**Electrical stimulation of cardiac adipose tissue-derived progenitor cells modulates cell phenotype and genetic machinery**

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Electrical stimulation of cardiac adipose tissue-derived progenitor cells
modulates cell phenotype and genetic machinery

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Abstract

**Aims** - A major challenge of cardiac tissue engineering is directing cells to establish the physiological structure and function of the myocardium being replaced. Our aim was to examine the effect of electrical stimulation on the cardioidifferentiation potential of cardiac adipose tissue-derived progenitor cells (cardiac ATDPCs).

**Methods and Results** - Three different electrical stimulation protocols were tested; the selected protocol consisted of 2-ms monophasic square-wave pulses of 50 mV/cm at 1 Hz over 14 days. Cardiac and subcutaneous ATDPCs were grown on biocompatible patterned surfaces, parallel and perpendicular to the electric field applied. Cardiomycogenic differentiation was examined by real-time PCR and immunocytofluorescence. In cardiac ATDPCs, MEF2A and GATA-4 were significantly upregulated at day 14 after stimulation, while subcutaneous ATDPCs only exhibited increased Cx43 expression. In response to electrical stimulation, cardiac ATDPCs elongated, and both cardiac and subcutaneous ATDPCs aligned following the linear surface pattern of the construct. Cardiac ATDPC length increased by 11.3%, while subcutaneous ATDPC length diminished by 11.2% ($P = 0.013$ and $P = 0.030$ vs. unstimulated controls, respectively). Compared to controls, electrostimulated cells aligned better to the patterned surfaces when the pattern was perpendicular to the electric field (89.71°±28.47° for cardiac ATDPCs and 92.15°±15.21° for subcutaneous ATDPCs).

**Conclusions** - Electrical stimulation of cardiac ATDPCs caused changes in cell phenotype and genetic machinery, making them more suitable for cardiac regeneration approaches. Thus, it seems advisable to use electrical cell training before delivery as a cell suspension or within engineered tissue.
Keywords: Electrical stimulation, Progenitor cells, Cardiac adipose tissue, Cardiac regeneration, Cardiac tissue engineering, Cardiac differentiation.
1. Introduction

Twenty-one days after conception, the human heart starts beating, a complex process that involves perfect synchrony between contractile, conductive, and vascular systems (Vunjak-Novakovic et al., 2011). Cardiomyocytes propagate electrical stimulus through intercellular junctions and across the syncytium to produce mechanical contractions and pump blood to the whole body within the vascular system. Spontaneous depolarization in pacemaker cells of the sinoatrial node induces electrical impulse propagation by electrical coupling. Direct currents are reportedly the main electrical currents that direct cell migration in embryonic cardiac development, while pulsatile signals are involved in the cardiac syncytium development (Nuccitelli, 1992; Ypey et al., 1979). The influence of electric fields on cell behaviour has been widely demonstrated in wound healing, electrotaxis (Rabey and Rozendal, 2010), neural stimulation (Logothetis et al., 2010), and stem cell differentiation (Serena et al., 2009).

Cardiac regeneration holds promise for repairing injured myocardium using methods that combine engineering, cell biology, and medicine. Ultimately, the hope is to produce functional cardiac-like tissue constructs exhibiting the properties of native cardiac tissue, including syncytium with contractile and electrophysiological functionality (Tandon et al., 2011). Tissue engineering involves the application of physiologically relevant chemical and physical stimuli to cultured cells, emulating the conditions of the in vivo environment (Tandon et al., 2009a). Thus, preconditioning of a chosen cell type could be a valuable tool for future regenerative medicine applications, e.g. a functional construct could combine trained cells within a scaffold. Numerous groups have attempted to make adult progenitor cells differentiate into cardiomyocytes by means of chemical (Fukuda, 2001) or electrical (Genovese et al., 2008) stimulation;
However, a definitive progenitor cell-derived cardiomyocyte-like cell has not yet been realized.

