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A high-content platform to characterise human induced pluripotent stem cell lines

Andreas Leha [c,1], Nathalie Moens [a,1], Ruta Meleckyte [a], Oliver J. Culley [a], Mia K. Gervasio [a], Maximilian Kerz [a,b], Andreas Reimer [a], Stuart A. Cain [e], Ian Streeter [d], Amos Folarin [b], Oliver Stegle [d], Cay M. Kielty [e], HipSci Consortium, Richard Durbin [c], Fiona M. Watt [a], Davide Danovi [a,*]

[a] HipSci Cell Phenotyping, Centre for Stem Cells and Regenerative Medicine, King's College London, Great Maze Pond, London SE1 9RT, UK
[b] NIHR Biomedical Research Centre for Mental Health Informatics Core, King's College London, De Crespigny Park, London SE5 8AF, UK
[c] Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SD, UK
[d] European Molecular Biology Laboratory, European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, UK
[e] Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, United Kingdom

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ABSTRACT

Induced pluripotent stem cells (iPSCs) provide invaluable opportunities for future cell therapies as well as for studying human development, modelling diseases and discovering therapeutics. In order to realise the potential of iPSCs, it is crucial to comprehensively characterise cells generated from large cohorts of healthy and diseased individuals. The human iPSC initiative (HipSci) is assessing a large panel of cell lines to define cell phenotypes, dissect inter- and intra-line and donor variability and identify its key determinant components. Here we report the establishment of a high-content platform for phenotypic analysis of human iPSC lines. In the described assay, cells are dissociated and seeded as single cells onto 96-well plates coated with fibronectin at three different concentrations. This method allows assessment of cell number, proliferation, morphology and intercellular adhesion. Altogether, our strategy delivers robust quantification of phenotypic diversity within complex cell populations facilitating future identification of the genetic, biological and technical determinants of variance. Approaches such as the one described can be used to benchmark iPSCs from multiple donors and create novel platforms that can readily be tailored for disease modelling and drug discovery.

1. Introduction

Human induced pluripotent stem cells (iPSCs) offer tremendous potential not only for cell therapy but also to develop platforms for medical research. In particular, patient-derived iPSCs can be used to obtain selected differentiated cell types to model diseases and discover new therapeutics [1]. Heterogeneity in gene expression has been described within a specific iPSC line [2], between different donors [3,4] and through the reprogramming process [5,6]. Furthermore, several studies have focused on the differences between a small number of lines from patients and controls or used isogenic lines [7]. However, despite recent examples in this direction [8] dissecting the phenotypic heterogeneity within one cell line and among lines derived from the same donor or diverse individuals is yet to be fully explored.

Nonetheless, a clear definition of the genetic and epigenetic variance and how each of these affects cell behaviour in large panels of iPSCs is crucial for stem cell biology. Moreover, assessing the phenotypic variance observed in cell populations from multiple donors will facilitate scaling up culture systems as well as the development of quality control and automation protocols with undoubted value for the maintenance of pluripotent stem cells and controlled differentiation towards specific cell types.

The human induced pluripotent stem cells initiative (HipSci) is generating iPSCs from hundreds of healthy individuals as well as patients diagnosed with selected diseases. This represents a powerful resource to evaluate and quantify cell responses to chemical, physical and biological stimuli using novel assays and artificial microenvironments. Within this framework, phenotypic data are being collated with genomics, epigenomics and proteomics data to discover the impact of their variation on the cellular phenotype. Here we describe the development of a simple assay (including methods, workflow and set-up) to capture and quantify phenotypic

* Corresponding author.
E-mail address: davide.danovi@kcl.ac.uk (D. Danovi).
[1] These authors contributed equally.

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features of iPSCs exposed to different extracellular matrix conditions.

2. Material and methods

2.1. iPSC quality control and maintenance

iPSCs are received from the Wellcome Trust Sanger Institute. There, cells are reprogrammed from fibroblasts using the Sendai virus method [9]. After reprogramming, each clone is genotyped and tested for copy number variations (CNVs). Pluripotency is assessed based on expression profiling [10], detection of pluripotency markers in culture and response to differentiation inducing conditions [11]. Data reported in this study refers to multiple replicate experiments of a single cell line [12] (Table 1, first line). iPSCs are passaged on Mitomycin-C inactivated mouse embryonic fibroblasts (MEFs) in Advanced DMEM/Ham's F-12 supplemented with 20% v/v KnockOut Serum Replacement (all Life Technologies), 1% v/v L-Glutamine, 1% v/v Penicillin–Streptomycin (all Sigma–Aldrich), 55 mM 2-Mercaptoethanol (Life Technologies) and 4 ng/mL human bFGF (Millipore). Cells are split every 3–4 days using enzymatic and mechanical dissociation and media changed daily. Briefly, cells are washed with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma–Aldrich) and incubated with dispase and collagenase (all Life Technologies) for 10 min at 37 °C. The enzyme solution is then replaced with fresh culture medium and pluripotent colonies are dissected manually. Colonies are selected based on morphological features typical of human pluripotent stem cells and are split approximately 1:3 onto a new feeder plate.

CF-1 MEFs (Amsbio) are cultured in Advanced Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 supplemented with 10% v/v Foetal Bovine Serum (all Life Technologies), 1% v/v L-Glutamine and 1% v/v Penicillin–Streptomycin (all Sigma–Aldrich). For iPSCs maintenance, inactivated MEFs are seeded as feeders on a 6-well microplate (Falcon) coated with 0.1% gelatin from porcine skin type A (Sigma–Aldrich) at a density of 10^6 cells per 6-well plate and allowed to attach overnight.

