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Membrane permeability of the human pluripotent stem cells to Me_2SO, glycerol and 1,2-propanediol

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ABSTRACT

Due to the unlimited capacity of self-renew and ability to differentiate into derivatives of three germ layers, human pluripotent stem cells, including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have a great potential in regenerative medicine. A major challenge we are facing during the long-term storage of human pluripotent stem cells with the conventional slow cooling rate is the low cell recovery rate after cryopreservation which cannot meet the requirements for the future clinical applications. Evaluating the cell membrane permeability and the corresponding activation energy of hESCs and hiPSCs for water and different cryoprotective agents (CPA), including dimethyl sulfoxide (Me_2SO), 1,2-propandiol and glycerol, is important for facilitating the development of cryopreservation protocol to enhance cell recovery after the cryopreservation. The osmotically inactive volume of hESCs and hiPSCs determined using the Boyle-van't Hoff model was $0.32V_0$ and $0.42V_0$, respectively. The membrane permeability was assessed from the volume changes of cells exposed to Me_2SO, 1,2-propanediol and glycerol at the temperatures ranging from 8 to 30 °C. These results showed the biophysical differences between hESCs and hiPSCs. Their activation energy for water and CPAs extrapolated from the Arrhenius relationship indicated that the water transport was probably not through the channel-mediated mechanism.

Introduction

Human embryonic stem cells (hESCs)[1] with the capability of self-renew and multi-linage differentiation into all types of somatic cell in human body [1] are considered to have an enormous potential for clinical applications, offering therapies for a wide range of degenerative diseases and disorders, such as diabetes and Parkinson's disease [2]. Due to numerous ethic issues of using hESCs derived from human embryos, human induced pluripotent stem cells (hiPSCs) reprogrammed from somatic cells to embryonic stem cell-like cells have become a promising cell source for tissue replacement therapies. However, the successful long-term storage of hESCs and hiPSCs is a prerequisite for their commercialization and clinical applications.

One of the challenges we are facing during the cryopreservation of hESCs and hiPSCs is how to maintain high cell viability and achieve high cell recovery rate. Currently, two kinds of freezing protocols, slow-freezing and vitrification, are applied to cryopreserve hESCs/hiPSCs. Slow-freezing, more practical than the vitrification method, has succeeded in the cryopreservation of human mesenchymal stem cells [3] and mouse embryonic stem cells [4], but is not successful in the cryopreservation of human pluripotent stem cells. hESCs and hiPSCs have been shown to be highly susceptible to freezing damages [5,6]. Although many attempts have been made to improve the cell viability and recovery rate after the cryopreservation [5–7], the cell recovery rate of hESCs and hiPSCs after the cryopreservation using 10% dimethyl sulfoxide (Me_2SO) as a cryoprotective agent (CPA) is still very low [3,8]. Hence, it is crucial to develop an efficient cryopreservation method to avoid damages caused by intracellular ice formation, recrystallization [9–13] and solution effects [14–16] for the widespread applications of human pluripotent stem cells.

A rational design of cryopreservation protocols for hESCs and hiPSCs can be facilitated using theoretical models. The mathematical models have been developed to describe the transport of water and CPAs across the cell membrane [17–22]. These models are

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[1] *Abbreviations used:* AP, alkaline phosphatase; BCIP–NBT, 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium; CPA, cryoprotective agent; hESCs, human embryonic stem cells; hiPSCs, human induced pluripotent stem cells; mESCs, mouse embryonic stem cells; Me_2SO, dimethyl sulfoxide.

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required to understand the cell membrane permeability changes during the cryopreservation procedures including CPA addition, freezing, thawing and CPA removal. Once the cell-specific hydraulic permeability and CPA-specific CPA permeability parameters are known, the cell responses during the cryopreservation can be predicted under different conditions. The protocols for the cryopreservation of hESCs and hiPSCs can be optimized using such theoretical models to avoid the lethal damages to cells. However, the information on the cell membrane permeability of hESCs and hiPSCs is little.

In this study, the membrane permeability properties of hESCs and hiPSCs in the presence of different CPAs at different temperatures were systematically investigated. The osmotically inactive volume (V_b) was extrapolated using the Boyle-van't Hoff plot when the cells were exposed to various hypo- and hyper-osmotic conditions. The cell responses to different common CPAs (approved by FDA), Me_2SO, glycerol and 1,2-propanediol, at the temperatures ranging from 8 to 30 °C were monitored and analyzed using an image analysis program. The cell membrane permeability parameters describing the transport of water (L_p) and CPA (P_s) across the cell membranes were derived using the Kedem–Katchalsky (K–K) model. Furthermore, to examine the temperature dependence of the cell membrane permeability, Arrhenius plots for both water and CPA permeability were created. The cell responses at different cooling rates were simulated. The optimal cooling rate for each CPA was predicted. The cell viabilities after the cryopreservation at 1 °C/min and at the predicted optimal cooling rate were determined. These data will enable us to predict the cell responses during the cryopreservation and also facilitate the rational design of the cryopreservation protocols for hESCs and hiPSCs.

