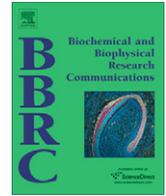




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Electrostimulation induces cardiomyocyte predifferentiation of fibroblasts

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ARTICLE INFO

Article history:

Received 18 March 2008

Available online 31 March 2008

Keywords:

Fibroblasts
Cell differentiation
Electrostimulation
Cell therapy
Myocardial infarct

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.

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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% NaHCO₃, 2 mM L-Glutamine, 100 U/ml Penicillin, and 100 µg/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/cm², and left overnight.

Cells were stimulated with a four-channel C-Pace chronic stimulation unit (Ion-Optics 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) (Biomedica, 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 37°C. 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).

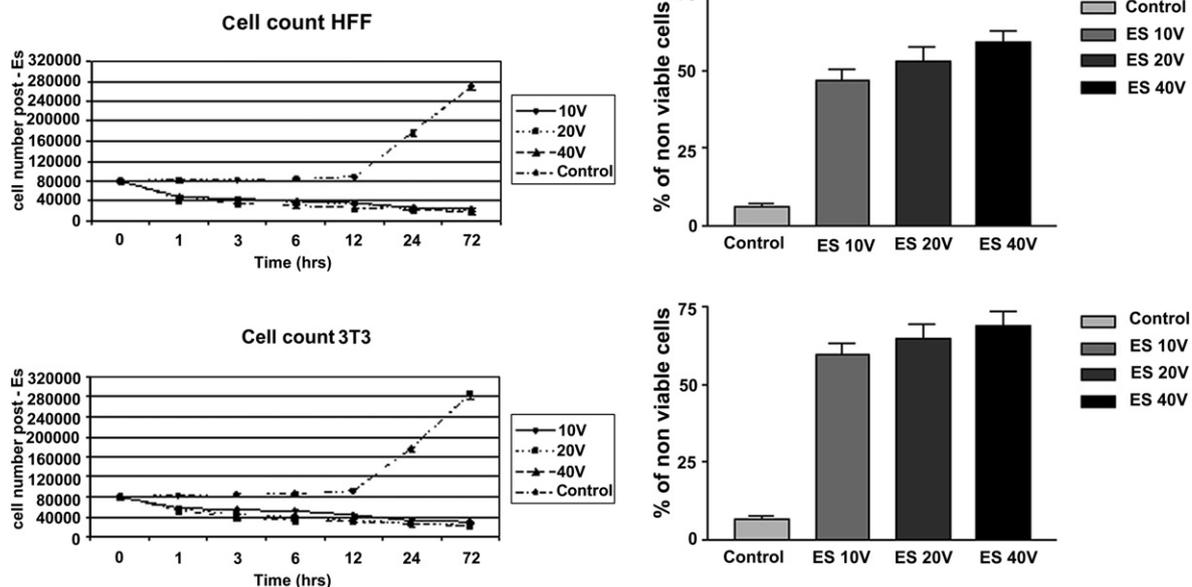


Fig. 1. Effects of ES on cell number and viability. Top panels: HFF-1 cell line. Bottom panels: 3T3 cell line. Left panels: Cell growth following ES at different times and stimulation intensities. Right panels: Percentage of non-viable cells after 24 h ES at different stimulation intensities. Control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

