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Fabrication and Evaluation of Pluripotent Stem Cells Expressing in Human iPS Cells as In-vitro Model

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Abstract

Our latest report details the production of human induced pluripotent stem cells (iPSC) master cell banks (MCB) that are produced by a therapeutically compliant approach that starts with cord blood. The two iPSC clones created by this method were thoroughly characterised in this publication, utilising whole genome sequencing (WGS), microarray, and comparative genomic hybridization (aCGH) single nucleotide polymorphism (SNP) analysis. We compare these profiles with lines created by a similar procedure from different donors, a reporter subclone, and a suggested calibration material. We think it is likely that several clinical products will be made using iPSCs. Furthermore, given their immortal state, we anticipate that the lines utilised as input material will be used for many years or even decades at various sites. As a result, assay development will be crucial to tracking the condition of the cells and their drift within the culture. We propose that a thorough description of the cells' starting state, a comparison with some calibration data, and the creation of reporter sublcones will aid in identifying the most beneficial set of tests for keeping an eye on the cells and defining standards for eliminating a line.

Keywords: Pluripotent Stem Cells, Human iPS Cells, cGMP, Manufacturing, etc.

Introduction

When it comes to their capacity for development, induced pluripotent stem cells (iPSCs) are like embryonic stem cells (ESC) [1, 2], but they differ from ESC in that they require a specific collection of proteins to establish pluripotency [3]. Even though they have the same functional characteristics as ESC, iPSCs may differ slightly from them due to factors such their epigenetic profile, environmental exposure, mitochondrial

makeup, and maybe X chromosomal inactivation [4]. These variations stem from the origin of the starting material and could be exacerbated by the process of pluripotency induction [5]. It is crucial to remember, though, that recent research has demonstrated that the inherent distinctions between ESC and iPSC do not always translate into functional utility. Rather, a number of extensive analyses have confirmed that the observed differences are more indicative of individual allelic diversity [6, 7]. The spectrum of variances observed across iPSC lines derived from distinct persons is also present between ESC and iPSC, as well as between iPSC lines derived from the same donor but distinct tissues [8, 9]. Not less significant, the alterations brought about by the nonintegrating methods of iPSC creation fall into the same range as those observed after extended cell culture [10].

Although significant to the scientific community, these variations would not affect the potency or effectiveness of the differentiated cells derived from these lines, making them essentially inconsequential to regulatory bodies and the development of an allogeneic or autologous product [11]. In fact, this idea has been applied to hematopoietic stem cell transplants in which many donors—differing in their allelic background and likely in their efficacy—have been transplanted without demonstrating through in vitro or in vivo testing that each sample was functionally equivalent [12]. It was assumed that the donor's cells were operating normally and that the harvesting procedure would not change the cells (i.e., the cells were only little altered), so it was reasonable to assume.

Subsequently, matched unrelated donors (MUD) transplants were added to this idea, along with sibling or related transplants. In this case, the idea of functional equivalence could be interpreted legitimately even if the transplanted cells were operating in a different environment. The notion of functional equivalency was expanded to include cord blood, which serves the same role as bone marrow but is regarded as undergoing more than minimum manipulation due to its distinct processing. The regulatory authorities reasoned that various groups employing different procedures and manufacturing at separate places might be permitted under a Biologics Licence Application (BLA) provided functional equivalency in in vivo experiments demonstrated that cells could be generated reliably and reproducibly. In fact, five public cord blood banks have received approval to offer MUD-type transplants to patients who meet widely recognised standards for functional equivalency. The approval process did not necessitate any animal research due to the abundance of accessible in vivo human data. It is significant to remember that American regulatory authorities acknowledged that autologous or related cord blood uses, like those suggested by private cord blood banks, should not be subject to the same BLA licensure requirements. In fact, those banks are exempt from these regulations. This reasoning has been expanded to include other autologous therapies, such as autologous T-cells, B cells, dendritic cells, NK cells, and macrophages, when cells are altered more than little [12]. Each lot of cells is produced in adequate quantities for a single human, and they are all fundamentally distinct from one another. The cells are transplanted into hosts in varying stages of sickness, presumably in environments that differ from one another. Unlike with an allogeneic product intended for use in hundreds or thousands of patients, the authorities have not mandated testing for each lot. Instead, they have requested that participants show that the processed final product is functionally identical [12]. Authorities have occasionally demanded that eight or ten produced samples demonstrate their efficacy in an animal model and, in other instances, have mandated minimal human safety research [12]. Having sufficient tests or comparable data that evaluate function has proven essential to such approval [13].

