Calcium Oscillations In Differentiating Mesenchymal Stem Cells: Analysis And Control Using Pulsed Electric Fields

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Abstract

Mesenchymal Stem Cells or Multipotent Stromal Cells (MSCs) are adult stem cells able to differentiate into many cell types such as osteoblasts, adipocytes or chondrocytes. These cells have become of particular interest for cell-based therapy these last decades. In another respect, calcium is a ubiquitous secondary cell messenger, known to encode important information for the cells in the form of oscillations. It has been shown that MSCs naturally exhibit calcium oscillations, whose frequency is varying over the course of differentiation processes. The main question addressed in this work is to assess whether, by manipulating the frequency of calcium oscillations, we could influence proliferation or differentiation events in MSCs. The technology used for this purpose involves the use of short high voltage pulsed electric fields capable of permeabilizing the cell membrane to calcium ions presents in the surrounding medium. Long-term exposure of the cells to the pulsed electric fields was performed using a programmable generator along with an original custom device to expose the cells under regular attached cell culture conditions. We report the experimental constraints that were found and how they were solved.

1 Introduction

Mesenchymal Stem Cells (MSCs) are adult stem cells able to give rise to various cells types constituting connective tissues in the body, such as osteoblasts, adipocytes or chondrocytes [1]. Also, a general excitement arose when some more recent studies showed that their multipotency might extend to muscle [2] or even neuron-like cells [3]. These cells have caught more and more interest for clinical application purpose not only due to their promising multipotency, but also to their secretory activity [4] as well as the rescuing functions that they can exert towards damaged cells in the body [5]. The purpose of our work focuses on the abilities of MSCs to differentiate into various cells types. It was observed that the MSCs naturally exhibit spontaneous calcium oscillations [6] whose frequency varies over the course of differentiation processes. It is well known that calcium is an important cellular secondary messenger and that variations in the frequency and/or in the amplitude of the calcium oscillations can be observed in response to some stimuli [7]. In our case, we are interested in the changes that might be triggered by stimuli inducing cellular proliferation or differentiation events. Downstream of these stimuli, calcium oscillation frequency and/or amplitude can embed important information, subsequently decoded by some proteins in the cell whose activity is calcium-sensitive, resulting in “direct” action of effector proteins or activation of gene transcription [7]. Thus, it is legitimate to wonder whether taking the control over calcium oscillations frequency and/or amplitude might mimic the effects of the physiological stimuli, bypassing them by directly triggering the secondary messenger response.

Therefore, the question was how to take the control over calcium oscillation frequency and/or amplitude? Previous studies in our laboratory have shown that applying high amplitude ultrashort electric pulses able to permeabilize the cell membrane to calcium ions can induce calcium oscillations similar to the naturally observed ones (in shape, amplitude and duration) [8]. The small amount of external calcium crossing the electropermeabilized membrane and penetrating the cell causes, through the “calcium-induced calcium-release response” mediated by IP3 receptors of the endoplasmic reticulum, the rising phase of the oscillation. The subsequent elimination of the cytoplasmic calcium excess, resulting from the activity of the SERCA ATPase that pumps the calcium back to the endoplasmic reticulum or of the activity of PMCA ATPase and NCX Na+/Ca+ exchanger that release the calcium to the extracellular space, explains the falling phase of the oscillation [8]. Thus, a weak electroporation to the calcium ions can produce physiological-like calcium oscillations.

MSCs were initially isolated from bone marrow, but they can actually be isolated from various sources such as dental pulp, placenta, umbilical cord or adipose tissue, the latter presenting the advantage to easily provide a high quantity of MSCs compared to the other sources [9]. For these reasons, we used adipose derived MSCs, also called Adipose Stem Cells (ASCs) in our study.

2 Materials and Methods

Cell culture: ASCs were isolated from a human lipoaspirate with the consent of the patient. Cells were grown in Dulbecco Modified Eagle Medium (GIBCO, Courtaboeuf,

France) supplemented with 10% Foetal bovine serum (Sigma Aldrich, Saint Quentin Fallavier, France) 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO) (complete DMEM) and incubated at 37°C in a humidified incubator with 5% CO_2.

