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In situ label-free quantification of human pluripotent stem cells with electrochemical potential

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ABSTRACT

Conventional methods for quantification of undifferentiated pluripotent stem cells such as fluorescence-activated cell sorting and real-time PCR analysis have technical limitations in terms of their sensitivity and recyclability. Herein, we designed a real-time *in situ* label-free monitoring system on the basis of a specific electrochemical signature of human pluripotent stem cells *in vitro*. The intensity of the signal of hPSCs highly corresponded to the cell number and remained consistent in a mixed population with differentiated cells. The electrical charge used for monitoring did not markedly affect the proliferation rate or molecular characteristics of differentiated human aortic smooth muscle cells. After YM155 treatment to ablate undifferentiated hPSCs, their specific signal was significantly reduced. This suggests that detection of the specific electrochemical signature of hPSCs would be a valid approach to monitor potential contamination of undifferentiated hPSCs, which can assess the risk of teratoma formation efficiently and economically.

1. Introduction

Because of recent success in establishing transgene-free induced pluripotent stem cells (iPSCs) [1] and nuclear transfer embryonic stem cells [2], immune rejection, which is one of the most serious obstacles to clinical application of pluripotent stem cell (PSC)-based therapy, could be avoided by autologous transplantation of stem cells derived from patient-specific cells [3]. However, the risk of teratoma formation from residual undifferentiated PSCs during cell therapy has not been fully resolved [4].

A wide range of approaches has been tested to reduce the risk of teratoma formation from undifferentiated human pluripotent stem cells (hPSCs, extensively summarized in a review article [5]), which aim to selectively isolate differentiated cells or to selectively induce the death of residual undifferentiated PSCs using a cytotoxic antibody [6,7], small molecules [8,9], or a suicide gene system [10,11]. After ablation of undifferentiated hPSCs, their successful elimination should be confirmed *in vitro* by fluorescence-activated cell sorting (FACS), real-time PCR analysis, or immunostaining [5,12,13]. However, especially, quantification based on FACS analysis requires pre-labeling process of a target protein with an antibody [14]. Besides, a large portion (more than 10^4 cells) or the entire population of cells, used for real-time PCR analysis or FACS analysis is not recyclable [15]. Therefore, currently available techniques allow neither the recycle of differentiated cells for clinical applications after safety assessment or frequent monitoring. Considering the strict clinical standard for stem cell therapy and the laborious protocols required to attain the desired level of differentiated cells from hPSCs, non-destructive and label-free tools should be developed to determine hPSC contamination in a mixed population.

An electrochemical cell-based biosensor (or cytosensor), which is a rapid, non-destructive, and label-free technique for monitoring

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various cell types (cancer cells and even bacteria), has been developed over the past decade [16,17]. For example, electric cell-substrate impedance sensing (ECIS) of the impedance of an electrode surface at specific frequencies is a well-established technique to monitor cell proliferation [18] and differentiation status of stem cells [19]. Alternatively, our group has established alternative biosensor technique based on cyclic voltammetry (CV) profile [20], which was used for determining redox state of a protein [21]. Because of the unique surface molecules (surface proteins and carbohydrates) of each cell type, cyclic potential changes at the cell/electrode interface generate a peculiar CV profile for each cell type [17], which can be used to determine the effects of anti-cancer drugs [20] and the extracellular redox state [20,22–24].

In this study, we identified a specific electrochemical signature of undifferentiated hPSCs, which is detectable using a simple CV technique on a cell-chip of a specific size. The intensity of the cathodic peak current (i_{pc}) of undifferentiated hPSCs showed clear linearity ($R^2 = 0.99$) to the number of undifferentiated hPSCs, implying that the signal intensity in the cell-chip can be used to determine the number of undifferentiated hPSCs. Moreover, smooth muscle cells differentiated from human induced pluripotent stem cells (hiPSCs) remained viable and their gene expression and karyotype were unaffected by electrochemical measurement. This technique could be applied multiple times for continuous monitoring to ensure the safety of hPSC-based therapy.

2. Materials and methods

2.1. Chemicals and commercial products

Dulbecco's Phosphate buffered saline (DPBS) was purchased from Stem Cell Technologies Inc. (Vancouver, Canada). 4-well plastic chamber (Lab-Tek(R)) suitable for cell culture was obtained from Thermo fisher scientific (USA). Other chemicals used in this study were obtained commercially and were of as reagent grade.

