QUANTITATIVE CYTOLOGICAL FEATURE ANALYSIS BY MICROFLUIDIC IMAGE CYTOMETRY REVEALS PHENOTYPIC DIFFERENCES AMONG HUMAN PLURIPOTENT STEM CELL LINES

Ken-ichiro Kamei[1], Minori Ohashi[2], Nicholas A. Graham[2], Yong Chen*, Amander T. Clark[2], Owen N. Witte[2], Thomas G. Graeber[2], April D. Pyle[2] and Hsian-Rong Tseng[2]

*Institute for Integrated Cell-Material Sciences, Kyoto University, JAPAN

**Department of Molecular and Medical Pharmacology, University of California Los Angeles, CA, USA

ABSTRACT

Human pluripotent stem cells (hPSCs) have a great potential for the application in clinical uses and basic developmental biology. Here, we introduce the new quantitative and multiparameteric methods based on microfluidic image cytometry to characterize individual hPSC behavior under a collection of self-renewing and differentiating conditions. In conjunction with microfluidic image cytometry and biostatistical analysis, we are able to evaluate and summarize the phenotypic differences among the hPSC lines.

KEYWORDS: human pluripotent stem cells; microfluidics; image cytometry; single cell profiling;

INTRODUCTION

Human pluripotent stem cells (hPSCs), including embryonic stem (ES) and induced pluripotent stem (iPS) cells hold a great potential for applications in cell-based therapy, drug screening and regenerative medicine as well as developmental biology due to their unique characteristics, such as (i) unlimited self-renewal at undifferentiated stage with normal karyotype and (ii) the ability to differentiate any cell types in a human body. However, recently, even though hiPSCs were generated to mimic hESC characteristics, there have been some studies reported that assess the similarities and differences between hiPSCs and hESCs. Indeed, some of the hiPSC lines appear to vary morphologically and behave differently in chemically defined media due to variables involved in the derivation/culture methods. Moreover, there are the additional concerns that hPSCs generate highly heterogeneous populations during culture and might affect the purity of targeted cells during induced differentiation. Therefore, it is necessary to develop new methodology to systematically assess the difference individual hPSC characteristics to address these issues.

Microfluidic image cytometry (MIC) (Fig. 1) [1] is advantageous over flow cytometry for profiling cell behavior at a single cell level due to its capabilities of (i) well-controlled cellular environments, (ii) higher-throughput and multiparametric assays for individual cells and (iii) *in situ* real-time cell monitoring. In these years, we have developed MIC platforms for characterizing individual hPSCs cultured in chemically defined medium and diagnosing glioblastoma patients *in vitro*. While genetic and epigenetic approaches can provide valuable information, in this study we have used MIC technique to indentify an alternative way to compare and distinguish the current state of a particular iPSC line from another iPSC line or other ESC lines and determine relative status of pluripotency by analyzing the unique cytological features of hiPSCs at a single cell level.

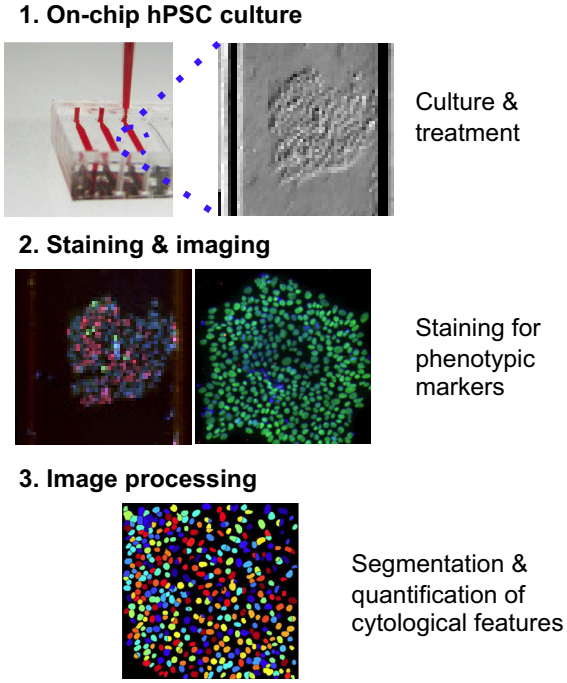

Fig. 1 Procedure of Microfluidic Image Cytometry (MIC) for quantitatively profiling individual hPSC behabior

THEORY

The microfluidic hPSC culture device (Fig. X) consists with 24 individually addressable culture chambers with a pair of an inlet and an outlet. We are able to introduce hPSCs, medium and staining solution into each cell culture chamber by using a conventional micropipetter, allowing a general biology lab to handle this microfluidic hPSC culture device without any special instruments.

For our proof-of-concept, we use an extensive collection of hES (HSA1, H1 and HSF6) and hiPS (iPS-2, iPS-18, iPS-A1 and iPS-B2) cell lines, and various defined culture conditions (StemPRO and mTeSR-1) to assess the difference of hPSC behavior under such conditions.

