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...
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 hiPSCs, which is detectable using a simple CV technique on a cell-chip of a specific size. The intensity of the cathodic peak current (ipc) of undifferentiated hiPSCs showed clear linearity (R² = 0.99) to the number of undifferentiated hiPSCs, implying that the signal intensity in the cell-chip can be used to determine the number of undifferentiated hiPSCs. 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 (H₂SO₄: H₂O₂ = 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 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 at a 8:1 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 1 hr.

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 d-ASMCs 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 (1 M KCl) as the reference electrode, and platinum wire as counter electrode. CV detection was performed between 0.6 V and −0.4 V at 0.1 V/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 3 days 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 follows: 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).
2.9. Growth curve and G-banded karyotyping

To monitor cell growth, the confluence of hASMCs with or without electric stimulation was determined at 3hr intervals for 3 days on the IncuCyte FLR (Essen Bioscience). For karyotyping, hAMSCs were incubated with 100 ng/ml colcemid for 2hr, and then collected. 1% Sodium citrate was slowly added for hypotonic treatment followed by fixation using Carnoy’s solution (75% methanol, 25% acetic acid). The karyotypes were determined using a standard G-banding procedure.

2.10. Statistics

Graphical data were presented as mean ± s.d. Statistical significance among three groups and between groups were determined using one- or two-way analysis of variance (ANOVA) following Bonferroni multiple comparison post-test and Student’s t-test, respectively.

3. Results

3.1. Establishment of a cell-chip for in situ detection of hPSCs

We designed an electrochemical cell-chip based on the conventional three-electrode system as reported previously [20]. The cell-chip was composed of a conventional reference electrode (Ag/AgCl) and a counter electrode (Pt) placed on a platinum-working electrode (Pt counter electrode) (Fig. 1A, left panel). hPSCs were cultured on top of the matrigel/gold-coated plate with a transparent plastic cover to prevent contamination (Fig. 1A, right panel). When either human embryonic stem cells (hESCs) or hiPSCs were plated onto the cell-chip, cells were well maintained in vitro in feeder-free conditions for at least 5 days, the time taken for the entire process (Fig. 1B). Using this cell-chip system, we attempted to determine a specific cathodic peak potential (Epc) of undifferentiated hPSCs by changing the electrical charge.

3.2. Electrochemical detection and quantification of hPSCs

Electrochemical properties can be strongly affected by the electrolyte composition; therefore, it is desirable to use phosphate-buffered saline (PBS) to minimize signal interruption due to the variety of supplements in the culture medium [23]. However, unlike other human cell lines, hPSCs are vulnerable to stress caused by sub-optimal culture conditions so that hPSCs cannot be maintained with PBS longer period of time [28]. Therefore, hPSCs were maintained in chemically defined mTeSR1 medium under conventional feeder-free conditions as described previously [29] and were then subjected to measurement. A clear Epc of hESCs (H9: left panel) and hiPSCs (SES8: right panel), of which ‘pluripotency’ was identified by their positive staining for alkaline phosphatase activity [30], was detected at −0.077 V (Fig. 2A). This specific signal was similarly observed when cells were measured under PBS conditions shortly, suggesting that this distinct Epc of hPSCs is not dependent on medium supplements (Fig. S1A). The ipc of three different hPSCs with different genetic background (hESCs: H9 [30] and CHA3 [25], and hiPSCs: SES8 [26]) proportionally increased with the number of cells. The linearity of the correlation between the ipc and cell number was 0.959 for hESCs (Fig. 2B), 0.986 for hiPSCs (Fig. 2C), and 0.979 for CHA3-hESCs, respectively (Fig. S1B), showing clear linearity of the ipc with cell number.

3.3. Disappearance of ipc of hPSCs after differentiation

The high linearity of ipc with cell number (over 0.98 in both hESCs and hPSCs shown in Fig. 2B and C) would be advantageous to determine the cell number by simply measuring ipc. To investigate this, we compared the ipc of several types of cells, namely, hiPSCs, human aortic smooth muscle cells (hASMCs), which hiPSCs were derived from, and smooth muscle cells derived from hiPSCs (i-dSMCs), which all share the same genetic background [26]. The same number of hiPSCs, hASMCs, and i-dSMCs (200,000 cells) were plated onto the cell-chip and the ipc was measured. The clear ipc was
detected in hiPSCs, not in hASMCs or i-dSMCs (Fig. 3A), suggesting that the ipc of hPSCs shown in Fig. 2A is specific to undifferentiated hPSCs. When large numbers of hASMCs and i-dSMCs were used (up to 200,000 cells), only negligible signals were detected as non-correlation to number of cells (Fig. 3A, right panel).

To exclude the possibility that the lack of an ipc in hASMCs or i-dSMCs is due to the distinct properties of smooth muscle cells, hiPSCs were subjected to spontaneous differentiation for 14 days (Fig. 3B, left panel) as described previously [9]. This resulted in differentiation of various types of cells from all three germ layers being simultaneously present, as determined by the expression of specific marker genes (Pax6 for ectoderm, Brachyury T for mesoderm, and Sox17 for endoderm) (Fig. 3B, right panel). The ipc at −0.077 V disappeared when hiPSCs were differentiated (Fig. 3C, left panel). Despite the large number of cells (550,000 cells), the signal intensity from differentiated cells remained low (Fig. 3C, right panel). Similar data were obtained in hESCs. After 7 days, the spontaneous differentiation of hESCs resulted in dramatic reductions in the levels of Oct4 and Nanog in a time-dependent manner (Fig. 3D, right panel). The ipc (shown in blue) was markedly decreased, while the total cell number (shown in grey) was increased (Fig. 3E). When same number of hESCs and hESCs derived mesenchymal stem cells (hESCs-MSCs) [31] was compared, electrochemical signature of hESCs-MSCs was different from that of hESCs (Fig. S1C). Therefore, we conclude that the ipc detected at −0.077 V in the cell-chip is specific to undifferentiated hPSCs.
3.4. Detection of ipc of hPSCs in mixed cell populations

Residual undifferentiated hPSCs are a serious risk factor in hPSC-based therapy because they can potentially lead to teratoma formation once engrafted in vivo [4]. Thus it is crucial to accurately assess the number of undifferentiated hPSCs following differentiation or even after cell sorting. Theretofore, additional processes using small molecules [5,9] or hPSC-specific antibodies [32] may be required to eliminate any residual hPSCs. To this end, the ipc of hPSCs should be detectable in mixed cell populations for hPSCs’ quantification. To prove that detection of the ipc of hPSCs in mixed cultures with differentiated cells is a valid means to assess the number of undifferentiated hPSCs, hiPSCs were co-cultured with hASMCs to mimic the existence of residual hPSC after differentiation. Undifferentiated hiPSCs could be clearly distinguished from co-cultured hASMCs by evident staining of the former cells for stage-specific embryonic antigen (SSEA)-4 (dotted line, Fig. 4A).

When the number of undifferentiated hiPSCs co-cultured with a given number of hASMCs was gradually increased, the ipc of hiPSCs proportionally increased with clear linearity (R² = 0.997, Fig. 4B, right panel), suggesting that this signal of undifferentiated hPSCs was not affected by the presence of differentiated cells.

