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Evaluation of inter-batch differences in stem-cell derived neurons

Gladys Morrison [a,1], Cong Liu [b,1], Claudia Wing [c], Shannon M. Delaney [c], Wei Zhang [d,*], M. Eileen Dolan [a,c,**]

[a] Committee on Clinical Pharmacology and Pharmacogenomics, The University of Chicago, Chicago, IL 60637, USA
[b] Department of Bioengineering, University of Illinois at Chicago, Chicago, IL 60612, USA
[c] Section of Hematology/Oncology, Department of Medicine, The University of Chicago, Chicago, IL 60637, USA
[d] Department of Preventive Medicine & The Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL 60611, USA

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ABSTRACT

Differentiated cells retain the genetic information of the donor but the extent to which phenotypic differences between donors or batches of differentiated cells are explained by variation introduced during the differentiation process is not fully understood. In this study, we evaluated four separate batches of commercially available neurons originating from the same iPSCs to investigate whether the differentiation process used in manufacturing iPSCs to neurons affected genome-wide gene expression and modified cytosines, or neuronal sensitivity to drugs. No significant changes in gene expression, as measured by RNA-Seq, or cytosine modification levels, as measured by the Illumina 450K arrays, were observed between batches relative to changes over time. As expected, neurotoxic chemotherapeutics affected neuronal outgrowth, but no inter-batch differences were observed in sensitivity to paclitaxel, vincristine and cisplatin. As a testament to the utility of the model for studies of neuropathy, we observed that genes involved in neuropathy had relatively higher expression levels in these samples across different time points. Our results suggest that the process used to differentiate iPSCs into neurons is consistent, resulting in minimal intra-individual variability across batches. Therefore, this model is reasonable for studies of human neuropathy, druggable targets to prevent neuropathy, and other neurological diseases.

1. Introduction

Neurodegenerative diseases and neuropathy are difficult to study due to lack of relevant human models (Phillips et al., 2009). Cell culture systems and primary rodent cultures have proven to be indispensable to clarify disease mechanisms and provide insights into gene functions. However, the current models have not provided much in terms of therapy for inherited neuropathies (known collectively as Charcot–Marie-Tooth disease) (Ekins et al., 2015), and the only effective treatments for diabetic neuropathy are glucose control and pain management (Callaghan et al., 2012). Chemotherapy-induced peripheral neuropathy (CIPN) is a common neurotoxicity affecting 20–40% of patients receiving chemotherapy (Smith et al., 2013). To truly understand and find relevant druggable targets that are causative, a cellular model that represents neuropathy is essential.

With recent advances in stem cell technology, the ability to differentiate human induced pluripotent stem cells (iPSCs) to neurons provides us with a new and potentially relevant human neuronal model. In addition, iPSC-differentiated neurons can be created from diseased individuals or individuals with severe sensitivity to neurotoxic chemotherapy to provide a model that will allow for the identification of *in vitro* phenotypic characteristics relevant to the disease or sensitivity to neurotoxic drug. These neurons may yield targets essential to overcoming and preventing symptoms associated with heritable neuropathy or CIPN. Stem cell technology has revolutionized the field of "in vitro disease modeling" (Sandoe and Eggan, 2013), as evidenced by the first set of drugs emerging into clinical trials from the use of iPSC derived neurons from patients with neurological diseases (Mullard, 2015).

Human fibroblasts were reprogrammed from an individual into nociceptor neurons without creation of iPSCs and the neurons exhibited sensitization to the chemotherapeutic drug oxaliplatin, modeling the inherent mechanisms underlying painful CIPN (Wainger et al., 2015); however, the advantage to creating iPSCs as an intermediate is that they can grow indefinitely, thus providing a ready source to create additional neurons of the same genetic background. Recently, our laboratory developed a potential model to evaluate CIPN by employing commercially available human neurons differentiated from iPSCs (Wheeler et al., 2015). We found reproducible differences in morphological characteristics including neurite outgrowth phenotypes, cellular viability and apoptosis following treatment with four distinct chemotherapeutic drugs: vincristine, paclitaxel, cisplatin and hydroxyurea. This