It is anticipated that the regulatory bodies will consider a comparable reasoning for more autologous or HLA-matched products, such as those originating from induced pluripotent stem cells [12]. Autologous or matched cells will therefore be regulated differently than allogeneic products created from iPSCs, like cord blood. We also reasoned that the community could specify the functional equivalency of these new lines if other parties wanted to create additional ones using the same procedure. We believed that no animal testing was necessary at the iPSC stage because the lines are essentially raw material to create fully differentiated cells. Instead, criteria for use in subsequent downstream processing could be established by in vitro differentiation assays and established quality control (QC) criteria for pluripotency. The finished product would undergo functional characterisation and equivalency testing with any relevant in vivo or human investigations. Comparability data will be crucial in both situations, even though we anticipate that the tests needed and the laws governing them will change depending on whether the product is used for autologous or allogeneic production. We started a programme to create therapeutically compliant cells because most groups were initially concentrating on allogeneic therapy. We have reported on the creation of two such lines [1], which we assume will be utilised to produce a range of products from an MCB. We reasoned that the cost of process

development would be amortised over many patients, even though it was costly and time-consuming [1,14]. According to our results, these lines might be employed to commercialise iPSC-based cell treatment by going through the typical IND (Investigational New Drug) process.

But as had been the case with other cell therapies (see above), it became apparent that this was not a feasible approach for autologous cells and haplobank-derived cells. One would have to cut expenses, and the authorities would have to create models like the ones they have created for cord blood banks. Consequently, we assessed the necessary steps to minimise expenses if cells were utilised for autologous therapy [13] or if a Haplobank was created [16]. Furthermore, we reasoned that additional characterization and a database to track changes might be required because these lines could be used by multiple people and generated into multiple different products, each of which would probably be manufactured in a different location by different companies. The comprehensive characterization of two cGMP-compatible iPSC lines utilising WGS, array-based analysis, and a CGH SNP analysis is presented in this publication. Two of these lines were created using the same GMP-compatible process previously described [1]: LiPSC-GR1.1 was generated during GMP manufacturing runs, while LiPSC-ER2.2 was generated during engineering runs. Our objective is to give end users the information they need to decide which subset of tests will be necessary for continuous monitoring, how to use these tests to assess the usage of subclones for preclinical research or cell therapy, and how to create comparability across manufacturing locations.

Material and Method

Generation of Human Induced Pluripotent Stem Cells

Human iPSC lines used in the analysis and standard analysis (Table 1.0) were generated at Lonza Walkersville, Inc. as described before [1]. Briefly, cryopreserved human umbilical Cord Blood (hUCB) CD34+ cells (Lonza, 2C-101) were thawed and expanded in a priming medium comprised of a basal medium [including IMDM (Iscove's Modified Dulbecco's Medium; Life Technologies, 12440–053), Ham's F12 (Life Technologies, 31765–035), Chemically Defined Lipid Concentrate (Life Technologies, 11905–031), Bovine Serum Albumin Fraction V (Life Technologies, 15260–037), and Insulin,-Transferrin-Selenium-Ethanolamine (ITS-X) (Life Technologies, 51500–056)] supplemented with 100 ng/ mL recombinant human (rh)SCF (PeproTech, AF-300-07), 100 ng/ml rhFlt3-ligand (PeproTech, AF-300-19), 20 ng/ml thrombopoietin (PeproTech, 300–18) and 10 ng/ml IL-3 (PeproTech, 200–03). The CD34+ cells were seeded at a density of 4-6 x 105 cells per well in 12-well plates (Corning, 3513). A day before nucleofection, confluent cells (about day 3 post-thaw) were passaged. Using the episomal plasmids encoding Oct4, