. A more revealing measurement may include quantification of the cells within the vicinity of the MEA, but simultaneous measurements of that with real-time PCR was unable to be preformed. The slew rate of $2.96 \mu\text{A}/\mu\text{s}$ was also more than adequate to ensure a proper biphasic waveform, as confirmed by the high-side current sense circuit. After 4 days of continuous stimulation, stimulation electrodes did not display signs of corrosion or other structural damage upon visual inspection with a microscope. The electrode impedance and phase angle measured over a range of frequencies (100 Hz to 100 kHz) did not display any notable changes. In addition, the voltage across the load is checked frequently in all experiments to verify a consistent amplitude under the threshold described for hydrolysis.²¹ Therefore, since the total amount of charge delivered is sufficiently small, this type of stimulation allows for operation primarily as non-Faradaic redistribution of charge,¹⁸ with controlled charging and discharging of the electrical double layer over the electrode surface. Faradaic reactions should be minimized to prevent the generation of cytotoxic byproducts that would destroy cell cultures, and to prevent corrosion that would compromise the experimental setup.

Real-Time PCR Analysis

The stimulation system was used to evaluate the effect of localized electrical current on the differentiation of embryonic stem cells at three differentiation stages (see Fig. 3). ES cells stimulated at the Early Stage across three amplitudes ($N = 6-8$) did not demonstrate much response at a lower amplitude stimulus of 10 or $30 \mu\text{A}$. However, stimulating at the highest amplitude of $60 \mu\text{A}$ yielded a statistically significant ($P < 0.05$) increase in the β -MHC levels. Otherwise, no significant changes were observed with troponin-T or nanog.

Intermediate Stage ($N = 6-8$) stimulation showed a similar increase in β -MHC from the Early Stage but was observed at $30 \mu\text{A}$ instead of $60 \mu\text{A}$. Troponin-T displayed a sixfold increase in relative expression compared with nonstimulated samples. However, high amplitude stimulation at $60 \mu\text{A}$ may have had a detrimental effect on cardiac marker expression, with lower values of these cardiac markers. Interestingly, the ES cell marker nanog increased significantly at this end.

In general, Terminal Stage stimulation ($N = 6-8$) increased cardiac expression as the stimulus amplitude increased, yielding up to a significant sixfold increase in troponin-T levels when stimulated at $60 \mu\text{A}$. Here too, expression of the embryonic stem cell marker showed an increase at the highest stimulation amplitude, although not statistically significant at $P < 0.05$.

To determine if continuous pacing was necessary to produce notable results, a partial stimulation experiment was performed (see Fig. 4). Intermediate Stage ES cells were cultured and stimulated for 2 days only at 30 μ A ($N = 7$), and then left without stimulation for an additional 2 days. In this case, the β -MHC levels statistically increased up to the same level as observed for continuous 4-day stimulation, while the troponin-T appeared unchanged. The stem cell marker nanog, however, was slightly increased, although without statistical significance compared to nonstimulated control samples. The test was then reversed where Intermediate Stage ES cells were plated and the 2-day stimulation at 30 μ A was delayed for 2 days ($N = 8$). In this situation, neither β -MHC nor troponin-T levels in the stimulated group showed any notable differences from control samples. At the same time, the nanog levels exhibited an increase, but not statistically significant at $P < 0.05$.

