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Characterization of calcium signals in human embryonic stem cells and in their differentiated offspring by a stably integrated calcium indicator protein

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ABSTRACT

Intracellular calcium signaling pathways play a major role in cellular responses such as proliferation, differentiation and apoptosis. Human embryonic stem cells (hESC) provide new possibilities to explore the development and differentiation of various cell types of the human body. Intracellular calcium responses to various ligands and the calcium signaling pathways, however, have not been thoroughly studied in embryonic stem cells and in their differentiated progenies. In our previous work we demonstrated that the use of the fluorescent calcium indicator Fluo-4 with confocal microscopy allows sensitive and reliable measurements of calcium modulation in human embryonic stem cells and stem-cell derived cardiomyocytes. Here we developed a human embryonic stem cell line stably expressing a genetically encoded Ca^{2+} indicator (GCaMP2) using a transposon-based gene delivery system. We found that the differentiation properties were fully preserved in the GCaMP2-expressing hESC lines and Ca imaging could be performed without the need of toxic dye-loading of the cells. In undifferentiated hES cells the calcium signals induced by various ligands, ATP, LPA, trypsin or angiotensin II were comparable to those in Fluo-4 loaded cells. In accordance with previous findings, no calcium signal was evoked by thrombin, histamine or GABA. Cardiomyocyte colonies differentiated from hES-GCaMP2 cells could be recognized by spontaneous contractions and Ca^{2+} oscillations. GCaMP2-expressing neural cells were identified based on their morphological and immuno-staining properties and Ca signals were characterized on those cells. Characteristics of both the spontaneous and ligand-induced Ca^{2+} signals, as well as their pharmacological modification could be successfully examined in these model cells by fluorescence imaging.

1. Introduction

Human embryonic stem cell (hESC) lines are unique from several points of view: these cells with a normal genetic background can not only be maintained and propagated in a pluripotent state but can also be differentiated towards selected human tissues [1,2]. These features make them a favorable model system, especially for drug screening and signal transduction research. Immortalized or tumor cell lines used alternatively in such experiments could have major alterations in their genetic background, especially in the signal transduction system pathways, while human primary cells cannot be properly propagated in vitro.

There is a rapidly increasing number of data on the signaling pathways in human pluripotent stem cells, underlying both self-renewal and differentiation towards various tissue types [3–5]. The transcriptional regulation of pluripotency and self-renewal in stem cells has been recently reviewed [6], and the key growth factors and morphogens maintaining pluripotency have been already identified [7–11]. At the same time, the exact mechanisms of signaling events have only been scarcely studied [12,13], and there is very little information about the calcium signaling events in human pluripotent stem cells.

The special culturing conditions required for hESCs, the existence of heterogeneous cell populations even in the pluripotent state [14], and the mixture of different cell types during differentiation make these studies challenging. In our previous paper (see [15]) we have used the fluorescent calcium indicator Fluo-4 and confocal microscopy to allow sensitive and reliable measurements of calcium modulation in hESCs. Besides the major methodological problems in estimating intracellular calcium we emphasized the drawbacks of calcium sensitive dyes, such as the reproducibility of the loading procedure, toxicity of the cleaved

Abbreviations: hESC, human embryonic stem cell; GECI, genetically encoded calcium indicators; MEF, mouse embryonic fibroblast; LPA, lisophosphatidic acid; GABA, gamma-aminobutyric acid.

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dye, variable distribution of the dye among the cells and inside a particular cell, bleaching problems, and the dynamic range variations.

One possible solution to overcome these problems is the usage of genetically engineered calcium sensors [16]. Light emitted from these fluorescent proteins varies with the amount of calcium present in the cells, thus changes in the levels of fluorescence can be directly used to determine the changes in calcium concentration. Genetically engineered calcium indicators (GECIs) offer unique opportunities to visualize cells during differentiation, or upon transplantation into a host system. The development of GECIs resulted in an array of indicators with different optical properties to enable in vivo measurements within selected tissues by using tissue specific promoters (see [17–20]). GECIs have also been used as quantitative calcium sensors in vitro in tissue preparations [21] or various intracellular compartments [22]. Although the GECIs are already widely used in biology (as reviewed in [23,24]) there is still only a limited amount of data available about their application in mouse pluripotent stem cells and differentiated tissues (see [25]), while no such data have been published for a human system.

In the present work we used a version of the GECI series, GCaMP2, and applied a highly efficient transposon (*Sleeping Beauty*) gene delivery technique to stably integrate this calcium indicator into human embryonic stem cells. Here we document the applicability of this approach for obtaining GCaMP2 cell lines, and measuring calcium signals evoked by well known calcium mobilizing agents both in pluripotent embryonic stem cells, as well as in stem cell derived cardiomyocytes and neural cells.

