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Electrical stimulation changes human mesenchymal stem cells orientation and cytoskeleton organization

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Abstract

Electricity, as a physical stimulus, is recently becoming an attractive tool for tissue engineering. In this study we simulated the electrical field delivered by a simplified electro-bioreactor, using finite element analysis. In addition, human mesenchymal stem cells (hMSCs) were cultured in an electro-bioreactor and an electrical field of 100 mV/mm for 1 hour per day was applied. The cell profile, orientation and cytoskeleton changes by CellProfiler was analysed at 1, 2, 3 and 7 days. The cytoskeleton texture of cells exposed to electrical stimulation was also compared with cells exposed to chemical stimulation during an early phase of osteogenic differentiation. Results showed that hMSCs orientation and cytoskeleton actin filaments reorganize perpendicular to the electrical field in the vicinity of the cathode area at day 7. This finding and analysis method has the potential to provide a framework for future studies of mechanism underlying the changes in cell profile in electrical fields.

Keywords: Electrical stimulation, Cell orientation, Computational modelling, Cytoskeleton reorganization

Introduction

Tissue engineering is a growing area of science, combining cells and stimuli, to direct the cells to differentiate into the desired phenotype within a specific time (Grayson, Martens, Eng, Radisic, & Vunjak-Novakovic, 2009). The concept of the endogenous electrical stimuli has been proven to be involved in important physiological processes such as endothelial tissue healing as well as complex tissue regeneration (Levin, 2009; Nuccitelli, 2003). Accordingly, exogenous electrical stimulation (ES) has been taken into account, as one of the efficient physical stimuli, which is able to increase the healing and/or regeneration capacity of the various tissues (McCaig, Song, & Rajnicek, 2009). To have a better understanding of the function of ES and to optimize the conditions for tissue bioengineering and/or tissue regeneration, it is essential to study the changes in stem cell behaviours in presence and absence of the electrical field (Titushkin & Cho, 2007). *In vitro*, electrical fields have been shown to influence the reorganisation of the cell membrane receptors (Griffin & Bayat, 2011), as well as the cytoskeleton (Sun, Titushkin, & Cho, 2006; Titushkin & Cho, 2009). Hammrick *et al.* showed that mouse adipose-derived stromal cells dramatically reorganize their cytoskeleton in response to the ES and these morphological changes lead to changes in function (Hammerick, Longaker, & Prinz, 2010; Kim et al., 2006). A cell's ability to adapt changes to cytoskeleton structure has been linked with differentiation commitment as shown in a study on mesenchymal stem cells and osteoblasts by Titushkin *et al.* (Titushkin & Cho, 2007, 2009). Several studies have recorded an increase in osteoblast lineage markers such as alkaline phosphatase and osteopontin in the stem cells that received daily electrical stimulation (Balint, Cassidy, Hidalgo-Bastida, & Cartmell, 2013; Mobini, Leppik, & Barker, 2016).

According to the effective role of cytoskeleton organization in cell mechanics, stability and motility which is able to promote subsequent functions such as the differentiation rate, we have investigated the changes in cytoskeleton actin filaments organization and orientation of human mesenchymal stem cells (hMSCs), in response to 100 mV/mm directly applied DC ES and compared it to the responses of chemical stress, applied by exposing the cells to the osteogenic supplies.

Materials and Methods

To apply ES to the cells, a simplified electro-bioreactor was utilized which was described previously (Mobini et al., 2016). Commercial finite-element analysis software, COMSOL Multiphysics® (version 5.1, COMSOL, UK) was used to model the electro-bioreactor and simulate the electrical field distribution. The detailed parameters are described in Table 1. Human mesenchymal stem cells from bone marrow obtained from (PT-2501, Lonza Group Ltd, Germany). Frozen vials of cells were thawed, cultured and expanded to reach the desired number, based on the cell provider's instructions. 32 mm diameter glass coverslips (VWR International, Radnor, PA, USA) were sterilised in 70% ethanol and rinsed twice with sterile phosphate buffer saline (PBS) (Gibco®, Gaithersburg, MD, USA) and placed in 6-well cell culture plates (TPP, Trasadingen, Switzerland) prior the seeding day. Cells of passage 6 were seeded on the glass coverslip at a density of 9×10^4 cells/well, in a cell growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM) high glucose + 10% Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin (10.000U/ml) all obtained from (Sigma-Aldrich, Hamburg, Germany), placed in a humidified incubator at 37 °C, 5% CO₂. The culture medium was changed initially, one day after seeding and then twice weekly. Electrical stimulation was applied using the described device (Mobini et al., 2016). Half of the samples in control group were exposed to 10^{-8} M Dexamethasone, 10 mM β -glycerophosphate, and 0.2 mM Ascorbic acid-2-phosphate (Sigma-Aldrich, Germany), to chemically induce osteogenic differentiation. The cells of the experimental groups were exposed to 100mV/mm of DC ES for 1 hour per day for 1, 2, 3 and 7 days. All evaluations and assays were performed immediately after last exposure to the electrical field. Cells were fixed in formaldehyde 10%, permeabilised with Triton-X100, stained with 100 μ l Phalloidin solution/cm² and carefully mounted with Prolong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific Inc, MA, USA) and viewed using fluorescence light microscopy (Nikon Eclipse 50i, equipped with Lucia GF -DXM1200 version 4.82- imaging software). Individual cell orientation (relative angle to the electrical field, Figure 1 C) and actin filament orientation of each image, captured from different locations (Anode, Centre and Cathode),