* Correspondence to: W. Zhang, 680 N. Lake Shore Dr., Suite 1400, Chicago, IL, 60611, USA.
** Correspondence to: M.E. Dolan, 900 E. 57th St, KCBD 7100, University of Chicago, Chicago, IL 60637, USA.
E-mail addresses: wei.zhang1@northwestern.edu (W. Zhang), edolan@medicine.bsd.uchicago.edu (M.E. Dolan).
[1] These authors contributed equally to this work.

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model was also used to demonstrate functional consequences of gene knockdown on neuronal sensitivity to chemotherapeutics of genes identified through clinical genome-wide association studies (GWAS) of CIPN (Wheeler et al., 2015; Leandro-Garcia et al., 2012; Diouf et al., 2015; Komatsu et al., 2015).

The potential of using the human iPSC-derived neuron model for larger genetic association studies requires an understanding of heterogeneity of cultures and to partition the variance associated with iPSC reprograming, culturing, and differentiation (Boulting et al., 2011; Rouhani et al., 2014; Thomas et al., 2015). A major concern in the field of stem cell technology is that techniques to reprogram cells could introduce variation that masks important genetic differences between individuals. A recent study demonstrated that the genetic background of iPSCs generated from peripheral blood mononuclear cells or fibroblasts accounted for more of the variation in gene expression between iPSC lines than any other tested factors such as cell type of origin or reprogramming method (Rouhani et al., 2014). These studies suggest that future studies should focus on collecting a large number of donors rather than generating large numbers of lines from the same donor. Since industrial grade cells can now be made, the evaluation of epigenetics, gene expression and phenotypic variation from batch to batch is an important consideration.

In this study, we obtained multiple batches of iCell® Neurons (iPSC-derived human cortical neurons) differentiated from a single iPSC originating from fibroblasts of an individual to evaluate inter-batch differences in gene expression, cytosine modification levels, and pharmacologic response to chemotherapeutics. To determine the utility of these cells for studies of neuropathy and other neurological diseases, we evaluated genes involved in hereditary neuropathy at different time points in culture as neurites were formed. We showed a consistent enrichment of genes with relatively higher expression levels among hereditary neuropathy associated genes over time.

2. Methods

2.1. ICell Neurons

Neurons (iCell Neurons®) were purchased from Cellular Dynamics International (CDI, Madison, WI, USA). iCell Neurons are an ~98% pure (Tuj1 +/Nestin −) pan-neuronal population of GABAergic and to a lesser degree glutamatergic neurons produced from human induced pluripotent stem cells. All batches of iCell Neurons are tested for sterility, viability, purity and morphology, and released according to strict manufacturer's standards. Four batches of iCell Neurons (batch numbers: 1366431, 1366825, 1369525, 1362632) were thawed and maintained according to the manufacturer's protocol. Each batch of iCell Neurons was mixed with 3.3 μg/ml laminin (Sigma-Aldrich) prior to seeding on poly-D-lysine coated 96-well Greiner Bio-One plates at a density of 1.33×10^4 cells/well. Approximately 1.1×10^6 neuron cells were pelleted immediately for the 0 h sample by spinning at 300 ×*g* for 10 min and either lysed using Quizol (Qiagen) for RNA extraction or media removed from pellet before storing both samples at −20 °C. For the cell collections to evaluate modified cytosine and gene expression, cells were pelleted at 0, 4, 28, and 76 h and then extracted DNA and RNA.

