DEFORMABILITY CYTOMETRY OF EMBRYONIC STEM CELLS REVEALS CONSISTENT MECHANICAL PROPERTIES ACROSS CELL LINES

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ABSTRCT

Due to high risks of genetic and phenotypic instability associated with cell culture, quality control strategies to ensure proper and consistent hESC functionality for potential use in regenerative medicine are vitally important. The expression of certain markers usually assessed by flow cytometry and the ability to form embryoid bodies (EBs) and teratomas in mice are two main categories of assays used for stem cell quality control. In addition to variability of these tests between different labs and their limited robustness, these assays are highly costly and labor-intensive. Recent studies using atomic force microscopy (AFM) and micropipette aspiration have shown that cell mechanical properties could be a promising label-free biomarker of stem cell pluripotency. Both human and mouse embryonic stem cells (ESCs) were shown to be significantly more deformable than differentiated progeny. However, AFM and other recent techniques are manual approaches severely limited in throughput and more suited for biophysics experiments rather than cell screening. Here we introduce microfluidic deformability cytometry as a screening tool for stem cell pluripotency that addresses the need for high throughput. The approach was previously demonstrated by our group to assay the deformability of cells in a variety of body fluids and cell lines.

KEYWORDS: Microfluidics, stem cell, pluripotency, label-free assay

INTRODUCTION

Due to high risks of genetic and phenotypic instability associated with cell culture, quality control strategies to ensure proper and consistent hESC functionality for potential use in regenerative medicine are vitally important [1]. The expression of certain cell surface markers such as SSEA3, SSEA4, Tra-1-60 and Tra-1-81 and transcription factors including Oct4, Nanog and Sox2 defines one category of assays used for stem cell quality control, usually assessed by flow cytometry. Cell morphology and the activity of alkaline phosphatase and telomerase are two other methods for validation of hESC and iPSC pluripotency . Another category assays maintenance of cell pluripotency, usually assessed by the ability to form embryoid bodies (EBs) and teratomas in mice, which shows the potential of cells to produce cells from all three germ layers. In addition to variability of these tests between different labs and their limited robustness, especially for EB and teratoma formation tests, these assays are highly costly and labor-intensive. Therefore, there is a need for a robust and cost-effective method to both screen differentiated cultures and ensure proper maintenance of pluripotent cultures.

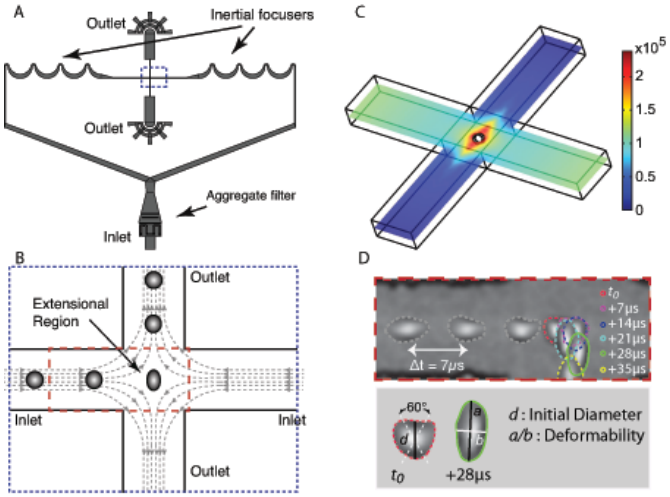

Figure 1. A) Deformability cytometry device. To ensure that all of the cells experience uniform stretching forces, inertial focusing is used to position the cells. B) The positioned cells arrive one at a time at an extensional flow, get stretched and leave the junction from either of the two outlets on the top or bottom. C) Comsol simulations show that the pressure applied on the cell in the extensional region is in the order of 10^5 Pa. D) The extensional region is continuously imaged using high-speed microscopic imaging and cell size and deformability are extracted by image analysis.

Recent studies have revealed that physical properties of cells change significantly during differentiation [2]. One study measured nuclei of human ESCs to be significantly more deformable than that of differentiated cells [3]. ESCs are known to have more open euchromatin than differentiated cells , which has been associated with a reduction in nuclear viscosity and stiffness [4]. Interestingly, chromatin and nuclear lamina, both being major determinants of nuclear mechanics, undergo extensive changes during differentiation. One of the major determinants, A-type lamins are not expressed in ESCs and are only present in differentiated

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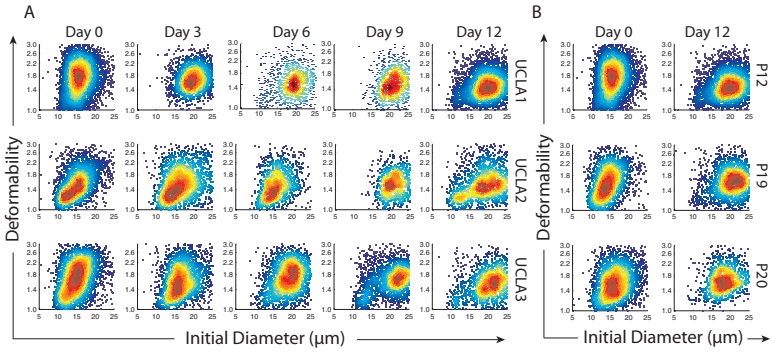