Figure 1 B, were analysed using CellProfiler, version 2.1.1, (The Broad Institute, MA, USA, available from: www.cellprofiler.org). Description of the pipelines is provided in the supplementary document. To visualize the complied data from the CellProfiler software, a custom-made application in the C# programming language was used.

Results and Discussions

Simulation results show that the average electric field strength on the whole cell culture surface was 85 mV/mm. In the anode, cathode and center areas, the average electric field strength was 110, 110 and 83 mV/mm, respectively. On the cell culture area, the electric field distributed more evenly between the electrodes with increasing electric field strength around the electrodes. In the peripheral areas parallel to electric field direction, the electric field strength diminished towards the edge. In the peripheral areas perpendicular to electric field direction, the electric field strength was suggestively smaller to average values as seen in Figure 1 A. There was no long term change in pH or temperature and these have been measured (data not shown).

More than 50 images of different regions, close to either the anode, the center or the cathode areas, were analyzed in different conditions and area of the cell culture well plate, to define the configuration of the cell orientation and actin filaments in each sample. While orientation and cytoskeleton of the cells has been changed significantly in the vicinity of the cathode area, changes were not distinguishable in the center and not detectable in the vicinity of the anode area. Figure 2 demonstrate hMSCs cytoskeleton texture, in the vicinity of the cathode area at day three. In these microscopic images, cells appear to show denser f-actin texture in electrically stimulated group when compared to the cells with no electrical stimulation. The cytoskeleton of the electrically stimulated cells is similar to those, which are not electrically stimulated, but were exposed to the osteogenic supplemented medium. Similarities in the cell response to the electrical and chemical stress, implies that both stress sources could trigger the same pathways in the cells. Differentiation can be estimated by morphological changes in cell cytoskeleton. It is reported that rearrangement

of the actin bundles into dense actin fibre networks is considered as a single of differentiation (Treiser et al., 2010).

Figure 3 A displays the outline of the cells in different conditions at day 7. Figures 3 B and C demonstrate the fluorescent images of the same cells at 10X and 60X magnification, respectively. These results only referred to the cells in the vicinity of the cathode area, since the most discriminated response of the cells have been observed in the cathode region rather than center and anode area. This could be explained according to the specific chemical environment near the cathode, due to attraction of the positively charged ions such as calcium. Calcium ion flux across the membrane is assumed to trigger various intercellular signalling pathways (Titushkin & Cho, 2009). Onuma *et al.* showed that cell shape and orientation changes have been linked to the calcium-dependant processes by observations on cytoskeleton redistribution in response to stimulus with or without calcium channel blockers and calmodulin antagonists (Onuma & Hui, 1988). Additionally, Sun *et al.* showed that Ca^{2+} oscillation has been directly linked with hMSCs' differentiation stage (Sun et al., 2006). This Ca^{2+} oscillation profile can be imminently changed by application of the electrical stimulation. Although the distinct chemical environment near the cathode could be the main reason to get the majority of the responses near the cathode, the higher voltage drop due to increased length of unit along the electrical field could be the reason why we cannot see the same effect in the center of the cell culture area (Tandon et al., 2009). Based on the temporal experiments we also speculated that more confluent cultures are more likely to be affected by electrical stimulation.

Figure 3 D, are colour-magnitude graphs, demonstrating the cell orientation in the vicinity of the cathode at day 7. These graphs were created using C# coding, to correlate the percentage of the cells to their specific angle (θ) relevant to the electrical field (orientation), Figure 1 C. θ was calculated for each cell (at least 70 cells per image), from the microscopic images, by CellProfiler software and visualized at each condition using the customized coding. The results show that the cells reoriented parallel the electrodes, which means cells aligned perpendicular to the electrical field. Robison mentioned the perpendicular alignment of the muscle cells in response to the DC electrical