We recently reported the identification of a novel cell source with cardiac regeneration potential — human cardiac adipose tissue-derived progenitor cells (cardiac ATDPCs). These cells exhibit an inherent cardiac-like phenotype under basal culture conditions; they express \textit{de novo} myocardial and endothelial markers, markedly reduced infarct area, and improved contractility when transplanted into myocardial infarction rodent models (Bayes-Genis \textit{et al.}, 2010).

In the present study, our aim was to assess the effects of electrical stimulation on progenitor cells derived from adipose tissue. Our approach involved electrical field simulation of cells cultured on patterned surfaces, which are known biophysical cues for cell differentiation (Ghafar-Zadeh \textit{et al.}, 2011). The responses of cardiac ATDPCs were compared to those of subcutaneous ATDPCs that lack the inherent cardiomyogenic traits reported in cardiac ATDPCs (Bayes-Genis \textit{et al.}, 2010).

2. Methods

2.1. Adipose tissue-derived progenitor cell (ATDPC) isolation and culture

ATDPCs were isolated from cardiac and subcutaneous adipose tissues obtained from patients undergoing cardiac surgery. Cardiac adipose tissue biopsy samples were obtained from fat pads surrounding the cardiac groove, near the base of the heart and around the aortic root. Subcutaneous samples were taken from fat pads between skin and sternum from the same patients (Bayes-Genis \textit{et al.}, 2010).

Adipose tissue biopsy samples were processed as previously described (Bayes-Genis \textit{et al.}, 2010; Martinez-Estrada \textit{et al.}, 2005). Briefly, samples were rinsed with PBS and cut into small pieces, removing visible blood vessels; thereafter, cells were
isolated by collagenase II digestion. Adhered cells were grown in α-MEM supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin, and cultured under standard conditions (37°C and 5% CO₂). This protocol was approved by the local Ethics Committee, and informed consent was obtained from all patients. The study protocol conformed to the principles outlined in the Declaration of Helsinki.

2.2. Electrical stimulation setup

The stimulation unit setup used in this study was custom made and consisted of a combination of a monophasic programmable electrical device, a printed circuit board (PCB) (FR-4 plastic laminate, designed using Ultiboard, National Instruments) that enabled electrical stimulation of up to six culture plates, and a biocompatible polydimethylsiloxane (PDMS) (Sylgard, 184, Dow Corning Corp.) silicone construct designed to provide structural support to cells and electrodes (Figure 1).

The PDMS constructs were built using a custom mould that was designed with commercial CAD software (Solidworks), fabricated with poly(methyl methacrylate) (PMMA), and constructed by the assembly of three different layers. Once the mould was disassembled, the PDMS construct included a cell pool (1cm×1cm×2mm) where cells were seeded and cultured. The cell pool surface area was imprinted with a regular pattern that could be perpendicular or parallel to the direction of the electric field to support cell alignment. The regular pattern was imprinted into the PDMS using a planed, ruled diffraction grating (1,250 grooves/mm; 05RG150-1250-2, Newport Corporation) using a high precision and consistent polyvinylsiloxane (Affinis, Coltène Whaledent). The structure also had dedicated rooms to accommodate the stimulation electrodes. These electrodes were made of a Teflon core (PTFE) (2 mm height, 1 cm length), which was the structure for the twisted platinum wire (0.5 mm, 30 cm; PTP201;
World Precision Instruments).

The PDMS compounds were made by mixing two liquid components in a ratio of 10 parts base to one part curing agent in a liquid state (10:1, by weight) (Sylgard, 184, Dow Corning Corp.). Air introduced into the mixture during stirring was removed by using a vacuum chamber at 700 mbar for 30 minutes. Then, the mixture was smoothly poured into the PMMA mould. To shorten the curing time, the mixture was cured in an oven (70°C, 2.5-3h). Once the mixture was cured, the mould was removed carefully to avoid damaging the structures. PDMS constructs were sterilised by autoclave prior to cell culturing.