Because point-stimulation techniques inject current into a localized area and do not stimulate the entire culture, the direct effect of physical coupling to an electrically active substrate could be measured by examining where the differentiation was taking place relative to the electrodes. It is unclear whether the observed differentiation is due to direct effects of the electrical source, or to secondary messengers that were up-regulated to elicit the differentiation through paracrine effects. Intermediate Stage ES cells were stimulated at 30 μ A for 4 days continuously, then fixed and stained for troponin-T. The resulting fluorescent images demonstrate that cardiac expression was not entirely isolated to the region around the stimulation electrodes (see Fig. 5). A separate set of stimulated ES cells were replated onto glass chamber slides and similarly stained for cardiac markers (see Supplemental Fig. 1). Immunofluorescence was analyzed by quantifying individual fluorescent pixels in Matlab (The Mathworks, Natick, MA), and normalizing it to fluorescence from a nuclei stain. On average, stimulated samples contained 3.5 ± 0.8 times the fluorescent area compared to nonstimulated samples ($N = 4$, $P < 0.05$). Further analysis of the fluorescent images showed that cells cultured directly over stimulation electrodes contained about a 25% higher concentration of troponin-T positive cells than the average concentration over the total area. Although qualitatively this is not a large difference, the consistent increase in concentration levels does indicate an effect, however small, on the spatial location of the electrodes and differentiation.

Phenotypically, cell cultures did not display significant differences in the number of spontaneously beating foci by the end of the stimulation period between stimulated and control samples, as recorded by electrodes and visual observation.

Genome Microarray

While PCR analysis of a limited number of gene transcripts in electrically stimulated ES cells is valuable, important genome-wide mRNA changes (the “transcriptome”) that define the specific regulatory networks of genes and pathways responsive to electrical stimulation will be missed. Global gene expression profiling of embryonic stem cells thus enables a systems-based analysis of the biological processes, networks, and genes that drive cell fate decisions. To understand these transcriptome changes, a genome microarray analysis of electrically stimulated Intermediate Stage ES cells at 30 μ A versus non-stimulated controls was performed. The real-time PCR results from these conditions (see Fig. 3) displayed upregulated cardiac markers with no significant change in the nanog levels.

Using a 1.4-fold change cutoff and $P < 0.05$, analysis revealed 495 upregulated genes and 729 downregulated genes in ES cells that had been electrically stimulated. Overall, the fold change in expression level of these genes was less than 3.5 compared to controls, so electrical stimulation did not dramatically alter the transcriptome. However, a number of interesting developmental-related genes exhibited altered expression, and a few are highlighted in Supplemental Table 1 (see Supplemental Text File 1 for full gene list).

Perhaps most significant is the downregulation of Oct4 (Pou5f1) and Foxd3 in electrically stimulated ES cells, with a corresponding upregulation of important differentiation and developmental-related genes such as Ncam1, Isl1, Foxc1, and Foxc2.

Genes associated with pluripotency are downregulated with electrical stimulation. ES cell self-renewal is dependent on a core set of transcription factors involved in the development of the embryo: Oct4, nanog, Sox2, and Foxd3.¹⁰⁻¹⁶ These embryonic transcriptional factors are essential for the formation and maintenance of the inner cell mass during mouse pre-implantation development and for self-renewal of pluripotent ES cells. Interestingly, two of these factors, Oct4 (Pou5f1) and Foxd3, were both downregulated 1.4-fold after electrical stimulation, indicating that important changes are occurring in the cellular pluripotency programs. A 1.7-fold downregulation of Lin28 was also observed, an important embryonic gene in mice and humans²⁸ that was recently used by Thomson *et al.* to reprogram fibroblasts into pluripotent stem cells.³¹

Mesoderm and cardiac development markers are upregulated with electrical stimulation. Mesoderm gives rise to cardiac, skeletal, and smooth muscle, as well as hematopoietic and endothelial cells. A number of early mesodermal genes were observed that were upregulated with electrical stimulation. Significantly, the mesodermal Foxc1 and Foxc2 genes, upregulated 1.6 and 1.5-fold, respectively, after electrical stimulation, are particularly important for heart development and morphogenesis. Another interesting finding was the upregulation of the transcription factor Islet1 (Isl1, up 1.5-fold), which has been reported to be a marker of cardiac progenitor cells.^{3,13} Though there is not yet total agreement in the field, it is generally accepted that Isl1, which is expressed in the secondary heart field, directly regulates cardiac precursor cells. Further, Isl1 has been shown to be expressed by progenitors of the outflow tract, right ventricle, and a majority of atrial progenitors.³ It should be noted that Isl1 is not cardiac-specific since it is also expressed in motoneurons and the pancreas during embryogenesis, as well as in normal adult islet cells,¹¹ so its initial activation and its actions on downstream targets likely require combinatorial mechanisms and influences.¹⁹