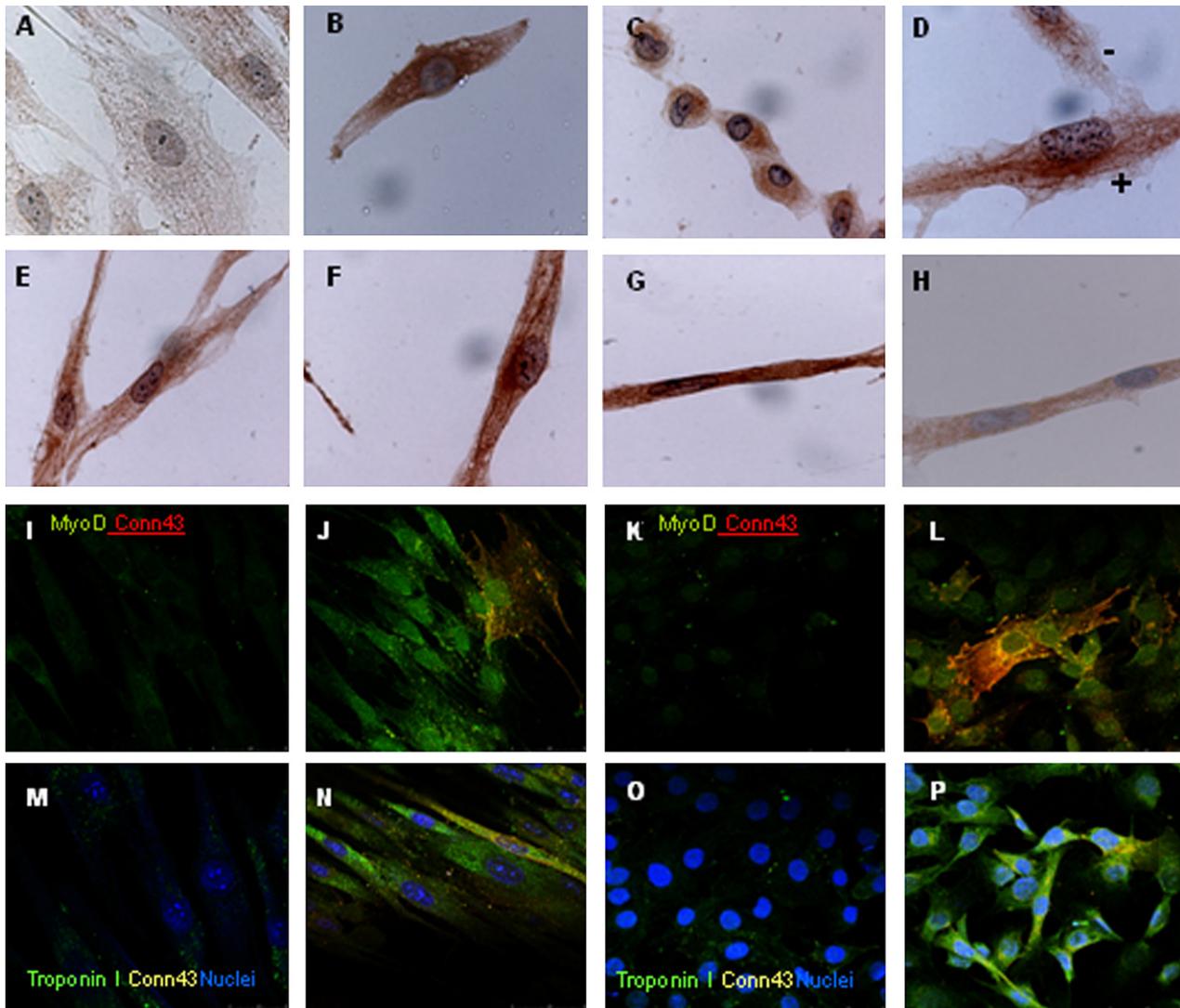


Fig. 2. Microscopy evaluation of phenotype after ES. Representative morphologies observed in both cell types after 20 V ES are shown. (A–G) cTnI immunostaining (H) sarcomeric actin immunostaining. (A) Non-stimulated HFF-1; (B) 3T3 fibroblasts stimulated for 3 h. *Note:* spindle shape and intense cTnI signal in the perinuclear region; (C) HFF-1 cells 24 h ES. *Note:* Longitudinal cell rearrangement of up to 5 cells; (D–E) HFF-1 cell 24 h ES. *Note:* cell projections indicating cell-to-cell interactions between cardiac markers expressing cells and positive and negative cells; (F–G) 3T3 cells 24 h ES. (H) 3T3 cells 24 h ES. (I–P) Confocal Microscopy Analysis of MyoD and cardiac markers expression. (I and M) HFF-1 control; (J and N) HFF-1 electrostimulated 24 h. In J *Note:* intense nuclear staining for MyoD and both membranous and perinuclear distribution of Cx43 antibody. In N *Note:* fiber-like arrangement of cTnI staining. (K and O) 3T3 control; (L and P) 3T3 electrostimulated 24 h. In P *Note:* cTnI showed a fiber-like arrangement of cTnI and cell elongation. Intense staining in the perinuclear region could may correspond to the Golgi apparatus (data not shown).

Short-time ES induces changes in cell morphology

In order to assess the ability of ES to induce differentiation, cell morphology and marker gene expression was examined in both cell lines. ES induced a spindle shape, characteristic of myoblasts (Fig. 2B). Anisodiametric, multinucleated, myotube-like structures with pole-to-pole cell fusion (Fig. 2C) and fiber rearrangements resembling a striated-muscle-like phenotype could be detected as early as 24 h after ES treatment (Fig. 2F and G). Cell-to-cell interactions via cellular projections were found between cardiac-marker expressing cells and also between the cells positive and negative for cardiac markers (Fig. 2D and E). Changes in cell morphology could also be detected by confocal microscopy, including a reorganization of cytoplasmic actin and a general elongation of the cells (Fig. 2I–P and data not shown).

Short-time ES induces muscle and cardiac protein expression

These morphological alterations were accompanied by increases in cardiac-specific marker expression. Polar fusion of the fibroblasts was associated with the expression of sarcomeric actin (Fig. 2H). Immunostaining for Cx43, associated to gap junctions, showed a membrane distribution of Cx43 following electrostimulation (Fig. 2J, N, L, P). Confocal microscopy of 3T3 and HFF-1 cells electrostimulated with 20 V for 24 h confirmed differential expression of cTnI, Cx43 and MyoD (Fig. 2I–P). Additionally, co-expression of cTnI and Cx43 could be detected, with the latter displaying a perinuclear distribution in addition to a typical membranous localization pattern (Fig. 2N and P).

A significant increase in MyoD expression was detected in both cell lines after 24 h of ES with 10 V and 40 V (Fig. 3A). MyoD upregulation was coupled with a decrease in vimentin, a