2. Materials and methods

2.1. Cell culture

The human embryonic stem cell line HUES9 (originally provided by Dr. Douglas Melton, Harvard University) and its GCaMP2 expressing stable derivative (HUES9-GCaMP2) were maintained essentially as described earlier, using cells from passage no. 35 [26]. Cells were cultured on mitomycin-C treated mouse embryonic fibroblast (MEF) feeder cells. The SB-CAG-GCaMP2 fluorescent calcium probe was generated from a DNA fragment encoding GCaMP2 (the pN1-GCaMP2 plasmid, a generous gift of Junichi Nakai, RIKEN Brain Science Institute, Saitama, Japan [27]). The plasmid used in this project contained the cDNA of the fluorescent marker GCaMP2 driven by the CAG promoter and a CAG promoter driven puromycin resistant gene (Fig. 1A). To create GCaMP2 transgene expressing clones, we applied our previously developed method for human embryonic stem cell lines, by using the *Sleeping Beauty* transposon system [28]. The copy numbers of selected GCaMP2 expressing clones were determined as described previously [29]. The copy number varied between 20 and 22 in the studied clones.

Pluripotency markers in the HUES9-GCaMP2 clones were examined by immunostaining (Oct4 and Nanog), and by flow cytometry measurement of the SSEA4 cell surface marker (see [26]). Spontaneous differentiation of the hESCs towards cardiac and neural directions was performed via embryoid body (EB) formation as described previously [28]. The neural differentiation was supported by addition of 4 ng/ml bFGF into culture media during EB formation.

2.2. Calcium signal measurements

The human embryonic stem cells (HUES9 and HUES9-GCaMP2) were cultured in a pluripotent state on mouse embryonic feeder (MEF) cells. Before calcium measurements, cells were seeded for two days onto eight-well Nunc Lab-Tek II Chambered Coverglass (Nalge Nunc International, Rochester, NY) covered by MEF feeder cells. For Fluo-4 studies the HUES9 cells were loaded for 30 min at 37 °C with 1.0 μM Fluo-4 AM in a serum free culture medium. Extracellular Fluo-4 AM was removed by changing the medium to Hanks' balanced salt solution supplemented with 20 mM Hepes (pH = 7.4) and 0.9 mM $MgCl_2$ (HBSS) — all the actual measurements were performed in HBSS. The ligand concentrations were chosen according to literature: ATP (100 μM), trypsin (2.5 μg/ml), lysophosphatidic acid (LPA — 10 μM), angiotensin II (10 μM), histamine (100 μM), caffein (4 mM), adrenalin (10 μg/ml), verapamil (1.5 μM), ionomycin (5 μM), thrombin (5 U/ml) and gamma-aminobutyric acid (GABA — 1 mM). For calibration procedures EGTA was used in 5× excess of calcium concentration in the medium. All the experiments were done in room temperature. Calcium signal measurements were carried out by following time lapse sequences of cellular fluorescence recordings and images were analyzed with the FluoView Tiempo (v4.3, Olympus, http://www.olympusmicro.com) software. For G-CaMP2 imaging cells were excited with the 488 nm laser line and emission was measured between 505 and 535 nm. Under these conditions the emitted fluorescence is proportional to the cytosolic free calcium concentrations [27]. In the confocal images we used the artificial coloring instead of green for better visualization of changes in calcium concentration.

3. Results

3.1. Generation and characterization of GCaMP2 expressing hES cells

Delivery of the GCaMP2 cDNA construct into HUES9 cells was carried out by using the *Sleeping Beauty* transposon system as described earlier [28]. The transfected cells showed low but visible fluorescence after 48 h of transfection. This fluorescence intensity did not give a solid basis for a cell sorting procedure, therefore we first enriched the transgene positive cells by puromycin selection using the resistance gene also present in the transposon construct (Fig. 1A).

The surviving cells were further propagated on mitomycin-C treated MEF cells and the appearing positive clumps were mechanically passaged to new feeder layers. By this procedure we managed to establish several GCaMP2 expressing HUES9 clones with different but homogenous fluorescence intensities in each clone. Three clones were chosen for further characterization of pluripotency and transgene copy number determinations. All the studied clones were in a pluripotent state after the cloning procedure, as testified by the flow cytometry analysis of the SSEA4 cell surface marker and by immunostaining of the key transcription factors Oct4 and Nanog (Fig. 1B and C). These clones also had the spontaneous differentiation capacity similar to the original HUES9 cells (see below).

3.2. Comparison of calcium signals measured by GCaMP2 calcium indicator and Fluo-4 the in pluripotent hESCs

In the following sets of experiments we compared the Ca signals in undifferentiated hESCs using confocal microscopy as we described earlier [15]. Parental HUES9 and HUES9-GCaMP2 cells were seeded into confocal microscopy chambers on MEF cells for 2 days before experiments. HUES9 cells were loaded with the Fluo-4 AM dye before the measurements (see Materials and methods). After the determination of baseline fluorescence, the cells were treated with specific calcium signal inducing agents. For determination of Fmax and Fmin, high concentrations of ionomycin (5 μM) and EGTA were used consecutively in each experiment.

As documented in Fig. 2A and Supplementary Fig. 1A and B, we could detect well measurable calcium signals induced by ATP, trypsin, lisophosphatidic acid (LPA) and angiotensin II both in GCaMP2 expressing and Fluo-4 loaded pluripotent HUES9 cells, while there were no calcium signals after histamine, thrombin or GABA treatment (data not shown). In these basic experiments we measured the average signal levels in independent hESC clumps, and during data processing we excluded the calcium level changes in the surrounding MEF cells (Fig. 2B). The development of the calcium signals in the hESCs was basically similar in the case of both fluorescent calcium indicators (GCaMP2 or Fluo-4) applied. However, there were apparent differences in the magnitude and the dynamics of these signals,