A number of studies have shown that stage-specific inhibition of canonical Wnt signaling is required for cardiac development, so the upregulation of the Wnt signaling pathway antagonist Dickkopf homolog 1 (Dkk1, up 2.0-fold) is of particular interest. Dkk1 is well known to specify cardiac mesoderm when expressed at the appropriate stage of development, and application of this factor has recently been shown to encourage *in vitro* differentiation of human ES cells into cardiac progenitors.²⁹ Other cardiac-related genes that were upregulated after electrical stimulation include the ATPase, Ca⁺⁺ transporting, cardiac muscle, fast twitch 1 (Atp2a1, upregulated 1.4-fold), and Sema6D (upregulated 1.7-fold), which regulates endocardial cell migration in the developing heart²⁴ and spinal cord.³⁰

Genes and pathways involved in nervous system development were also observed to be upregulated after electrical stimulation. Perhaps the most striking example is the upregulation of neural cell adhesion molecule 1 (Ncam1, upregulated 1.5-fold), a common marker of primitive neuroectoderm. Another gene, Delta/Notch-like EGF-related receptor (DNER, upregulated 1.7-fold), is a neuron-specific transmembrane protein that functions as a ligand of Notch during cellular morphogenesis of glia in the mouse cerebellum.⁹

DISCUSSION

By utilizing current-controlled electrical stimulation for ES cells physically coupled to a microelectrode, a representative model was created to study the electrical effects on ES cells in the myocardium. One of the main advantages of using a current source was its ability to

create a precisely controlled charged balanced waveform, minimizing electrochemical effects. Together with the microcontroller-based active grounding, this assured optimal performance for long-term stimulation, with safe and accurate delivery of current. This is to be contrasted with field stimulation systems, which typically require complex salt bridges to segregate cytotoxic byproducts generated at the electrodes and resulting from the much higher required voltages.

In continuous stimulation, embryonic stem cells at different differentiation stages of development displayed varying responses. In general, later stages tended to have larger changes in both cardiac and embryonic gene expression. At the Terminal Stage, stem cells exhibiting higher cardiac gene expression showed a positive correlation to the stimulation amplitude. However, higher amplitude stimulation was not always correlated with increased expression, as demonstrated in stimulating at the Intermediate Stage, where β -MHC and troponin-T were downregulated and nanog increased significantly. The increase in nanog levels was not expected, and it is possible that at this stimulation amplitude the ES cells remained in an embryonic state while control samples were differentiating. Such inflections of trends away from a neuronal and muscular lineage due to high amplitude stimulation were also observed in field stimulation studies.²⁷ In any case, these results demonstrate the sensitivity of ES cells at various differentiation stages to their local electrical environment, and in particular to a stimulation pattern mimicking endogenous physiological pacing¹⁴ to yield statistically significant differences. Of particular interest is the fact that pacing Intermediate Stage cells at 30 μ A can possibly increase the troponin-T expression by up to sixfold. Unlike field stimulation studies that uniformly depolarize whole volumes, simulating the physiological micro-environment by stimulating from a point-source reveals the importance of cell coupling (both electrical and chemical). Although quantitatively observing increases in the cardiac gene expression of electrically stimulated cells does not necessarily imply cardiac differentiation, it does give special insight into how ES cells first respond to their new environment. This work especially highlights their sensitivity, and suggests greater changes as the ES cells develop. Nonetheless, direct assessment of differentiation was seen through immunostaining, where cardiac markers appeared about three and a half times as prevalent compared to nonstimulated samples.