2.2. Fabrication of platinum working electrode

Platinum working electrode was prepared by sputtering of 5 nm thick titanium (Ti) layer on glass, and then sputtering of 50 nm thick gold layer on Ti. The electrode was carefully washed by sonication in absolute alcohol and distilled water for 5 min, and then immersed in a piranha solution (H_2SO_4: H_2O_2 = 7:3) for 5 min at 65 °C. The gold electrode was thoroughly cleaned with 100% alcohol and distilled water, and finally electrochemically cleaned in 10 mM PBS until the stable cyclic voltammogram was obtained. A plastic chamber (2 cm width × 2 cm length × 0.5 cm height) was fixed with polydimethylsiloxane (PDMS) on the working electrode. Matrigel (BD Biosciences) was coated on the working electrode at 1:80 dilution in hESC basal medium (DMEM/F12 supplemented with 1% non-essential amino acids, 0.1% β-mercaptoethanol, and 0.1% gentamycin) for at least 1hr.

2.3. Cell culture

hESCs (H9; Wicell Research Institute, CHA3-hESC; as described previously [25]) and hiPSCs (SES8; as described previously [26]) were cultured in mTeSR1 medium (StemCell Technologies) under feeder-free condition. hASMCs and i-dSMCs were maintained in SMC-specific medium, SMCM (ScienCell Research Laboratories). Human dermal fibroblasts (hDFs) were cultured in high-glucose DMEM medium (Gibco) supplemented with 10% FBS and 0.1% gentamycin. Alkaline phosphatase (AP) staining was performed according to the direction included with the Alkaline Phosphatase Kit (Sigma).

2.4. Electrochemical detection

Cyclic voltammetry (CV) method was carried out with a CHI−660C Potentiostat (CH Instruments, Austin, TX, USA). A fabricated chip consisted of Au electrode served as a working electrode, Ag/AgCl (1M KCl) as the reference electrode, and platinum wire as counter electrode. CV detection was performed between 0.6V and −0.4V at 0.1V/s in mTeSR1. All measurements were replicated at least three times and the cell number was calculated following detection.

2.5. Spontaneous differentiation

Spontaneous differentiation was performed by either embryoid body (EB) formation or direct FBS differentiation. To generate EBs, dissociated hiPSCs were maintained in hESC basal medium containing 20% serum replacement (SR) under suspension condition. EBs were attached on cell-culture plate and maintained in DMEM medium at the indicated days. For direct FBS differentiation, mTeSR1 medium was replaced with DMEM 3days after hESCs seeding, and the hESCs were differentiated until the indicated times.

2.6. FACS analysis and apoptosis assay

Cells were stained with different fluorescent-labeled antibodies, and measured by flow cytometry on a FACSCalibur (BD Biosciences), and then analyzed with FlowJo software. The following conjugated antibodies were used: OCT4 (Abcam), FITC Rat Anti-SSEA-3 and PE Mouse Anti-Human TRA-1-60 (BD Pharminogen). For apoptosis assays, cells were stained with Annexin V and 7-AAD using the PE Annexin V Apoptosis Detection Kit I (BD Pharminogen) and stained using the PE Active Caspase-3 Apoptosis Kit (BD Pharminogen) according to the manufacturer's direction.

2.7. RNA extraction and real-time PCR

Total RNA was isolated using Total RNA Extraction Kit (Intron), and 500 ng total RNA was converted to cDNA using Prime Script RT Master Mix (Takara) in accordance with the manufacturer's instruction. Real-time PCR was performed using SYBR Premix Ex Taq (Takara) on the LightCycler 480 instrument II (Roche). Gene-specific primers used in this study were represented in Table S1.

2.8. Immunocytochemistry and immunoblotting

Briefly, cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100. After blocking with TBS-T containing 3% BSA, they were incubated with the indicated primary antibody overnight. Cells were then washed and incubated with either Cy2- (Jackson ImmunoResearch Laboratories) or Alexa 594- (Life Technologies) conjugated secondary antibody. Nuclei were counterstained with DAPI. Cell images were captured and analyzed using a BX53 research microscope. Primary antibodies used were as followed: SSEA-4 (1:400, R&D Systems), α-Smooth Muscle Actin (1:100, Sigma). Immunoblotting analysis was performed as described previously [27]. Primary antibodies used in present study were as listed: PCNA and PARP-1/2 (Santa Cruz), Cleaved Caspase-3, P-Histone H2A.X (S139), and alpha/beta-Tubulin (Cell Signaling), α-Smooth Muscle Actin (Sigma).