2.2. Extracellular matrix coating conditions

To develop our assay, we first sought to identify favourable extracellular matrix substrates. We screened and examined a total of 74 diverse conditions from two sources (see Table 1). A customised array plate acquired from Orla protein technologies (Sarstedt cat. No. 02XECM-96) contained 9 conditions derived from single ECM proteins including fibronectin, laminin, collagen, vitronectin, osteopontin, tenascin C and bone sialoprotein and 17 conditions presented as a mixture of different ECMs. For this set, triplicates of a single concentration of approximately 25 μg/ml per condition were coated on wells. Additionally, we created an array plate containing in duplicate fragments of fibrillin-1, fibrillin-2 and agrin as well as cellular and plasma fibronectin at a range of 1, 10 and 25 μg/ml. The plate also contained single concentrations of the following ECM proteins: LTBP-1 C-terminal fragment, MAGP-1, syndecan-2 extracellular domain, syndecan-4 extracellular domain and fibulin 4. Extracellular matrix proteins diluted in 80 μl PBS were incubated overnight on 96 well μClear black tissue culture plates (Greiner cat. No. 655090) at 4 °C. The supernatant was removed and well-coating blocked by the addition of 10 mg/ml BSA for 1 h. Upon removal of the BSA solution, the plates were stored at −80 °C prior to use. Bovine Serum Albumin (BSA) and uncoated tissue culture plastic (TCP) were used as controls.

For the fibronectin assay, 96-well μClear plates (Greiner) are coated with 1, 5 and 25 μg/ml human plasma fibronectin (Corning) and stored at 4 °C (overnight or up to 14 days). We will refer to these conditions as Fn1, Fn5 and Fn25, respectively (whereas Fn10 was only used in the screening). Each is present in a technical triplicate on the same vessel randomised per column using diverse patterns (*i.e.* Fn1-Fn5-Fn25, Fn1-Fn25-Fn5, Fn5-Fn25-Fn1, Fn5-Fn1-Fn25, Fn25-Fn1-Fn5, Fn25-Fn5-Fn1). Border wells are avoided to reduce edge effects. Before use, fibronectin is removed and wells are washed with DPBS (Sigma–Aldrich).

2.3. Assay set-up: cell seeding, fixation, staining and image acquisition

When iPSCs cultures reach approximately 80% confluency, cells are washed with DPBS and dissociated with collagenase and dispase for 45 min at 37 °C. Pluripotent colonies detach from the microplate surface and are further dissociated with Accutase (Innovative Cell Technologies) for 5 min at 37 °C. The single-cell suspension is centrifuged for 3 min at 400 rpm after which the supernatant is removed and cells re-suspended in fresh culture medium (Section 2.1) supplemented with 10 μM Y-27632 Rho-associated protein kinase (ROCK) inhibitor (Enzo Life Sciences). Cells are then counted using a Scepter 2.0 automated cell-counting device (Millipore) and seeded onto the fibronectin-coated 96-well plate using Viaflo (INTEGRA Biosciences) electronic pipettes.

At 23.5 h after seeding cells are labelled with EdU (Click-iT EdU kit, Life Technologies) for 30 min. For fixation, 8% paraformaldehyde (PFA, Sigma–Aldrich) is added to an equal volume of medium for a final concentration of 4%, and left at room temperature for 15 min. After fixation, cells are washed with DPBS (Sigma–Aldrich) and stored at 4 °C. Cells are then blocked and permeabilised with 0.1% v/v Triton X-100 (Sigma–Aldrich), 1% w/v bovine serum albumin (BSA, Sigma–Aldrich) and 3% v/v donkey serum (Sigma–Aldrich) for 20 min at room temperature. After washing with DPBS, cells are stained with Click-iT EdU kit (Life Technologies) according to the manufacturer's instructions except the azidofluoride reaction buffer halved in DPBS. After 1 h cells are washed with DPBS, stained for 1 h at room temperature with CellMask plasma membrane stain (1:1000, Life Technologies) and DAPI nuclear stain (1:5000, 1 μg/ml final concentration, Life Technologies). Plates are then washed with DPBS and stored at 4 °C. EdU was used according to manufacturer's instructions except for the concentration of the azide reagent halved. A period of half hour was chosen in line with the cell cycle period described in the literature for human iPSCs [6]. As a control, cells were exposed to the same reagents in the absence of EdU incorporation showed comparable background intensity values to the cells considered EdU negative by our analysis. Acquisition parameters and image analysis pipeline are described in details in Section 3.1 and Section 3.3 respectively. For endpoint analysis, stained plates are imaged using an Operetta® (Perkin Elmer) high content device. Images are acquired in wide field mode using 4 channels (DAPI, 488, 647, Brightfield as control). On Greiner μClear plates, we optimised heights focal settings for brightfield, DAPI, EdU and CellMask (respectively 11, 20, 9 and 10 μm) following the sharpest focal plan guided by the highest intensity of signal. Times of exposure (respectively 100, 200, 300 and 10 millisecs) were chosen to minimise the time of acquisition and the amount of reagents used. Incucyte (Essen Bioscience) images were acquired largely as described in [13].

3. Results and discussion

We first aimed to obtain a robust read out to evaluate response of undifferentiated iPSCs to controlled changes in the microenvironment. Furthermore, we aimed to develop a set of procedures to effectively extract from images relevant phenotypic features,