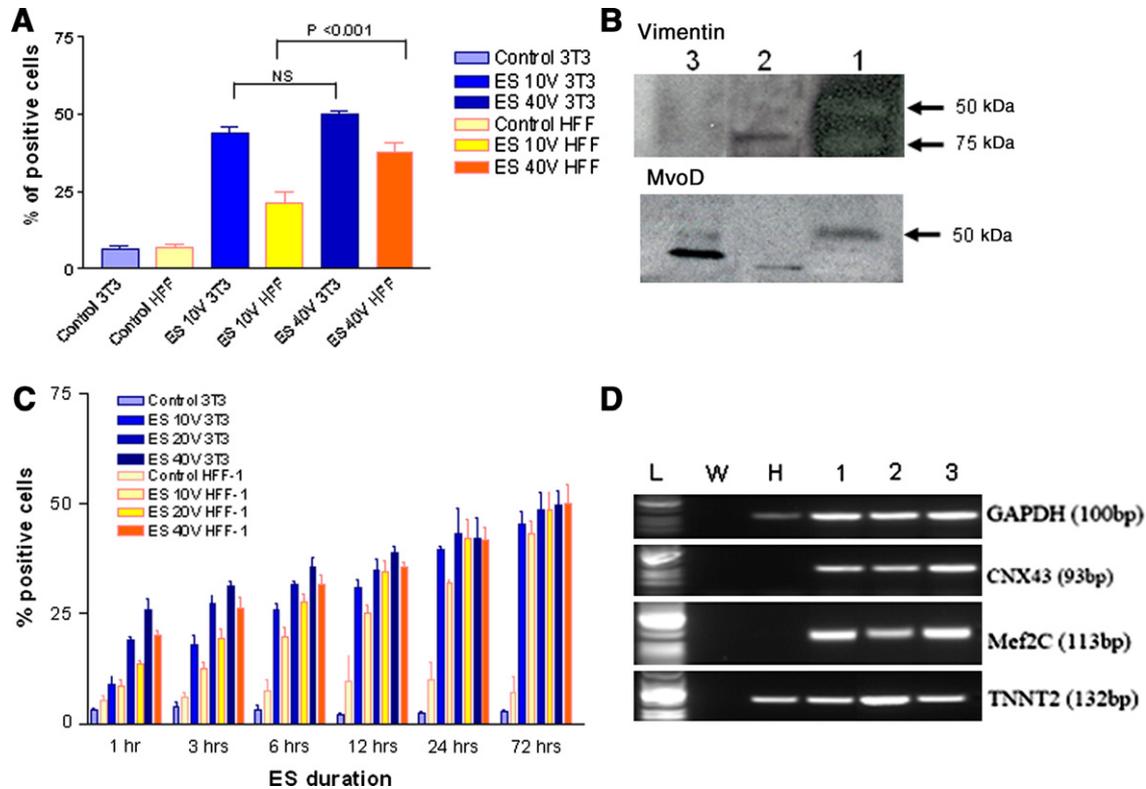


Fig. 3. Expression of muscle and cardiac marker genes following ES. (A) Percentage of MyoD positive cells following ES at different time points and voltages. (B) Western blot analysis showing MyoD (45 kDa) (bottom) and Vimentin (60 kDa) (top) expression in 3T3 cells. Lane 1, molecular weight marker; Lane 2, 3T3 cells control; Lane 3, 3T3 cells electrostimulated 12 h at 20 V; Lane 4, 3T3 cells electrostimulated 24 h at 20 V. (C) Percentage of cTnI positive cells following treatment with ES at different time points and voltages. (D) RT-PCR analysis of short-time (3 h) electrostimulated 3T3 cell line.

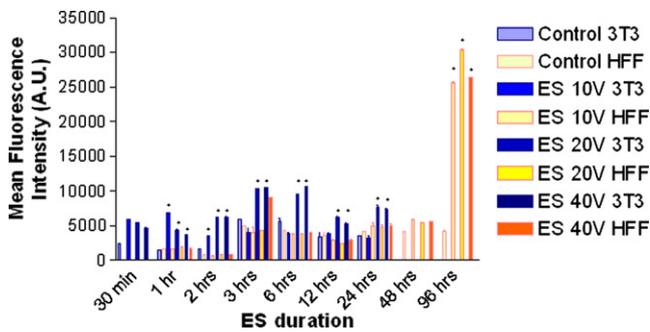


Fig. 4. Cytofluorimetric analysis of intracellular cTnI staining in 3T3 and HFF-1 cells following treatment with ES at different time points and voltages. Results are reported as mean fluorescence intensity expressed as arbitrary unit (AU) ± SD.

fibroblast-lineage marker (Fig. 3B), providing additional evidence of a shift toward a muscle-like phenotype. Cardiac troponin I (cTnI) expression increased in a time- and dose-dependent fashion in the ES group but not in the control group (Fig. 3C). Because there was little difference between the groups stimulated at 20 or 40 V for 24 or 72 h in both cell lines, an optimal condition, after which differentiation can no longer be increased, may exist (Fig. 3C).

ES induces muscle-related transcription factor expression in embryonic fibroblasts

Using RT-PCR, we detected the early upregulation of Mef2C, a muscle cell related transcription factor, in the 3T3 cells. ES also induced expression of the GATA-4 transcription factor in 3T3 cells (data not shown). The mRNAs for cTnI and Cx43 were upregulated

as well, with a statistical significant peak reached after 3 h stimulation at high voltages (Fig. 3D and data not shown). This early upregulation of a muscle-related transcription factors may be unique to embryonic cells as it was not seen in the HFF-1 cells.