2.3. Electrical stimulation equipment and protocol

The applied electrical field was intended to mimic the electrophysiological heart environment. For a given electric field amplitude applied to the cells using two stimulation electrodes, a fraction of the applied electric field was located near the electrodes due to the electrode impedance resulting from the medium-electrode interface. Consequently, the effective electric field applied to the cells was variable, depending on the electrode material and the medium volume. To verify the effective electric field applied to the cells, we conducted a set of experimental validation measurements using a four-electrode measurement technique. These measurements were carried out as follows: the high (HCUR) and low (LCUR) current electrodes were connected to the PCB electrode connector, while the high (HPOT) and low (LPOT) potential recording electrodes were connected to a digital oscilloscope (DSO6034A; Agilent Technologies). The distance between HPOT and LPOT electrodes was fixed to 1 cm. Then, the amplitude of the monophasic current pulses generated by the electrical stimulator was modified according to the electric field recorded (Figure 1).
We tested three different electrical stimulation protocols, modified from those described in the literature (Tandon et al., 2009a; Tandon et al., 2009b; Tandon et al., 2010). At day 0, 3x10^4 cells were seeded on the silicone-patterned surface, previously coated with 0.1% gelatin, and the medium was refreshed three times per week for 14 days.

2.4. Quantitative real-time PCR

Total RNA was isolated from cardiac and subcutaneous ATDPCs using the AllPrep RNA/Protein Kit (Qiagen). cDNA was synthesized using random hexamers (Qiagen) and the iScript™ One-Step RT-PCR Kit (BioRad Laboratories) according to the manufacturer's protocol. cDNA was preamplified with the TaqMan® PreAmp Master Mix Kit (Applied Biosystems) and then diluted 1:5 with RNAse-free water.

Real-time PCR amplifications were performed with 2.5 µL of cDNA in a final volume of 10 µL, containing 5 µL TaqMan 2× Universal PCR Master Mix, 2 µL RNAse-free water, and 0.5 µL of FAM-labelled primer/probe purchased from Applied Biosystems (Foster City), including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1), myocyte-specific enhancer factor 2A (MEF2A) (Hs01050409_m1), GATA-binding protein 4 (GATA-4) (Hs00171403_m1), sarcomeric α-actinin (Hs00241650_m1), cardiac Troponin I (cTnI) (Hs00165957_m1), connexin43 (Cx43) (Hs00748445_s1), and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2) (Hs00544877_m1). Data were collected and analysed on the LightCycler® 480 Real-Time PCR System (Roche); each sample was analysed in duplicate. The Livak method (2^{ΔΔCT}) (Livak and Schmittgen, 2001) was used to quantify absolute and relative expressions of each gene between electrostimulated and control samples, using GAPDH as an endogenous reference, as previously described (Prat-Vidal et al., 2007).
2.5. Immunocytofluorescence

Cells were fixed with 10% formalin, permeabilised, blocked in 10% normal horse serum for 2h, and incubated for 1h at room temperature with primary antibodies against Cx43 (1:100; Sigma), sarcomeric α-actinin (1:100; Sigma), GATA-4 (1:50; R&D), MEF2 (1:25, Santa Cruz) and SERCA2 (1:50; Santa Cruz). Secondary antibodies conjugated with Cy2 and Cy3 (1:200; Jackson ImmunoResearch), and actin fibres were stained with Phalloidin Alexa 568 (1:40; Invitrogen). Nuclei were counterstained with Hoechst 33342 (1:10^4; Sigma); images were acquired with an Axio Observer Z1 inverted microscope (Zeiss).

2.6. Measurement of cell elongation and alignment

Phalloidin-stained images were randomly acquired at 200× magnification, and cells with clearly defined outlines were manually traced using NIH ImageJ software (Supplementary Figure 1). The cell major axis was measured, and the cell orientation angle was calculated as the angle between the cell major axis and the direction of the electric field (perpendicular to the surface pattern alignment). A minimum of 90 cells per group were examined from four independent experiments.