Differentiation: Prior to differentiation, cells were seeded at 8 000 cells/cm^2 and let in culture for 2 to 3 additional days to reach confluency. All chemicals used to supplement culture media were purchased from Sigma Aldrich. Cells were induced in adipogenic differentiation with an induction medium composed of complete DMEM supplemented with 1 µM of dexamethasone, 200 µM of indomethacin, 500 µM of IBMX and 10 µg/mL of insulin used in alternance with a maintenance medium composed of complete DMEM supplemented with 10 µg/mL of insulin. An induction/maintenance cycle lasted 5 days with 3 days in induction phase followed by 2 days in maintenance phase. This cycle was repeated over the 22 days of differentiation.

Calcium oscillations monitoring: Calcium oscillations were monitored in ASCs induced in adipogenic differentiation at different time points. Prior to microscopy recording, cells were incubated with Fluo-4 AM ($\lambda ex = 494$ nm / $\lambda em = 506$ nm) (Invitrogen, Courtaboeuf, France) at 5 µM and Hoechst 33342 ($\lambda ex = 361$ nm / $\lambda em = 497$ nm) (Sigma Aldrich) at 0,2 µg/mL for 30 min at 37°C, 5% CO_2 in the conditioned medium to avoid inducing any perturbations. Time lapse microscopy analysis was performed with Axio Observer microscope (Zeiss, Marly le Roi, France). Still in the optics to avoid perturbations of the system, cells were maintained at 37°C, 5% CO_2 during the acquisition. Acquisitions lasted 15 minutes with 1 picture recorded each 10 seconds (excitation filter = 475 nm FWHM 35 nm / dichroic filter transmission band = 508-675 nm)

Calcium oscillations analysis: Time lapse microscopy movies were analyzed with CellProfiler image analysis software to provide raw data of Fluo-4 fluorescence intensities linked to cytosolic calcium concentrations. These data were subsequently analyzed with a custom version of the "Spectral Analysis of calcium oscillations" Matlab program made by Uhlén [10] to extract principal frequencies of calcium signals.

Electric pulses generator: The generator used in this study was built in University of Zaragoza, Spain. It is constituted of several capacitor banks working as multiplicators able to multiply the voltage of the DC source. This generator can deliver impulsions whose duration ranges from 1 to 100 µs. The output voltage can reach 1000 V.

Electrodes / exposure chamber: The first sets of electrodes were built at desired dimensions from stainless steel sheet (RS PRO, Beauvais, France). The second sets of electrodes in grade 2 titanium were purchased from Titanium Services France, Vourles, France.

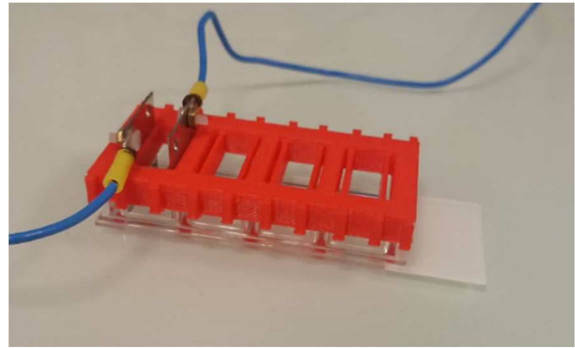

Figure 1 First exposure chamber system composed of Sarstedt 4 well tissue culture chamber along with a custom lid used as electrode guide.

The first type of exposure chambers was composed of 4 well tissue culture chamber (Sarstedt, Marnay, France) with a custom lid used as electrode guide (Figure 1).

The second type of exposure chambers was composed of p35 petri dish (Corning, Boulogne-Billancourt, France) in which a custom PDMS mold was used to create a rectangular chamber with desired dimensions. A custom lid was as well used as electrode guide. This chamber is based on the setup described in reference [11].