Next, we compared the reliability of the cell-chip approach in comparison with currently available approaches to quantify undifferentiated hPSCs, namely, real-time PCR and FACS analysis. Lin28 homolog A (Lin28a), which encodes a highly conserved RNA-binding protein, was suggested to be a highly sensitive marker of undifferentiated cells in comparison to retinal epithelial cells [14], which are currently in clinical trials to treat macular degeneration [33]. Although levels of Lin28a and other typical pluripotent cell-specific markers, namely, Oct4, Sox2, and Nanog, gradually increased while the percentage of undifferentiated hPSCs in the cell mixture increased, the linearity of the fold change in these mRNA levels was lower than the linearity observed with the cell-chip (R² as high as 0.88) (Fig. 4C, left panel). Rather, the fitting of the fold change in mRNA levels was apparently close to the sigmoid curve (R² as high as 0.9981) (Fig. 4C, right panel). FACS analysis using Oct4 for hiPSCs (Fig. 4D) and SSEA-3 for hESCs (Fig. 4E), another typical surface marker of undifferentiated hPSCs [30], was performed to determine the linearity of the size of the Oct4- or SSEA-3-positive population with the percentage of undifferentiated hPSCs in the mixed culture. The linearity of FACS analysis was over 0.95 (R² = 0.997 for Oct4 and 0.958 for SSEA-3) (Fig. 4D and E), which was equivalent to the linearity of the cell-chip approach. Similar linearity was obtained for TRA-1-60, a hPSC-specific keratin sulfate antigen, and SSEA-3 with the percentage of hiPSCs in mixed populations with hASMCs (R² = 0.995 for TRA-1-60 and 0.934 for SSEA-3 respectively) (Figs. S2A and B).
3.5. Smooth muscle cells are not damaged after measurement

Unlike the large number of cells that are unrecoverably used in FACS and real-time PCR analyses, cells on the cell-chip remained viable even after measurement, which would be suitable for further use. There was no distinct apoptotic response in hASMCs following the electrical charge (Fig. 5A). hASMCs still actively proliferated after the electrical challenge, suggesting that the electrical charge used during the measurement procedure minimally affected hASMC growth and survival (Fig. 5B). Next, the cellular stress response to the electrical charge, which may trigger undesirable effects on cellular functions, was further examined by determining the phosphorylation level of Histone H2AX (H2AX), which is increased by a variety of stresses such as DNA damage (e.g., ultraviolet and ionizing radiation), oxidative stress (e.g., reactive oxygen species), osmotic stress [34–36], and the formation of active caspase 3. The level of neither active caspase 3 nor phosphorylated H2AX increased following application of the electrical charge (Fig. 5C). Furthermore, there were no noticeable changes to the morphology of hASMCs after application of the electrical charge: the level of α-smooth muscle actin, a typical molecular marker of smooth muscle cells, remained constant (Fig. 5D and E) and the karyotype remained normal (Fig. 5F). Therefore, we conclude that hASMCs remain normal after cell-chip-based measurement, allowing their further usage for cell therapy.

3.6. Validation of in situ monitoring of undifferentiated hPSCs as a safety assessment

We previously reported two small molecules, namely, quercetin and YM-155, which can both selectively induce the death of undifferentiated hPSCs (as ‘stem-toxic’ activity) [9]. Because of the clear induction of cell death by nanomolar range of YM-155 [9], it was hypothesized that treatment with YM-155 would decrease the number of hPSCs and thereby lower the ipc. As predicted, YM-155 treatment markedly reduced the number of cells and the ipc in a dose-dependent manner for both hiPSCs (Fig. 6A) and hESCs (Fig. 6B). Label-free cell-chip-based quantification of residual undifferentiated hPSCs may be applicable at the end of the differentiation process to determine whether any additional process is required to eliminate any residual hPSCs, which is critical to ensure tumor-free cell therapy. To mimic this, hiPSCs were co-cultured with hASMCs. In this co-culture, hiPSCs maintained the specific colony morphology of hPSCs on the cell-chip (Fig. 6C, right panel, black dotted line). After 24 h of YM155 treatment, there was clear shrinkage of the typical colony morphology of hiPSCs, whereas hASMCs were not affected (Fig. 6C, right panel, red dotted line). After YM155 treatment, an apoptotic population, as determined by the production of active caspase 3, was only present among SSEA-3-positive cells (e.g. undifferentiated hPSCs), not SSEA-3-negative cells (e.g. hASMCs) (Fig. S3). This indicates that the cell morphological changes observed on the cell-chip after YM155 treatment (Fig. 6C, red dotted line) were due to the death of hPSCs but not hASMCs. Under this condition, the ipc in a mixture of hiPSCs and hASMCs gradually decreased in a dose-dependent manner (Fig. 6D). Following treatment with 40 nM YM155, the ipc was as low as that of hASMCs, suggesting that hPSCs were markedly ablated, consistent with previous studies [9].