To quantitatively characterize individual hPSCs, we established a set of phenotypic markers for cytological feature analysis collected at single cell level. General cytological features evaluated in the experiments include, but not limited to, the size, texture, shape and maker intensity of nucleus, cytoplasm or both and the distance between neighbor cells. In terms of phenotypic markers, this set contains 4 markers aimed to check the stem cell status, such as i) DAPI for DNA contents, ii) OCT4 and iii) SSEA1 for pluripotency and differentiation, respectively.

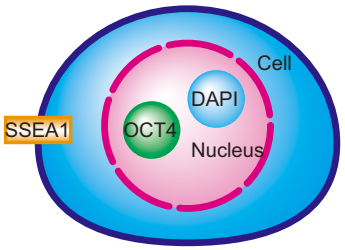

Fig. 2 Phenotypic markers for hPSC phenotypic profiling for cellular states of pluripotency and differentiation.

EXPERIMENTAL

A microfluidic hPSC array chip was fabricated using soft lithography. The mold of the fluidic channels was first produced through photolithographic processes (channel width: 700 mm, length: 1000 mm and height: 100 mm). A 100-mm-thick negative photoresist (SU-8 2100) was spin-coated onto a silicon wafer (Silicon Quest). After UV exposure and subsequent development, rectangle-profiled patterns for the culture chambers were obtained. The mold was exposed to trimethylchlorosilane (TMSCl, Aldrich-Sigma) vapor for 5 min. A well-mixed PDMS pre-polymer (Sylgard 184, A and B in 10 to 1 ratio respectively) was poured onto the mold located in a Petri dish to give a 6-mm-thick fluidic layer. The fluidic layer was cured in an 80˚C oven for overnight. After incubation, the PDMS layer was peeled off from the mold, and holes were punctured into the fluidic layer for access of reagent solutions. The PDMS layer was then trimmed and cleaned. A PDMS-based microfluidic component and a glass slide were treated with oxygen plasma, and then assembled by baking in 80˚C for overnight.

All hPSC lines were cultured on γ-irradiated mouse embryonic fibroblast (MEF) cells in hPS medium consisted with DMEM/F12 medium supplemented with supplemented with Knockout Serum Replacement (KSR, 20%, Invitrogen), non-essential amino acids (NEAA, 1%, Invitrogen), β-mercaptoethanol (0.1 mM, Invitrogen), and bFGF (10 ng mL^{-1}, Invitrogen). Media was changed daily, and these cells were passaged with collagenase IV (1 mg mL^{-1}, Invitrogen) weekly.

hPSC culture in a microfluidic hPSC device were carried out as follow. A new microfluidic hPSC array was sterilized under UV light for 15 min prior to on-chip hPSC culture. Afterwards, hESC qualified Matrigel diluted with DMEM/F12 were introduced into cell culture chambers and incubated at 4˚C overnight away from light. After coating, cell culture chambers were washed with DMEM/F12 and incubated at 37˚C until hPSC loading. To start culturing in a microfluidic hPSC array, hPSCs cultured in a conventional 6-well plate were passaged mechanically with StemPRO EZPassage kit (Invitrogen) and separated from the MEF feeder cells by gravity. Harvested hPSC colonies, each with diameter of 70 mm, and roughly 200 cells were suspended in hPS medium and gently loaded into a microfluidic hPSC array. Each cell culture chamber contained four to ten hPSC small colonies. Three hours later, StemPro (Invitrogen)[4] and mTeSR (Stem Cell Technology)[5, 6] were separately introduced into their respective sets of cell culture chambers. A microfluidic hPSC culture was conducted in the static culture conditions and after hPSC loading, medium was changed every 12 hours using a micropipette.

To perform phenotype assay, we utilized On-Chip immunocytochemistry was carried out to detect OCT4, SSEA1 and DAPI. hPSC colonies were fixed by loading paraformaldehyde (4% in PBS, Electron Microscope Science) into the chambers and incubated at room temperature for 15 min. After permeabilization with Triton X-100 (0.5%, Fluka) in PBS for 30 min, a blocking solution containing normal goat serum (5%, Vector Laboratory), normal donkey serum (5%, Jackson ImmunoResearch Laboratories), bovine serum albumin (3%, Fraction V, Sigma) and *N*-dodecyl-b-D-maltoside (0.1%, Pierce) was loaded into the chambers, and the chambers were incubated at room temperature for overnight. After rinsing with PBS containing 0.1% Tween 20 (PBS-T), the hPSC colonies were incubated with human specific antibodies for OCT4 (2 mg mL^{-1}, mouse monoclonal IgG, Santa Cruz Biotechnology), SSEA1 (2 mg mL^{-1}, mouse monoclonal IgM, Santa Cruz Biotechnology) at 4˚C for 24 hr. After rinsing the chambers with PBS-T, a mixture of the respective secondary antibodies: FITC-conjugated donkey anti-mouse IgG (H+L) (3.75 mg mL^{-1}, Jackson ImmunoResearch), or Rhodamine Red-X (RRX)-conjugated goat anti-mouse IgM (0.375 mg mL^{-1}, Jackson ImmunoResearch) in a blocking solution was loaded into the chambers to detect the bound primary antibodies. One hour aster incubation at room temperature, the chambers were rinsed with PBS-T. Finally, 300 nM of DAPI solution was loaded and incubated at 25˚C for 30 min for nuclear staining.