3 Results and Discussion

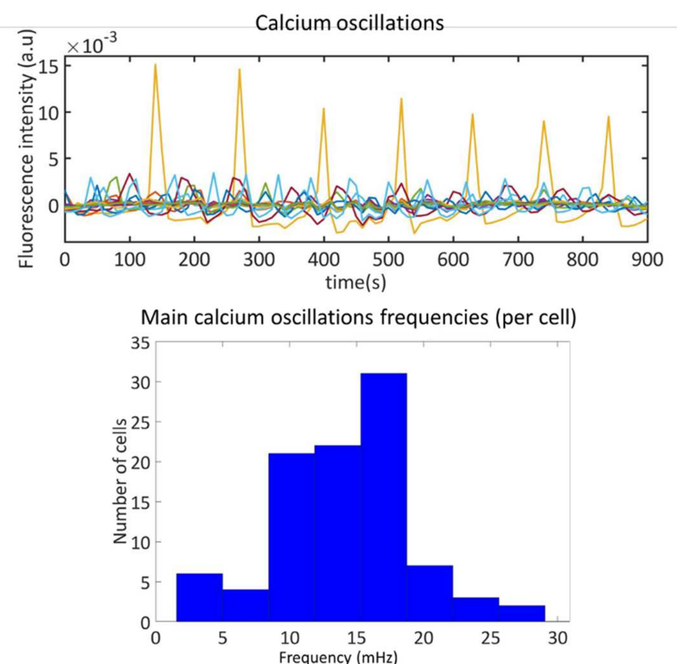

Figure 2 Calcium oscillation of ASCs (non differentiated) in standard culture conditions (upper panel, data on only 20 cells for the clarity of the image) and main frequencies extracted from the spectral analysis of each cell reported as histogram (lower panel, data shown on all cells, here on 129 cells).

As a first piece of the puzzle, we analyzed the variations of calcium oscillations frequencies when the differentiation is induced by classical chemical stimuli. To that end, Fluo-4 AM cell-permeant calcium indicator was used to monitor calcium level variations by means of time lapse microscopy acquisitions at different time points of the differentiation. Hoechst 33342 staining was also performed in order to facilitate the analysis, allowing to easily delimitate cell nuclei whose calcium oscillations are the same than the cytoplasmic ones. Nuclei trimming and fluorescence intensity measurements were performed with the help of CellProfiler image analysis software. Spectral analysis was subsequently performed on the raw data to retrieve the principal frequency of the calcium signal for each analyzed cell (up to several hundred cells per experimental point). This analysis allowed us to identify changes in calcium oscillation frequencies. Indeed, we observed that at the end of the differentiation process, most of the ASCs induced in adipogenic differentiation did not display calcium oscillations in the time window of analysis as non-differentiated ASCs did as shown in figure 2 and 3. Of note, the cells that were still oscillating after 22 days of adipogenic differentiation were the ones that did not exhibit adipocyte morphological features (data not shown).

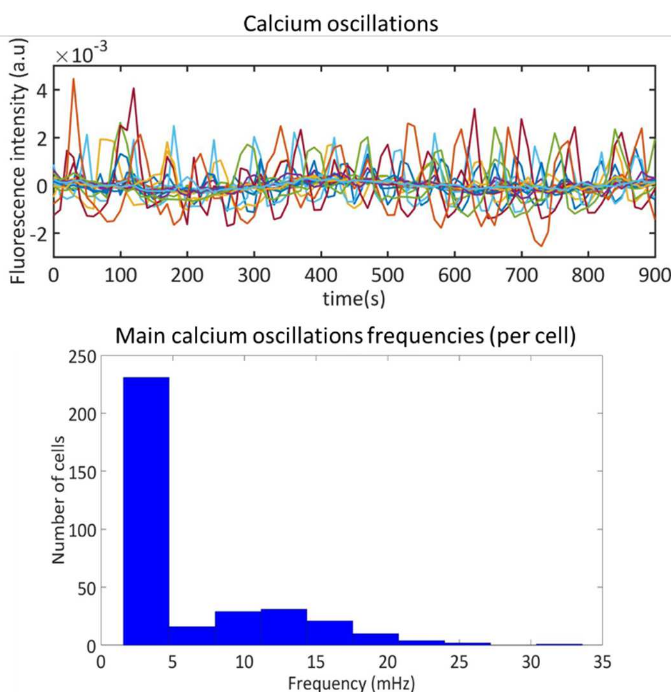

Figure 3 Calcium oscillation of ASCs induced in the adipogenic differentiation for 22 days (upper panel, data shown on only 20 cells for the clarity of the image) and main frequencies extracted from the spectral analysis of each cell reported as histogram (lower panel, data shown on all cells, here on 345 cells). 65 % of cells were not displaying oscillations.