These data suggest that assessment of the ipc of hPSCs at the end...
of the differentiation process is a valid in vitro approach to ensure the absence of undifferentiated hPSCs and to lower the risk of undesirable teratoma formation.

4. Discussion

There have been many attempts to resolve the risk of teratoma formation by selectively inducing the ablation of residual hPSCs after differentiation using hPSC-specific antibodies and small molecules [5]. However, even following an attempt to eliminate hPSCs, an additional step is required to ensure all hPSCs have been successfully removed prior to clinical applications. Current techniques to determine the presence of hPSCs, such as real-time PCR and FACS analysis, consume a large number of cells, which should be rather used for cell therapy.

The unique electrochemical signature of a given cell type may result from the specific electrochemical potential of the protein with different redox status [37,38]. Given that hPSCs have a wide
range of specific surface proteins in comparison to their differentiated counterparts, it is readily speculated that hPSCs have a particular electrochemical signature in the undifferentiated state [32,39]. As predicted, we observed a specific electrochemical signature of hPSCs, the intensity of which was highly proportional to the cell number (linearity of over 0.99) (Fig. 2) and dramatically disappeared after differentiation (Fig. 3). Most importantly, the signal intensity remained high even in co-culture condition (Fig. 4B). The hPSC-specific $i_{pc}$ was monitored in mixed cell population after differentiation to assess whether teratoma-forming cells (undifferentiated hPSCs) were present (Fig. 6E). Once a particular $i_{pc}$ level is detected, indicating the presence of residual hPSCs, an additional step can be performed to selectively remove hPSCs using 'stem-toxics', as reported in several studies including ours [5,9], to ensure the safety of the preparation (Fig. 6E).

Unlike FACS and real-time PCR analysis, which comprise multiple steps, assessment of $i_{pc}$ on a cell-chip is relatively simple, takes only a few minutes, and requires neither additional labeling, special buffer nor chemical to obtain highly reproducible results with clear linearity. Alternatively, determining the cycle threshold value of real-time PCR for Lin28 is highly sensitive and can detect even a single hPSC [14]. However, according to the data shown in Fig. 4C, real-time PCR analysis of hPSC-specific markers, including Lin28a, has lower linearity than the cell-chip-based assay and FACS (Fig. 4), suggesting real-time PCR analysis is less suitable to accurately determine the number of hPSCs in a mixed population. Recent studies reported hPSC-specific fluorescent probes [40] and the secretion of hyperglycosylated podocalyxin by hPSCs [15,41], which may also be applicable to detect hPSCs in mixed population. Indeed, the $i_{pc}$ of hPSCs showed high linearity and reproducibility (Fig. 4), which may be applicable to readily develop an electrical device that can be used to measure the number of hPSCs. In this
regard, it will be important to improve the sensitivity of iPG measurement by optimizing the components of the cell-chip used for hPSCs as described previously [42,43]. The iPG was currently detectable in as few as 30,000 hPSCs (Fig. 4B), which is close to the minimum number of hPSCs required to develop teratoma in a rodent model [44]. Taken together, we demonstrate that the intensity of an electrochemical signature of hPSCs shows high linearity to the cell number in a cell-chip-based assay. Measuring iPG after cell differentiation or an additional step to eliminate hPSCs would be an important safety assessment for teratoma-free engraftment of hPSC-derived cells in the future.

5. Conclusions

In this study, we identify a specific electrochemical signature of hPSCs, of which intensity is highly corresponding to the cell number. Thus, real-time measurement of cathodic peak current on the cell-chip achieves simultaneous quantification of hPSCs even in the mixed condition without additional labeling procedure. As the electrical charge in the procedure appears to be safe to the differentiated cells, the in situ cell-chip system would be beneficial to monitor potential contamination of hPSCs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.10.038.

References