2.7. Statistical analysis

Relative fold expressions of cardiac and subcutaneous ATDPCs were compared using Student’s t-test, and the statistical difference was determined for the samples from six separate experiments. The results are presented as mean±SEM. Statistical analyses of the cellular length and angle measurements in all groups were performed using ANOVA followed by Tukey’s tests. Results are shown as mean±SEM. P values < 0.05 were considered significant. All analyses were performed with SPSS (15.0.1 version, SPSS, Inc.).
3. Results and Discussion

3.1. Electrical protocol performance

After an exhaustive search, we adapted three different electrical stimulation protocols from the literature: direct current of 5 V/cm over 2 hours (Tandon et al., 2009a); alternating current of 2-ms monophasic square-wave pulses of 5 V/cm at 1 Hz over 14 days (Tandon et al., 2009b); and alternating current of 2-ms monophasic square-wave pulses of 50 mV/cm at 1 Hz over 14 days (Tandon et al., 2010). The electrical parameters were adapted to our device requirements, and protocols were tested consecutively according to the results obtained.

The first and second protocols induced cell growth arrest and cell death in a repetitive and reproducible manner (not shown). Use of protocol 3 achieved normal cell appearance and growth, and this protocol was chosen for further experiments and used to collect the data reported in this manuscript.

3.2. Effects of electrical stimulation on cardiac and subcutaneous ATDPCs

At baseline, cardiac ATDPCs exhibited significantly higher constitutive expression of cardiac markers, such as Cx43, sarcomeric α-actinin, SERCA2, and GATA-4, compared to subcutaneous ATDPCs (data not shown) (Bayes-Genis et al., 2010).

Preliminary experiments with cardiac ATDPCs and patterned surfaces (parallel and perpendicular) were executed to obtain information about their effect on electrical stimulation. Studies comparing cardiac gene expression profiles in both surfaces were performed. Analysis of cardiomyogenic marker expression showed no significantly differences in gene expression between both groups (all $P > 0.05$). Accordingly, we decided to develop all subsequent experiments with perpendicular surfaces because, as
it was previously described (Tandon et al., 2009b) the electric field gradient across the cell is minimized with a perpendicular cell disposition to the electric field.

Electrical stimulation on perpendicular patterned-surfaces modulated gene expression in cardiac and subcutaneous ATDPCs, as shown by real-time PCR results in Figure 2. Data are presented as relative fold expression (electrostimulated versus non-electrostimulated control) as measured through real-time PCR analysis following the Livak method (Livak and Schmittgen, 2001).

After 14 days of electrical stimulation, cardiac ATDPCs expressions of MEF2A and GATA-4 transcription factors were upregulated by more than 4.5- and 2-fold, respectively (Figure 2). Student’s t-test revealed MEF2A upregulation \((P = 0.05)\) and a very significant increase of GATA-4 expression \((P = 0.03)\) in cardiac ATDPCs with respect to subcutaneous ATDPCs. In contrast, the electrostimulated subcutaneous progenitors only exhibited higher expression of Cx43 (2-fold), the major protein involved in cardiomyocyte gap junction structures.

