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Electrostimulation induces cardiomyocyte predifferentiation of fibroblasts

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ABSTRACT

Stem-cell therapy has become a promising therapeutic tool for myocardial repair. Cardiac pre-committed cells, which complete their differentiation in the myocardium, may reduce fibrosis and restore muscle function. However, many questions concerning a precise, functional integration of injected cells remain unanswered. Fibroblasts regulate the cardiac extracellular matrix and are the most abundant cell population in an infarcted area. Electrostimulation is a well-known trophic factor and can induce phenotypic changes in myoblasts. The objective of this study was to evaluate the effectiveness of electrical stimulation to induce pre-commitment of fibroblasts into cardiomyocytes *in vitro*. Using short-time electrostimulation in a cytokine-free culture system, we induced pre-commitment of two fibroblast cell lines to a cardiomyocyte phenotype. This partial differentiation *in vitro* may facilitate further differentiation within the cardiac environment and result in better electro-mechanical integration of the therapeutically introduced cells.

Sudden suppression of the oxygen supply following an acute myocardial infarction triggers cascades of cellular processes [1]. Cell death, migration of inflammatory cells and remodeling of the extracellular matrix are induced to stabilize the region and restore ventricular wall function. However, cardiomyocyte loss and replacement by fibrous elements frequently results in a pathological process evolving from progressive left ventricular remodeling to congestive heart failure [2]. Because of its potential to restore myocardial cellularity and regenerate the contractile tissue, stem-cell therapy has become a promising therapeutic tool [3]. However, the ultimate fate of many injected cell lines and whether they precisely, functional integrate remains unknown. Introduction of cardiac pre-committed cells might overcome these uncertainties. Following completion of their differentiation within the myocardium, the pre-committed cells could effectively and selectively reduce fibrosis and restore muscle function.

Fibroblasts are major regulators of the progression of cardiac ischemic pathology. Immediately after an infarction, the process of wound-healing begins with recruitment of cardiac fibroblasts and restructuring of the extracellular matrix [4,5]. The resident fibroblasts give rise to myofibroblasts, which play important roles in scar contraction and tissue repair [6,7]. Even after the acute phase of injury, the persistence of fibroblasts with the capacity to avoid apoptosis or acquire a fibrocytic phenotype may be important, as these may generate an elastic extracellular matrix which affects ventricular compliance [8].

Fibroblasts are the most numerous cell types in the heart [9]; therefore, they are attractive, therapeutic targets. They can be converted to a myogenic phenotype by co-culture with myoblasts or by treatment with conditioned medium, secreted growth factors and by the introduction of muscle-specific transcription factors [10–14]. Myogenic conversion of cardiac fibroblasts could be a useful therapeutic tool for the treatment or prevention of myocardial infarction. *In vitro* conversion of fibroblasts to a myocardial fate could yield an abundant source of cells for cardiac therapy. However, using conditioned medium, cytokines or ectopically expressed genes to drive differentiation precludes clinical translation.

Electrical stimuli play pivotal roles in muscle cell function, as cell fate and function are tightly regulated in response to environmental factors and intercellular signals. Compensation, by means of controlled electrical stimulation (ES), increases muscle mass in the absence of physiologic electrical stimuli [15]. ES induces muscle cell hypertrophy, regeneration and apoptosis [16,17], although the underlying mechanisms are unclear. Latissimus dorsi muscle flaps, preconditioned by long term pacing for subsequent use in cardioplasty, undergo anatomopathological differentiation [18] and it has been suggested that ES drives bone marrow stem cells to differentiate into cardiac-type [19].

In this study we hypothesized that ES could induce phenotypic changes in fibroblasts *in vitro*, resulting in the pre-commitment of these cells to a myocardial fate. We developed a cytokine-free system using ES to induce pre-commitment of fibroblasts to a myocardial phenotype.