Furthermore, in addition to understanding the sensitivity of stimulated cells, the temporal patterns of stimulation appear vital. Continuous pacing of ES cells during the 4-day experiments was a requisite to promote the levels of differentiation observed. When ES cells were stimulated for 2 days and then left alone for an additional 2 days, the cardiac markers were only a fraction of the levels when continuously paced for 4 days. When the ES cells were paced for only the last 2 days of the 4-day period, cardiac markers were relatively unchanged while nanog levels increased by almost four times. A progression was found that started with an upregulation of nanog followed by β -MHC, and then troponin-T due to pacing.

The differences found in stimulated groups have raised the issue of spatial distribution of differentiated cells with respect to stimulation electrodes. Since the undifferentiated tissue is not capable of supporting the propagation of a depolarization wave, electrically induced differentiation would occur only in the direct neighborhood of electrodes and fully differentiated, electrically active cells. By fluorescently labeling a cardiac marker following stimulation, the distribution of the cardiac expression did not appear specifically confined to the stimulation electrodes, although did remain in the general vicinity. Although the concentration of cardiac marker troponin-T was higher over stimulation electrodes compared to the total average, it was not a very large difference. In addition to the theory of simply increased differentiation is the idea that cell-cell contact density, proliferation, and distribution over the stimulation electrode is altered within the stimulation period, and will

be the subject of future investigations. So far, the direct role of electrical pacing does appear to play a part in differentiation, but this result points toward a joint paracrine signaling pathway, where electrical stimulation locally triggers the release of markers which may then promote cardiac differentiation throughout the culture. Although the stimulation conditions are different, this hypothesis aligns well with experiments done by Sauer *et al.*,²² which proposed the idea that reactive oxygen species act as secondary messengers as a mechanism to promote cardiac differentiation with application of a brief DC voltage field across the entire culture.

Of particular interest too is the fact that while cardiac differentiation may be triggered by electrical stimulation, this stimulation promotes differentiation into other tissues. This may help explain why statistical significance was not always observed in the real-time PCR analysis. In some field stimulation techniques on ES cells which affect the entire culture rather than only localized areas, major cardiac differentiation was not always elicited,²⁷ but have seen a greater activation of neural pathways instead. In order to achieve a broader understanding of the effects of electrical stimulation, whole genome microarray analysis was performed. Simultaneous to the mild upregulation of mature gene programs is the apparent downregulation of important self-renewal and pluripotency genes, including Oct4 and Foxd3. The alteration in developmental gene programs of electrically stimulated mESCs is clearly seen with gene ontology (GO) overrepresentation analysis, which categorizes genes based on their annotations into functional groups (Supplemental Table 2, see Supplemental Text Files 2 and 3 for full lists). The overrepresented GO terms in stimulated ES cells reveal a number of processes related to embryonic development, pattern specification, and tissue and organ development and morphogenesis. In contrast, downregulated GO processes included cell organization and biogenesis, RNA and DNA metabolism, cell cycle and cytoskeletal organization, and microtubule biogenesis. Ingenuity Pathway Analysis (Ingenuity Systems Inc, Redwood City, CA) also demonstrates significant changes in developmental processes after electrical stimulation (Fig. 6, see also Supplemental Table 3). Based on Ingenuity's database of gene networks, the functional categories that are most significantly changed by electrical stimulation include gene expression, organismal and organ development, cellular development, cell cycle, and specific physiological systems such as cardiovascular, neurological, hematological, and musculoskeletal development. Notably, the cardiovascular, neurological, and musculoskeletal systems are all electrically excitable tissues. Strangely, neither troponin-T nor β -MHC appeared on the microarray gene list, but that may only be because it did not reach statistical significance. Microarray analysis can often have issues with sensitivity, and this does not negate the real time PCR results but simply means the microarray cannot draw accurate conclusions about these specific genes.