Protein expressions with and without electrical stimulation are shown in Figure 3. Control and electrically stimulated cultures of cardiac ATDPCs expressed similar distribution of Cx43 protein (Figure 3A), in accordance with real-time PCR data. Increased Expressions of GATA-4, sarcomeric \(\alpha\)-actinin, MEF2A, and SERCA2 were also confirmed by immunocytofluorescence in electrostimulated cardiac ATDPCs (Figure 3C, E). As for subcutaneous ATDPCs, analysis of Cx43 and sarcomeric \(\alpha\)-actinin protein expressions in control and treated cells confirmed gene expression observations (Figure 3B). Absence of GATA-4 and minimal MEF2A expression observed by real-time PCR were verified at the protein level (Figure 3D, F).
According to the results shown in Figure 2, electrical stimulation on cardiac ATDPCs caused relevant genetic changes in main cardiac transcription factors, such as MEF2A and GATA-4, involved in heart development and early cardiac differentiation (Heikinheimo et al., 1994). MEF2 is a MADS-box transcription factor involved in several biological processes, including cardiac conduction, ventricular cardiac myofibril and vascular development, and muscle cell differentiation; specifically, MEF2 controls the differentiation of cardiomyoblasts into cardiomyocytes (Karamboulas et al., 2006). Its downstream targets include ventricular myosin light chain, cardiac troponin T, cTnI, α-myosin heavy chain, desmin, and ANF, with other targets and mechanisms yet to be elucidated (Morin et al., 2000). GATA-4, on the other hand, is a zinc finger transcription factor that is essential for proper endodermal differentiation and ventral morphogenesis; like MEF2, GATA-4 is one of the earliest transcription factors expressed in the developing heart (Heikinheimo et al., 1994). GATA-binding motifs are present in enhancer regions of cardiac genes, including sarcomeric proteins, cardiac-specific enzymes, ANP, BNP, and several others (Pikkarainen et al., 2004). Evidence of direct physical interaction between MEF2 and GATA-4 through their DNA binding domains indicates that MEF2 proteins are recruited by GATA-4 transcription factors to activate cardiac promoters. The synergy between GATA-4 and MEF2 means that MEF2 proteins are GATA coactivators in cardiac myogenesis, and both could displace corepressors of either or both factors (Morin et al., 2000). Therefore, increased levels of those important markers could induce further cardiac differentiation of treated cells, as Alexander and Bruneau described (Alexander and Bruneau, 2010), yet a combination of cardiogenic transcription factors, such as GATA-4 and MEF2, could induce direct transdifferentiation of adult cell types into functional cardiomyocytes.
On the other hand, connexins are transmembrane proteins that oligomerize to form connexons or hemicannels, called gap junctions, to connect with neighbouring cells (Morel and Kwak, 2012). In cardiac electrophysiology, gap junctions promote cell connections through a hydrophilic pathway, and contribute to electrical signal transference during action potential propagation, facilitating cell contraction synchronization (Delmar et al., 2004). Thus, increased Cx43 expression is directly related to the electrical field stimulation and its involvement in electrical charge transference, and suggests that a cell is able to adapt to the electrical environment.

With regard to cell phenotype and organization, cardiac ATDPCs elongated, and both cardiac and subcutaneous ATDPCs aligned following the linear surface pattern of the construct (parallel or perpendicular) when electrical stimulation was applied. Figure 4 shows that actin filaments at baseline already showed a certain alignment following the direction of surface pattern. Cellular organization of electrically stimulated cultures expressed a remarkably more pronounced cell alignment (Figure 4). Measurements of cell elongation and angulation were performed. Indeed, after 14 days of stimulation, the length of cardiac ATDPCs increased by 11.3%, while the length of subcutaneous ATDPCs diminished by 11.2% ($P = 0.013$ and $P = 0.030$ vs. unstimulated controls, respectively). Although no statistically significant differences were detected in cell alignment, measurements showed that when the pattern was perpendicular to the electric field, electrostimulated cells aligned better to the patterned surfaces than controls ($89.71^\circ \pm 28.47^\circ$ for cardiac ATDPCs and $92.15^\circ \pm 15.21^\circ$ for subcutaneous ATDPCs).

This phenomenon was previously described by Tandon et al., who suggested that perpendicular cell disposition to the electric field minimizes the electric field...
gradient across the cell (Tandon et al., 2009b). However, cells aligned parallel to the electric field when they were cultured on parallel-patterned surfaces, suggesting that the surface pattern is a strong modulator of cell orientation; similar results were previously described (Tandon et al., 2009a). In addition, our present data verify that electrical field stimulation enhances this alignment. On perpendicular-patterned surfaces, cardiac ATDPCs significantly elongated and adapted in a more organized way as a response to electrical field stimulation. It is plausible that this morphology may facilitate cell connections and thus allow electrical signal propagation. In fact, myocardium is formed by parallel fibres with perpendicularly intercalated discs (adherens junctions, desmosomes, and gap junctions) that connect single cardiomyocytes to an electrochemical syncytium. Following electrostimulation, subcutaneous ATDPCs shortened and adopted a more random organization, rendering this cell source less suitable for cardiac regeneration purposes compared to cardiac ATDPCs.

In summary, the present results indicate that electrostimulation contributes to enhance expression of several cardiac-specific genes that are of great value for achieving a certain degree of cardioidifferentiation. This phenomenon is particularly relevant in cardiac ATDPCs, since upregulation of the early cardiomyogenic transcription factors MEF2A and GATA-4 could result in a further cascade of cardiac proteins. We also found that cell disposition was based on the surface pattern and emphasized by the electrical stimulation (particularly in cardiac ATDPCs). Interestingly, we were able to enhance cell orientation with perpendicular patterned surfaces, diminishing cell opposition to the electrical current flow.