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Materials and methods

Cell culture and ES protocol. HFF-1 newborn human fibroblasts (ATCC: SCRC-1041) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum, 15%, $NaHCO_3$, 2 mM L-Glutamine, 100 U/ml Penicillin, and 100 ug/ml Streptomycin. Balb/3T3 clone A31 mouse embryonic fibroblasts (ATCC: CCL163) were cultured in high-glucose DMEM containing 10% calf-serum, 2 mM L-glutamine, 100 U/ml Penicillin, and 100 μg/ml Streptomycin. Both cell lines were cultured at low passage till reaching 80% confluence in order to avoid spontaneous differentiation. For each experiment, the cells were seeded in 4-well plates (NUNC Apogent, Rochester, NY) at a density of 5000 cells/cm^2, and left overnight.

Cells were stimulated with a four-channel C-Pace chronic stimulation unit (IonOptics Co., MA). In order to construct a voltage dose–response curve, the cells were stimulated starting at voltages within the highest range used to stimulate latissimus dorsi muscle prior to ventricular cardiomyoplasty [20]. Voltages of 10, 20, and 40 V were used in 0.5 Hz, 5 ms pulses. Cells were analyzed after 1–96 h of stimulation. An equal number of non-treated cells were recovered at same time points as controls.

Cell viability. Assessment of cell viability was performed by Trypan Blue Exclusion according to manufacturer's instructions (Whittaker Cambrex, Verviers, Belgium). The LIVE/DEAD Cell-mediated Cytotoxicity Kit (Molecular Probes/Invitrogen, CA) was used to assess cell death. Percentage of dead and non-viable cells was calculated by counting at least 200 cells in five randomly chosen microscopic fields.

Immunocytochemical analysis. Expression of cardiac troponin I (cTnI), sarcomeric actin, connexin 43 (Cx43), and Myo-D was evaluated by immunocytochemistry and immunofluorescence as previously described [21–23]. For immunofluorescence, mouse monoclonal anti-cTnI (at the dilution of 1:1000), anti-sarcomeric actin (1:1000), and Cx43 (1:500) (Biomeda, CA) antibodies were used. For triple-labelling experiments, cells were incubated with Alexa-Fluor 488-conjugated anti-mouse IgG and Alexa-Fluor 546-conjugated anti-rabbit IgG (Invitrogen, CA) for 30 min at 37 °C. All cell preparations were counterstained with TO-PRO3 (Invitrogen) and cytoplasmic actin was counterstained with phalloidin red (Invitrogen). Slides were mounted in fluorescence anti-fade, mounting medium (Vectashield, CA). Slides, with their identities masked, were viewed under fluorescence (Leica CMR) and confocal (Leica TCS-SPE) microscopes by two independent observers. Immunohistochemistry was performed and processed as described above. Briefly, cells were fixed and incubated with mouse anti-cTnI (at a dilution of 1:1000), anti-sarcomeric actin (1:1000), anti-Cx43 (1:500), anti-Myo-D (1:250) antibody (Sigma, MO) at 37C. 3-3′-diaminobenzidine was used to detect antibody binding. Slides, with their identities masked, were viewed under light microscope (Nikon F100) and positive cells were counted by two independent observers.

Western blotting. Both stimulated and control cells were recovered and processed for Western blotting analysis as previously described [24]. Troponin-T (NeoMarkers/LabVision, CA), and cTnI, Cx43, Myo-D, GATA4 antibodies (all from Santa Cruz Biotechnology, CA) were used. Densitometry analysis was performed using Image-Pro 6.0.

Flow cytometry. Cells were stained using antibodies against cTnI (Abcam Inc., MA) as previously described [25] and run through a FACSDiva flow cytometer (Becton–Dickinson, CA). A minimum of 50,000 events were collected and acquired in list mode with the FACS Diva software (Becton–Dickinson, CA). The same cell type was stained for Vimentin as a positive control (Santa Cruz Biotechnology) Acquired data were analyzed using CellQuest software (BD BioSciences, CA).

RT-PCR. Total cellular RNA was isolated using RNeasy mini kit (Qiagen Inc., CA) according to the manufacturer's protocol. To eliminate DNA contamination, the samples were treated with 2.0 U DNase-I at 37 C for 15 min followed by inactivation with 2 mM EDTA at 65 C for 10 min. The samples were concentrated by ethanol precipitation and resuspended in RNase-free water. Prior to cDNA synthesis, the samples were screened for genomic DNA contamination by PCR on RT-controls. Total RNA was transcribed into cDNA using the Omniscript RT kit (Qiagen) and later purified with the QIAquick PCR purification kit (Qiagen). For each PCR reaction, 20 ng of cDNA template was used in a 25 μL reaction volume with HotStar Taq Plus and Quantitect Primers (Qiagen). All targets were amplified at an annealing temperature of 55 C for 30 cycles.