field was interesting as it produces an approximation of the geometrical relationship between neuritis and muscle that occurs *in vivo* (Robinson, 1985). Cooper and Keller also have observed that the perpendicular alignment is a result of the cells attempt to minimize the perturbing effect of the field on the membrane potential (McCaig et al., 2009). Schmidt and co-workers, showed the alignment of human dermal fibroblast is enhanced by a DC current (Hardy et al., 2015). We also observed cell alignment results are quite similar to the distribution of actin filament orientation, in the same condition. Figure 3 E, shows another set of color-magnitude graphs, created in same way as the figure 3 D, to visualize actin filaments orientation. Similarities between two set of graphs (cytoskeleton and the cell orientation) suggests orientation of the cells directly linked to the actin cytoskeleton reorganization (Titushkin & Cho, 2007). It is also shown that depolymerisation of the actin cytoskeleton induces nuclear trafficking of regulatory proteins and global effects on gene transcription. The continued presence of intra-nuclear actin, which forms rod-like structures that stain with phalloidin, is associated with induction of robust expression of the osteogenic genes leads to acquisition of osteogenic phenotype (Sen et al., 2015).

Conclusion

In conclusion, human mesenchymal stem cells react to the 100 mV/mm DC electrical field. This response is quite evident in cell and cytoskeleton reorientation perpendicular to the electrical field in the vicinity of the cathode area. In addition, the changes in the texture of actin filaments are comparable to the changes forced by chemical stress. In this study, the chemical stress applied by osteogenic differentiation ingredients. The results of this study may help our understanding of the mechanism underlying this phenomenon, which could give a clue to optimization of osteogenic differentiation using electrical stimulation for tissue engineering applications.

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Figures:

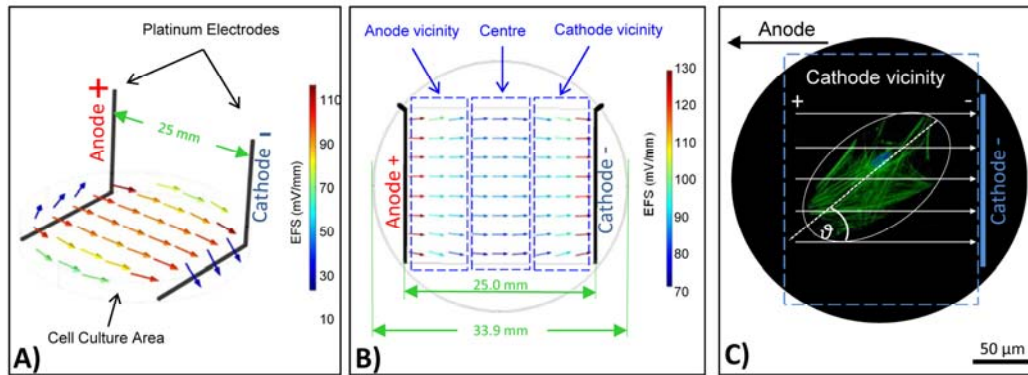


Figure 1. Computational modeling and simulation analysis. A) Computational model of the electro-bioreactor and simulation of the electrical field within the cell culture area, B) Electrical field strength distribution in the anode, center and the cathode area of the cell culture well. C) The (θ) angle of the cells relevant to the electrical field, which is measured by the CellProfile software to determine the orientation.

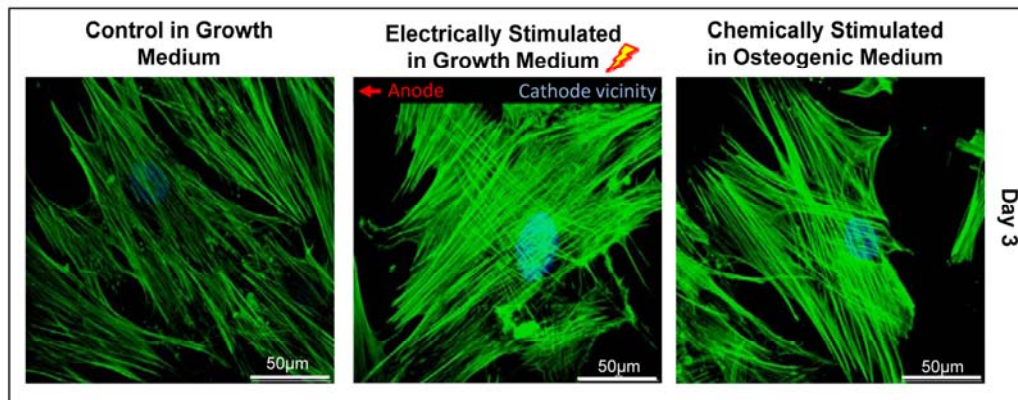


Figure 2. Cell profile analysis at day 3 in the vicinity of the cathode area. Immunofluorescence images of the cytoskeleton (F-actin) and nuclei of human mesenchymal stem cells (60X), in different conditions; Non-electrically stimulated in growth medium (control group), electrically stimulated in growth medium (electrically stimulated group) and non-electrically stimulated in osteogenic induced medium (chemically stimulated group).