Overall, this study describes a novel system used to electrically stimulate ES cells safely over long periods of time, and is capable of demonstrating subsequent changes in differentiation. Although some of the results were not statistically significant, it may be due to the limited area that the stimulation electrodes cover relative to the total culture area. Originally conceived as a feature to probe the spatial effects of stimulation, the particular size and configuration of the stimulation electrodes may not be optimal for differentiation. Another similar limitation of the system is the configuration of the recording electrodes in the center of the culture. The area that it covers (~4%) was unable to properly map any differences in the electrical activity between stimulated and nonstimulated samples. Differences were nonetheless quantified through real-time PCR, immunofluorescence, and whole genome microarray analysis.

While understanding the gene expression and subsequent differentiation is a first and necessary step toward functional repair, understanding integration with host tissue

(participation to electrical conduction and mechanical contraction) is also important. Additional future work using the presented system will use the full potential of MEAs to monitor the development of action potentials and propagation patterns (including conduction mismatches responsible for increased risks of arrhythmias) in co-cultures, and study the impact of electrical stimulation on these parameters. Especially given the transient effects observed in these pacing experiments, continuous pacing may be necessary and one might even suggest the coordinated effort of stem cell implantation and cardiac pacing in the region of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Microelectrode arrays (MEA). Top: Micrograph of the MEA. An array of 6×6 electrodes is located in the center used for electrical sensing. The larger electrodes on the periphery contained in the dashed boxes are used for stimulation.¹⁵ Bottom: Image of an assembled microelectrode array chip, which is glued and wire-bonded to a printed circuit board carrier.

FIGURE 2.

Two operational amplifiers were configured as a voltage-controlled current source to deliver a precise current at each pulse. In addition, a high side current sense was applied using an instrumentation amplifier to monitor the current delivered to the load (i.e., electrodes and cell culture).

FIGURE 3.

Dose-response curve of electrically stimulated ES cells. ES cells at three different differentiation stages were locally stimulated using the MEAs over three stimulation amplitudes. Early Stage ES cells were relatively unchanged following low and mid-level amplitude electrical stimulation, but were still able to significantly up-regulate β-MHC at the highest level of stimulation. Intermediate Stage ES cells demonstrated increased sensitivity, and while cardiomyocyte markers were increased up 30 μA, higher levels of stimulation may have the opposite effect, as seen by the decrease in β-MHC and troponin-T, and the increase in nanog. Stimulation at the Terminal Stage, on the other hand, showed an increase in both cardiomyocyte and stem cell markers. $N = 6-8$ per differentiation stage; $*P < 0.05$.

FIGURE 4.

Partial stimulation of ES cells. To investigate the role of continuous stimulation, ES cells at the Intermediate Stage were stimulated for only half the typical 4-day period at 30 μ A. ES cells that were stimulated for the first 2 days and then incubated for another 2 days were able to demonstrate the same amount of β -MHC as continuous 4-day stimulation. However, troponin levels were relatively unchanged, and the stem cell marker nanog levels were slightly higher. In the reverse situation where ES cells were not stimulated until the second half of the experiment, both cardiomyocyte cell markers did not show significant changes, while nanog levels were increased, although not significantly. $N = 6-8$ for each experimental condition; * $P < 0.05$.

FIGURE 5.

Immunostaining of electrically stimulated cells. Following 4 days of continuous stimulation of Intermediate Stage ES cells at 30 μ A, samples were fixed for fluorescent analysis. In both stimulated and control cases, cells appeared confluent, and were confirmed with nuclei staining (top two panels). In addition to that, cells were stained for troponin-T (lower two panels). Qualitatively, stimulated samples displayed higher amounts of troponin-T and is repeatedly observed in other samples ($N = 4$). The increase in troponin-T due to stimulation was not limited to the areas around the electrodes (highlighted in the dotted regions), but nonetheless stayed within the general vicinity.

FIGURE 6.

Categories of altered gene expression following electrical stimulation. Ingenuity Pathway Analysis displays the function categories that exhibited the highest level of statistically significant changes following electrical stimulation. Among many changes in development, many specific physiological systems such as the nervous, hematological, musculoskeletal, and cardiovascular development were noted.