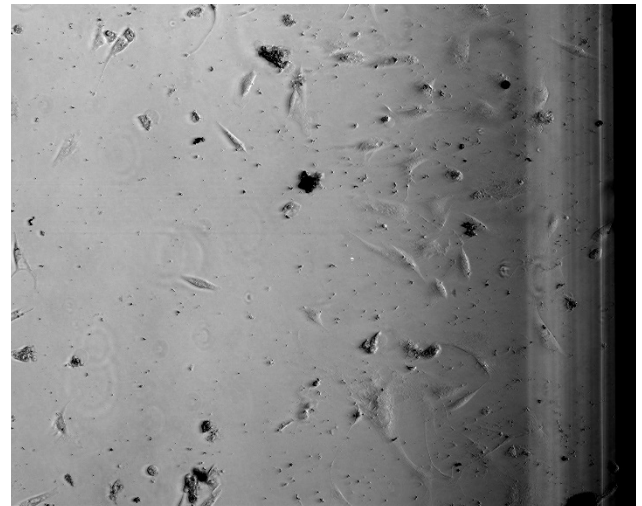

Figure 4 Microscopy picture showing the release of metallic particles (black spots) in the cell medium subsequent to electrode degradation.

In a second step, our study was aiming at taking the control over calcium oscillations by using pulsed electric fields permeabilizing MSCs membranes to calcium ions. These pulsed electric fields were delivered with plate electrodes sets, arranged on both sides of the culture wells as shown in figure 1. The first sets of electrodes used in our study were made of stainless steel, however, this material turned out to be unsuitable in the context of our experiments. Indeed, our experimental design implies long-term exposure of the cells to pulsed electric fields, what promoted occurrence of highly unwanted electrochemical reactions, with progressive deteriorations of the electrodes (pitting corrosion) and release of metallic particles in the cell medium (as shown in figure 4). In order to reduce to the maximum these unwanted effects that might have consequent cytotoxic effects, we chose to use electrodes made of titanium, material known for its high capacitance, allowing to slow down the charging process of the double layer capacitance at the surface of the electrodes, and subsequently electrochemical reactions [12].

Using the first exposure system that we designed, we also dealt with issues linked to capillarity phenomena. Indeed, when the electrodes were inserted in the hollow spaces of the lid fitting on the wells, the culture medium penetrated this narrow space by capillarity and leaked from the wells. To fix this problem, we designed new exposition systems inspired by the setup described in reference [11].

This work is still in progress, but the targeted changes in the calcium oscillations are now defined and the tools to achieve the desired oscillation patterns are developed. In this context it is important to note that we have established that ASCs can be treated with pulses generating a weak electroporation to calcium in a long-term fashion.

4 Conclusion

Our goal is to modify biological cells physiology by electric means, namely, to control cells differentiation through the delivery of short and intense electric pulses for long periods. The means to achieve this goal is the perturbation of the rhythm of the spontaneous calcium oscillations through the penetration of external calcium in transiently electropermeabilized cells. We previously reported the proof of principle, but exposures were limited to a few minutes, insufficient to provoke long-term changes in the cells.

In our efforts to achieve our goal, we have found physical, chemical and biological limitations due to the long duration of the cell exposure to electroporating electric pulses. To overcome these limitations, a new generator has been developed, able to control and to deliver the pulses over well defined, long periods. Because of electrochemical reactions at the electrodes, we had to elaborate electrodes in more inert materials such as titanium. Temperature changes were not observed, as expected considering the very low average power deposited in the exposure chambers. The numerical methods for quick analysis of the calcium oscillations in a large number of cells have been developed. The methods and set-up for the exposure of the cells for weeks are now set and the cell physiological reactions can be studied.

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