Materials and methods

Cell culture without feeders

The hESCs (line H9) from WiCell Research Institute and the hiPSCs (line SiDSH) from SIDANSAI Biotechnology were used to assess the cell membrane permeability properties. The cells were cultured on tissue culture plates coated with matrigel (BD Biosciences, UK) at the dilution ratio of 1:100 in Essential 8™ medium (Gibco, UK) at 37 °C with 5% CO_2. The culture medium was completely changed daily. When the cells were approximately 90% confluent, they were digested by TrypLE™ SELECT (Gibco, UK) at 37 °C for 5–7 min into single cells and collected after centrifugation at 1000 rpm for 5 min. Then the cells were re-suspended in the fresh media at the density of 10^6 cells/ml for the further experiments.

Experimental solutions

To evaluate the osmotically inactive volumes of hESCs and hiPSCs, a series of non-permeating saline solutions at the concentrations of 199, 300, 593, 1492 and 2271 mOsm were made with NaCl dissolving in ultra pure water. The CPA solutions were prepared by adding CPAs to phosphate basal saline (PBS). Three different CPAs, 10% and 20% (v/v) of Me_2SO, 10% (v/v) glycerol and 1,2-propanediol, were used at the osmolarities of 2250, 4500, 1930 and 1880 mOsm. The osmolarity was measured using a freezing point depression osmometer (Osmomat 030, Gonotec, Germany).

Stem cell characterization

For alkaline phosphatase (AP) staining, both hESCs and hiPSCs were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, washed with PBS for three times and stained with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP–NBT) (Sigma–Aldrich, USA) at room temperature for 2–3 h in the dark.

Immunostaining was performed using two stem cell markers, Oct4 and SSEA3. For intracellular staining of Oct4, the cells were rinsed with PBS for three times, then fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, permeabilized with 1% (v/v) Triton X-100 (Sigma, USA) supplemented with 1% (w/v) bovine serum albumin (BSA, Sigma, USA) in PBS for 30 min at room temperature, rinsed by PBS and blocked with 1% BSA in PBS for 60 min at 37 °C. For SSEA3 staining, the cells were only blocked with 1% BSA. Primary antibodies, goat anti-Oct4 polyclonal antibody (Santa Cruz, USA) and mouse anti-SSEA3 monoclonal antibody (R&D, UK), were added to the cells at a final concentration of 5 and 10 μg/ml in PBS, respectively, at 4 °C overnight, then rewarmed at 37 °C for 60 min followed by PBS rinsing to remove the unbounded primary antibody. Secondary antibodies, Alexa Fluor® 594 conjugated-donkey anti-goat IgG for Oct4 and Alexa Fluor® 594 conjugated-donkey anti-mouse IgG for SSEA3 (Molecular Probes, USA) were diluted at 1:200 in PBS containing 1% BSA. The diluted secondary antibody was added to the cells for 60 min at 37 °C, and then washed with PBS for three times. The slides were mounted with 50% (v/v) glycerol in PBS. All images were visualized by a fluorescence microscopy (Nikon, Japan) connected to a CCD (Tucsen, China).

Cell viability assay

After the CPA loading or cryopreservation, the cells were stained with propidium iodide (PI, Fanbo Biochemicals, China) at 1 μM for 1–2 min in the dark. The cell viability was quantified using a flow cytometer (Cube 6, Partec, Germany). The cells without the CPA loading were used as the negative control. In each measurement, around $2–5 \times 10^4$ events were measured.

Measurement of the cell volumes

The cells were immobilized on a 15 mm diameter cover slip coated by poly-L-lysine (Sigma, USA), which was placed in a 6 cm tissue culture dish. 100 μl of cell suspensions was loaded on the cover slip for up to 10 min. Once the cells were immobilized on the cover slip, the medium was completely replaced by the prepared CPA solution to achieve a rapid extracellular osmotic change [23]. The cell responses during this procedure were monitored and recorded via an inverted microscope (XSP-19C, Shanghai, China) with a digital camera (JVC, Japan) attached to the microscope.

A series of images at the discrete times were obtained by converting the video into individual pictures using the KMPlayer software. The cell area was measured using a digital image processing method programmed in MATLAB with batch processing. Due to the spherical shape of hESCs and hiPSCs during the experiments, the three-dimensional volume can be extrapolated from the two-dimensional area according to the equation, $\left(\frac{A_1}{A_2}\right)^3 = \left(\frac{V_1}{V_2}\right)^2$. The cells without round shapes were not included during the image analysis by confining the circularity as 0.85. The data of cell volumes at discrete times were inputted into a numerical simulation program using the MATLAB for the further estimation of the membrane permeability properties.

Determination of the equilibrium osmotic responses

The V_b is defined as the entire internal cell volume except that occupied by free water [24], which is unable to participate in transmembrane flux. The equilibrium cell volume was measured under the microscope via the attached digital camera after the cells were incubated under different osmotic conditions. The analysis of V_b