ES Increases cellular cardiac troponin I

To more quantitatively assess changes in cell shape and cTnI expression following ES, we subjected both cell lines to different ES conditions, immunostained for cTnI and analyzed by flow cytometry. ES caused significant changes in cell morphology represented by cell elongation and increases in cytoplasmic density. When intracellular expression of cTnI after ES was analyzed using flow cytometry, the 3T3 and HFF cells had different expression patterns. cTnI protein rapidly increased in the 3T3 embryonic fibroblasts and was significantly higher in the treated group than the control, especially when a low voltage was applied ($p < 0.001$) (Fig. 4). As stimulation time was increased, higher ES voltages resulted in significant increases in cTnI expression while no statistically significant difference between the ES cells and the control cells could be seen at the lowest voltage. HFF-1 cells showed no significant difference in cTnI expression, compared with the non-treated group, at the time points when the 3T3 cells got a maximal response. Therefore, the stimulation time was extended to 96 hrs for the HFF-1 cells and a dramatic effect was detected ($p < 0.001$) (Fig. 4).

Discussion

Because fibroblasts play pivotal roles in scar formation and in the colonization of the infarcted area, we tested whether delivering ES via pacing would induce fibroblast commitment towards a

cardiomyocyte phenotype. We demonstrated that ES induced a pre-differentiation process in fibroblasts, reflected at both the morphological and biochemical levels, toward a myocardial phenotype. This partially differentiated phenotype might allow a gradual, ongoing differentiation within the cardiac environment providing time for electro-mechanical integration.

Mimicking physiological stimulation provides the correct signals to maintain and improve muscle structure and function. For example, ES prevents immobilization-induced muscle atrophy [26] and induces conversion of fast twitching muscle fibers to slow contracting cells [27]. Our data suggest that ES can be used to convert fibroblasts to multinucleated myotubes-like structures. If these myotubes can be made to contract, systolic function could be improved. If they do not contract, as perhaps occurs with most of the cells injected into the myocardium, a passive cardiac improvement could still be expected through increased compliance, hence diastolic function, of the infarcted myocardium.

A dose-dependent effect of ES on the expression of cardiomyocyte-specific markers was observed. However, this was coupled with a dramatic decrease in cell number and availability, supporting the idea that ES induces cell death or inhibits cell growth. The cell population was reduced by as much as 50% in the first 3 h. This early cell loss appears to be a cytotoxic effect triggered by ES which may result in selection of an ES-resistant population of cells that will later undergo phenotypic changes. This cell population could have clinical application because of the relative ease of acquiring and culturing fibroblasts as compared with other cells used in therapy.

We observed the development of cell projections indicative of cell-to-cell interactions not only between cardiac marker expressing cells, but also between positive and negative cells and the membrane distribution of Cx43 following ES suggests the formation of new intercellular junctions. This morphology resembled the fusion of cardiomyocytes with surrounding non-cardiomyocytes seen in co-cultures [28] and may recapitulate *in vitro* the functional coupling of cardiomyocytes and fibroblasts described *in vivo* [29]. These types of interactions may occur in ischemic scar tissue acting as bridges between myocyte islands embedded in the necrotic tissue [30]. Understanding the significance of these cell-to-cell interactions requires further investigation.

In conclusion, ES induces both morphological and biochemical changes in fibroblasts resulting in a shift toward a striated muscle cell phenotype. These changes are associated with a significant cell loss initially and careful adjustment of stimulation parameters will be required reduce this event. Remarkably, these phenotypic changes could be achieved without any additional treatment, media supplementation or co-culturing. The partially differentiated phenotype achieved by this method could allow a gradual, ongoing differentiation within the cardiac environment providing time for electro-mechanical integration, improving treatment efficiency and realizing a convenient alternative for clinical applications.

Acknowledgments

We thank Francisco Silva and Jane Pham for their expert assistance. These studies were supported by the Heart, Lung, and Esophageal Surgery Institute, University of Pittsburgh Medical Center and McGowan Institute for Regenerative Medicine, a State Grant from Pennsylvania and the Gamal and Cheryl Tawfik Foundation.

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