2.2. Drug preparation and treatment

Paclitaxel (Sigma-Aldrich) was prepared in the semi-dark by dissolving powder in 100% DMSO and filtered to obtain a stock solution of 58.4 mM. Control wells were treated with 0.17% final concentration of DMSO to match drug treatments. Cisplatin (Sigma-Aldrich) was prepared in the semi-dark by dissolving powder in 100% DMSO and filtered to obtain a stock solution of 20 mM. Control wells were treated with 0.2% final concentration of DMSO to match drug treatment. Vincristine (Sigma-Aldrich) was prepared on ice in the dark by dissolving powder in cold PBS and filtered to obtain a stock solution of 100 mM. Vincristine stocks were each diluted independently then added into the media and onto the cells before proceeding to the next dilution. All stock drugs were serially diluted in media for final dosing concentrations ranging from 0.01 μM to 100 μM, increasing by factors of ten. Cells were plated and 4 h later treated with increasing concentrations of drug for 72 h.

2.3. High content imaging and neurite outgrowth analysis

After drug treatments of 72 h, neurons were stained for 15 min at 37 °C with 1 μg/ml Hoechst 33342 (Sigma-Aldrich) and 2 μg/ml Calcein AM (Molecular Probes, Life Technologies Inc., Carlsbad, CA, USA) then washed twice using dPBS without calcium or magnesium (LifeTechnologies). Imaging was performed at 10× magnification using an ImageXpress Micro (Molecular Devices, LLC, Sunnyvale, CA, USA) at the University of Chicago Cellular Screening Core. Individual cell measurements of total neurite outgrowth (sum of the length of all processes), number of processes and number of branches were calculated using the MetaXpress software Neurite Outgrowth Application Module (Molecular Devices, LLC). At least 500 cells per dose were quantified in triplicate for three independent experiments.

2.4. Nucleic acid isolation

At each time point, DNA was extracted by adding 50 μl per well of tissue digestion buffer (100 mM NaCl, 50 mM Tris 8.0, 100 mM EDTA, 1% SDS) along with 2 mg/ml Proteinase K (Denville Scientific; South Plainfield, NJ, USA). The plate was sealed with parafilm and agitated overnight at 55 °C using 200 RPM in an Innova orbital shaker (Eppendorf; Enfield, CT). The digested cells from each well were combined and DNA extracted with equal volume of phenol: chloroform (Invitrogen), vortexing for 10 min (Lab Vortex-setting of 4) and the samples were centrifuged for 5 min at 14,000 ×*g*. The aqueous phase was collected, extracted with equal volume of chloroform and repeated two times. The DNA was precipitated with equal volume 100% ethanol and centrifuged for 10 min at 14,000 ×*g*. Two washes with 70% ethanol produced a pellet that was air dried for 10 min at room temperature and finally dissolved in 50 μl of 10 mM Tris (pH 8.0) for 3 days at 4 °C then stored at −80 °C.

RNA isolation was performed after removal of media and addition of 50 μl Qiazol per well plate for 5 min at room temperature to lyse cells. After vigorously pipetting each well several times, each batch was collected and stored for further processing at −80 °C. When all the time points had been collected, the RNA was purified using the Ambion RNA protocol (15596026.PPS), substituting Qiazol as the lysing reagent after consulting with the company. The final pellet was resuspended in 50 μl of RNase-free water, dissolved at 55 °C for 10 min, aliquoted, and stored at −80 °C.

Nucleic acid quantification was performed using the Quibit dye for RNA or dsDNA kits (Molecular Probes, Life Technologies), as per the manufacturer's specifications.

2.5. RNA-Seq and analysis

1 μg RNA from each time point was submitted to the University of Chicago Genomics Core. RNA quality was then checked on the Agilent Bio-analyzer 2100. RNA-Seq libraries were generated in the core using Illumina RS-122-2101 TruSeq® Stranded mRNA LT libraries and the final libraries checked again on the Agilent bio-analyzer 2100, which was followed by sequencing on the Illumina HiSeq2500. High quality sequencing paired-end reads of 100 bp in each (76.4 to ~83.7%) were mapped to the human genome reference (hg19) using TopHat2 (Kim et al., 2013). Estimated genome coverage is 33.6% to ~52.1%. Cufflinks (Trapnell et al., 2010) were then used to quantify gene expression levels of the assembled transcripts. Fragments per kilobase of transcript per