The microfluidic hPSC cultured device containing stained cells was mounted on a Nikon TE2000 fluorescent microscope with a CCD camera to take bright-field and fluorescent images. CellProfiler (Broad Institute)[10] was used to quantify fluorescence intensity of markers in individual cells. A set of data was loaded into CellProfiler and quantified for individual cells.

RESULTS AND DISSCUSSION

For our proof-of-concept of this study, we evaluated the effects of culture conditions on individual hPSC behavior. First, we perform on-chip hPSC culture with various conditions, including KSR/MEF conventional hPSC culture condition, StemPRO and mTeSR defined conditions, and differentiation condition supplemented with serum. The micrographs in Fig. 3 represent the H1 hESCs cultured in tested conditions. The cells cultured in KSR/MEF, StemPRO and mTeSR showed the strong and uniform expression of OCT4 pluripotent marker, but didn't express SSEA1 differentiation marker. On the other hands, we found that the cells treated with differentiation condition expressed SSEA1 heterogeneously. Even though the cells were treated a same condition, individual cells responded differently. Thus, we need to have a technique for quantitatively profiling hPSC characters.

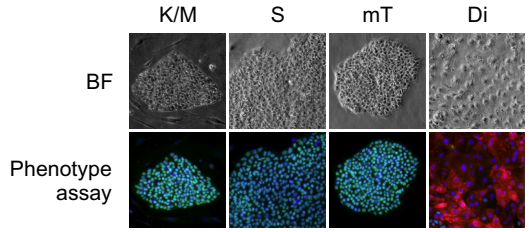

Fig. 3 Bright field (BF) and fluorescence micrographs of H1 human embryonic stem cells treated with KSR/MEF (K/M), StemPRO (S), mTeSR-1 (mT) and differentiation (Di) conditions. DAPI (blue), OCT4 (green) and SSEA1 (red).

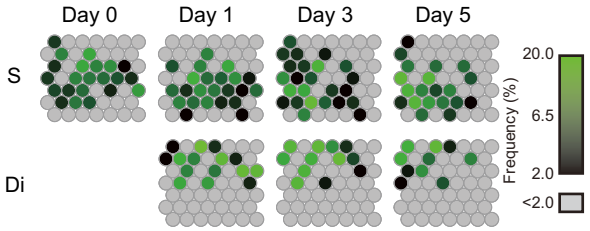

Fig. 4 Quantitative hPSC profiling maps (Self-organizing map analysis) for visualizing distribution of differences in hPSC characters.

Finally, based on the obtained images, we have established the multi-parametric microfluidic phenotypic assays combined with biostatistic analyses (i.e., common factor analysis, self-organizing map analysis and hierarchical clustering) that provide a collection of cytological features. By utilizing biostatistical analyses, we were able to visualize the heterogeneity of hPSC characters determined with the obtained cytological features (Fig. 4). Interestingly, unsupervised hierarchical clustering of SOM data revealed that hESC lines and hiPSC lines were distinguished, and hiPSC lines was categorized into a same group with IMR90 somatic fibroblast cell line (Fig. 5).

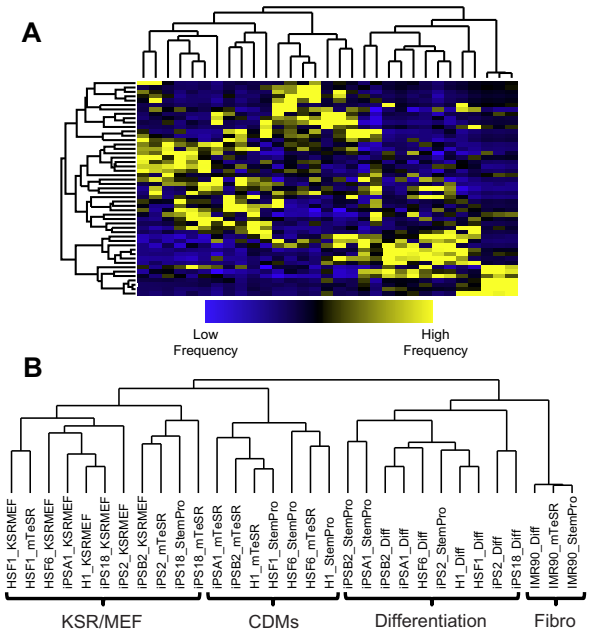

Fig. 5 Hierarchical clustering of phenotypes of hPSCs treated with a standard culturing (KSR/MEF), chemically defined culturing (StemPro and mTeSR-1) and differentiation conditions evaluated by MIC cytological feature analysis. (A) Heatmap of a collection of hPSC lines cultured in various conditions. (B) Cluster dendrogram of hPSC phenotypes.

REFERENCES:

[1] K. Kamei et al., *Microfluidic Image Cytometry for Quantitative Single-Cell Profiling of Human Pluripotent Stem Cells in Chemically Defined Conditions*, Lab Chip, **10**, pp. 1113-1119 (2010)