It is widely known that the electrical activity present in the heart is a physical phenomenon that plays a key role in contractile behaviour of the myocardium. Efforts
towards cardiac regeneration have focused on finding a cardiomyocyte-like cell with electromechanical activity, a cell attribute required for cardiac regeneration approaches. Electrostimulation is likely a useful tool for cell training for further cardiac applications, e.g. enhancing functional assembly of cardiomyocyte-like cells into a synchronously contracting cardiac bioimplant suitable for cardiac regeneration purposes. These modifications exert an adaptation to a hostile heart environment and the selection of well-trained cells. Moreover, electrical stimulation has the advantages of being a simple and reproducible method of mimicking a part of the cardiac physiology in vitro.

Further investigation in this field is necessary. Changes in the expression of ion channels in ATDPCs must be studied, as they have been reported in baseline conditions (Bayes-Genis et al., 2010). Additionally, cell phenotype modulation should be detected at different time points after electrical stimulation; longer experiments could reveal intercellular communications and cell reorganization. Finally, the contractile function in electrically stimulated culture could be modified; further research should investigate the effects of changes in contractile function as well as electrical signal transduction.
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Conflict of Interest: none declared.
References


Figure 1 - Electrical stimulation setup. (A) Detail of the PDMS biocompatible construct, supporting the Platinum-PTFE electrical stimulation electrodes connected to the PCB. (B) Experimental setup for the electrical stimulation of 6 wells of culture. The electrical stimulator is connected to the PCB through an isolator stage. (C) Schematic of the four-electrode measurement to verify the effective electric field applied to the cultured cells. The electrical stimulator is connected to the external injection electrodes, HCUR and LCUR, respectively. The inner detection electrodes, HPOT and LPOT, are separated by 1 cm and connected to an oscilloscope. The amplitude (A) of the monophasic alternating current pulses generated by the electrical stimulator was modified until the desired electric field amplitude (E) (5 V/cm or 50 mV/cm) was observed in the oscilloscope.

309x289mm (96 x 96 DPI)
Figure 2- Real-time PCR of cardiomyogenic genes in cardiac and subcutaneous ATDPCs. Relative fold expressions of cardiomyogenic markers in electostimulated versus non-treated controls are shown in both cardiac and subcutaneous ATDPCs. Cardiac Troponin I (cTnI) transcripts were not detected in either cell types. Values were normalized to GAPDH expression and are shown as mean±SEM for six independent experiments, performed in duplicate; * P ≤ 0.05 versus subcutaneous ATDPCs group.
Figure 3 - Protein expression in cardiac and subcutaneous ATDPCs on a vertical patterned surface. Phalloidin staining (red) and Cx43 expression (green) in cardiac (A) and subcutaneous (B) ATDPCs. Sarcomeric α-actinin (red) and GATA-4 (green) expressions in cardiac (C) and subcutaneous (D) ATDPCs. SERCA2 (red) and MEF2 (green) expression in cardiac (E) and subcutaneous (F) ATDPCs. Nuclei were counterstained with Hoechst 33342 (blue) in A, B, D and F. Scale bars = 50µm.
355x329mm (96 x 96 DPI)
Figure 4 - Cells elongated and aligned following the patterned surface. Phalloidin staining (red) in control and electrostimulated cultures of cardiac (left) and subcutaneous (right) ATDPCs on perpendicular- (upper panel) and parallel- (bottom panel) patterned surfaces. Nuclei were counterstained with Hoechst 33342. Scale bars = 50µm.

359x239mm (96 x 96 DPI)
Cardiac ATDPCs cell length measurements. Representative immunocytofluorescences images with Phalloidin staining (red) in control (left) and electrostimulated (right) cardiac ATDPCs. Nuclei were counterstained with Hoechst 33342 (blue).

Supplementary Figure 1

Cardiac ATDPCs

Control

Electrostimulated

254x190mm (72 x 72 DPI)