Figure 2. Stem cell differentiation associated with deformability changes. A) Color density plots from three stem cell lines UCLA1, UCLA2 and UCLA3 (rows 1, 2 and 3 respectively) show gradual decreases in deformability and increases in cell size after initiation of differentiation. B) Comparing the deformability of UCLA1 cells from three different passage numbers before and after 12 days of differentiation shows slight differences between the profiles. Initial diameter axis spans 5 to 25μm and deformability axis

cells. Additionally, several epigenetic modifications including global DNA hypermethylation and a decrease in acetylation of histones H3 and H4 lead to substantial chromatin reorganization during differentiation. Cellular and nuclear deformability associated with specific nuclear properties and chromatin organization could therefore be used as an indicator of pluripotency.

Cell mechanical properties have been mainly measured using methods such as atomic force microscopy (AFM), or micropipette aspiration, which are single-cell based and manual approaches with a limited throughput. Deformability cytometry provides the mechanophenotype of thousands of cells per second in a similar manner to traditional flow cytometry. The approach was previously demonstrated by our group to assay the deformability of cells in a variety of body fluids and cell lines [5]. Here we make use of deformability cytometry to screen the quality and differentiation state of stem cells and stem cell-derived cultures.

EXPERIMENTAL

The microfluidic device is shown in Figure 1 [5]. Microfluidic devices were fabricated using common polydimethylsiloxane (PDMS) replica molding processes. The device contained 20μm filters to avoid the entrance of cell clusters or dust followed by curving channels to ensure inertial focusing and a junction that provided an extensional flow (Fig.1A). The positioned cells arrived one at a time at an extensional flow, were stretched and left the junction from either of the two outlets on the top or bottom (Fig.1B). The extensional region was continuously imaged using high-speed microscopic imaging. Functioning in an inertial regime (channel Reynolds number $Re \sim 100$), inertial focusing positions cells precisely before stretching [6], which ensures a more uniform three-dimensional force on cells of the same size. Cell viscoelastic properties then determine to what extent a cell deforms. This deformation is continuously imaged using high-speed microscopy and automated image analysis is conducted on the gathered images to extract cell size and deformability parameters. Individual cells are plotted based on these parameters as points on color density plots. Cells must only be brought into suspension in an identical manner and measured after a conserved amount of time from sample to sample. Simulations show that for a simplified model of the system the force applied to a cell at the junction is on the order of 10^{-4} N (Fig. 1D), which is almost three orders of magnitude higher than that applied by conventional methods like AFM or micropipette aspiration . Deformability cytometry allowed measurement of the mechanophenotype of tens of thousands of cells throughout the differentiation process.

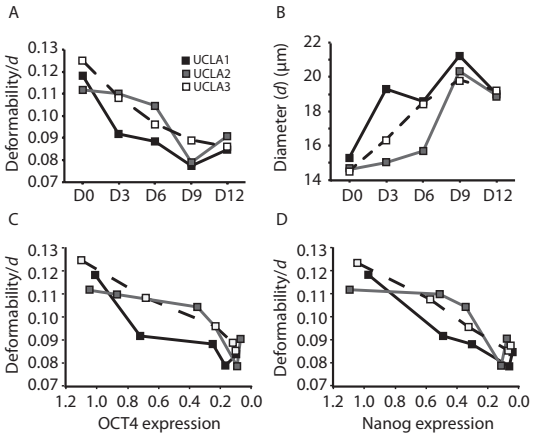

Figure 3. Deformability correlated with pluripotency markers. A) Deformability values normalized by cell initial diameter (d) decreases as cells differentiate, B) Cells become larger as they differentiate. C) Correlation between the mechanical measure (Deformability/d) and expression of OCT4 is 0.80 for UCLA1, 0.76 for UCLA2 and 0.98 for UCLA3 cell line. D) The same trend was observed for Nanog. Correlation between Deformability/d and Nanog expression is 0.93 for UCLA1, 0.61 for UCLA2 and 0.99 for

Three lines of human ESCs, (UCLA1, UCLA2 and UCLA3) were maintained in DMEM high glucose with 20% knockout serum replacer supplemented with 20ng/ml of bFGF, and grown on mitomycin-treated mouse embryo fibroblasts. Culture on 1% gelatin coated dishes without feeder cells in DMEM high glucose with 20% FBS resulted in a gradual differentiation. Single cell suspensions were prepared by 5 min treatment with 1X trypsin EDTA followed by detachment from the dish aspiration and suspension in culture media. For each condition and each replicate three sets of samples were prepared for (i) live cell flow cytometry analysis, (ii) RT-PCR and (iii) deformability cytometry. For deformability cytometry the cell suspension was prepared immediately prior to the test (<1 hour). Cell suspensions were injected into the device, at a concentration of 200,000

to 500,000 cells/mL, using a syringe pump (Harvard Apparatus PHD 2000) and a plastic syringe (Becton), at flow rate of 1000 μL/min.