Statistical analysis. All the experiments were performed in triplicate. Data are reported as means ± standard deviation. Data were processed using SPSS (Statistical Package for Social Sciences) release 13.0 for Windows (SPSS, IL). One-way ANOVA was performed, followed by multiple pair-wise comparison procedure (Tukey test). Assumptions of normality were checked and met. Holm–Sidak method was used to increase the power of the analysis. Pearson's product-moment *r* coefficient was calculated to evaluate correlations. Significance was at the 0.05 level.

Results

ES reduces cell population number

The effects of ES on HFF-1 human newborn fibroblasts and Balb/3T3 clone A31 mouse embryonic fibroblasts were evaluated. Electrostimulation, over a range of voltages, was applied, in 5 ms pulses with a frequency of 0.5 Hz for 1–72 h. In both cell lines, ES significantly reduced cell number, in a manner dependent on both intensity and duration of stimulation (Fig. 1). ES affected both cell viability and cell loss, with the largest impact on cell survival at the highest voltage stimulation intensity. An early inflection point represented the initial delivery of the electrical stimuli, and was followed by a more gradual decrease in total and viable cells throughout the later time points (Fig. 1).

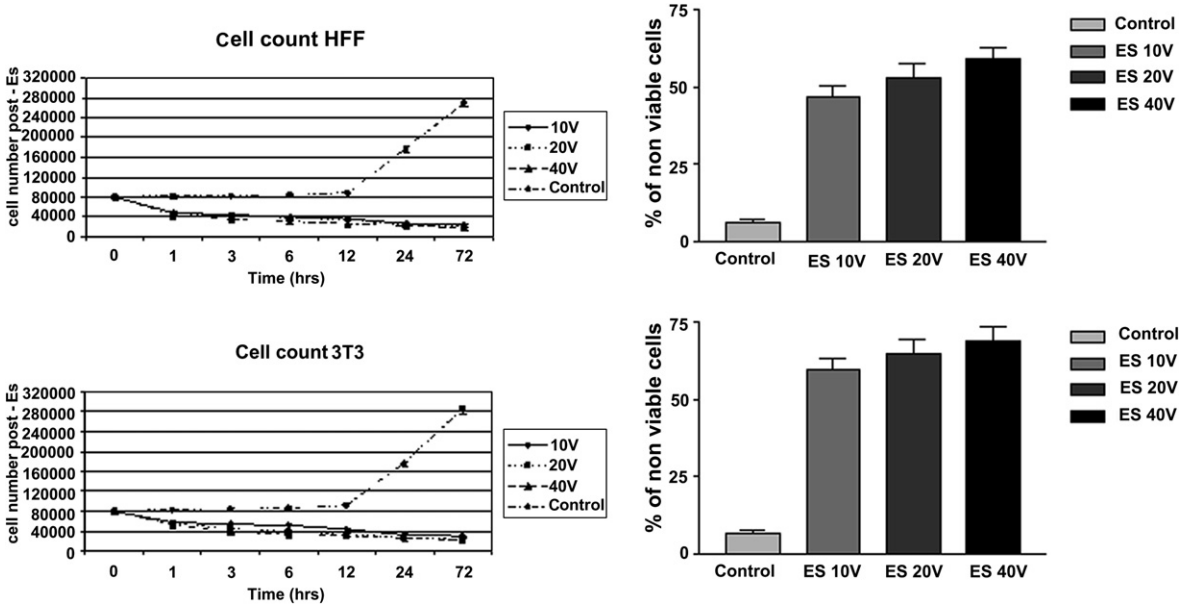

Fig. 1. Effects of ES on cell number and viability. Top panels: HFF-1 cell line. Bottom panels: 3T3 cell line. Left panels: Cell growth following ES at different times and stimulation intensities. Right panels: Percentage of non-viable cells after 24 h ES at different stimulation intensities. Control groups are non-ES cells.