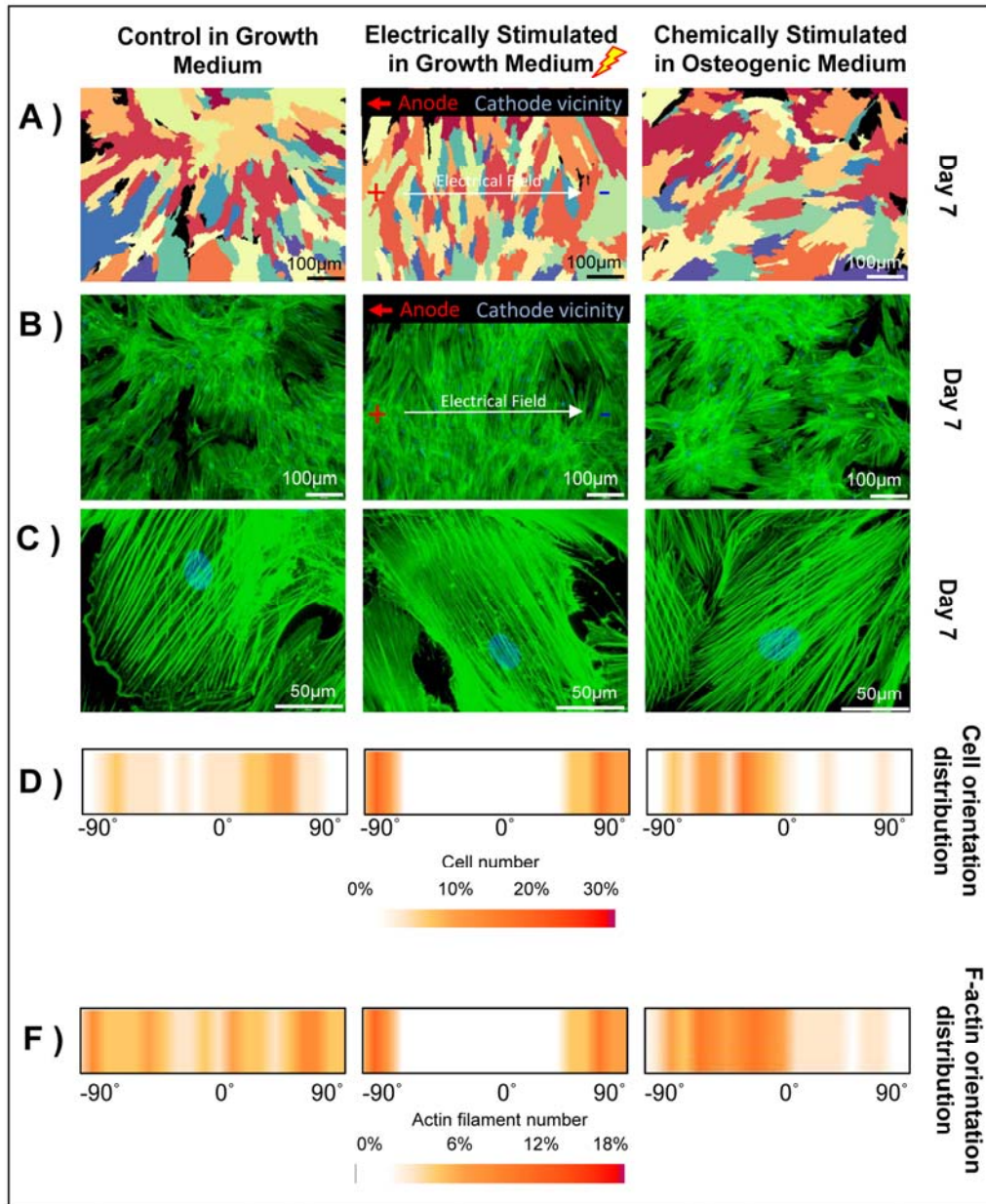


Figure 3. Cell profile analysis at day 7 in the vicinity of the cathode area.

A) CellProfiler analysis of the cell outlines based on the intensity and orientation of the cytoskeleton and distance from the nucleus. B) Immunofluorescence images of the cytoskeleton (F-actin) and nuclei of the human mesenchymal stem cells (10X), C) (60X). D) Distribution of the cell orientation, and E) actin filament orientation. All experiments assessed in three different conditions; Non-electrically stimulated in growth medium (control group), electrically stimulated in growth medium (electrically stimulated group) and non-electrically stimulated in osteogenic induced medium (chemically stimulated group).