RESULTS AND DISCUSSION

The deformability of human embryonic stem cells (hESCs) (day 0) was analyzed before and after feeder and serum-free non-specific induction to differentiation for up to 12 days. Fig. 2A shows the color density plots of deformability versus cell diameter for over time for three different hESC cell lines. Gradual changes in the density plots are visible with the progression of differentiation. While the pluripotent cells exhibit a deformable, small diameter profile mostly concentrated in upper-left quadrant of the plots, they switch to a larger, less deformable state as they lose their pluripotency. Although we observe the same trend of cell stiffening upon differentiation, the density plots indicate a difference between UCLA2 and the other two cell lines. The decrease in deformability and increase in size is consistently observed, even though some slight variations were detected at later passage numbers of UCLA1 (Fig. 2B). The median value of the ratio between cell deformability and diameter was used as a simple metric to show this relative change of cell physical properties with time. The range of deformability/*d* parameter for each cell line upon differentiation is depicted in Fig. 3A. Cell size gradually increased upon differentiation (Fig. 3B).

We assessed pluripotency of live cells using conventional pluripotency markers including Tra-1-85, Tra-1-81, and SSEA4 by immunofluorescence flow cytometric analysis at day 0 (Fig. 4A). The expression of Oct4 and Nanog was analyzed by RT-PCR as well (Fig. 4B). The results consistently show down regulation of pluripotency markers following differentiation as expected. The high correlation (≥ 0.9 for UCLA1 and UCLA2, for the expression of Oct4 and Nanog) between our mechanical measure and these commonly used pluripotency markers indicates the potential of deformability cytometry as a label-free assay of pluripotency (Fig. 3C and 3D).

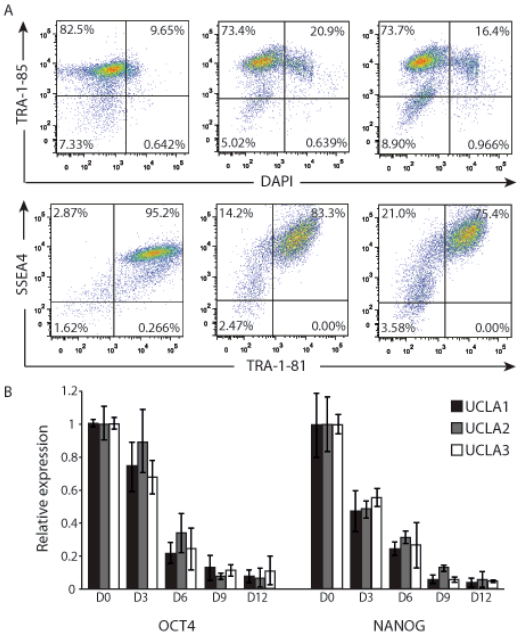

Figure 4. A) Flow cytometry analysis for the expression of pluripotency markers TRA-1-85, TRA-1-81 and SSEA4 and viability analysis by DAPI for UCLA1, UCLA2 and UCLA3 (column 1,2 and 3 respectively) shows high expression of the three markers at day 0. B) RT-PCR analysis confirms the gradual downregulation of these OCT4 and Nanog expression for up to 12 days of differentiation.

CONCLUSION

The high correlation between our deformability measure and commonly used stem cell markers indicates the potential of deformability cytometry as a label-free assay to characterize cell pluripotency. The high information content, low cost, and high throughput of deformability cytometry technology provides further evidence for the feasibility of using deformability as a biomarker for routine screening of ESC quality and differentiation for research and clinical use.

REFERENCES:

1. Knoepfler, P.S., *Deconstructing stem cell tumorigenicity: a roadmap to safe regenerative medicine.* Stem Cells, 2009. **27**(5): p. 1050-6.
2. Tse, H.T.K., et al., *Strategies for Implementing Hardware-Assisted High-Throughput Cellular Image Analysis.* Journal of the Association for Laboratory Automation, 2011. **16**(6): p. 422-430.
3. Pajerowski, J.D., et al., *Physical plasticity of the nucleus in stem cell differentiation.* Proc Natl Acad Sci U S A, 2007. **104**(40): p. 15619-24.
4. Dahl, K.N., A.J. Ribeiro, and J. Lammerding, *Nuclear shape, mechanics, and mechanotransduction.* Circ Res, 2008. **102**(11): p. 1307-18.
5. Gossett, D.R., et al., *Hydrodynamic stretching of single cells for large population mechanical phenotyping.* Proc Natl Acad Sci U S A, 2012. **109**(20): p. 7630-5.
6. Di Carlo, D., et al., *Continuous inertial focusing, ordering, and separation of particles in microchannels.* Proc Natl Acad Sci U S A, 2007. **104**(48): p. 18892-7.

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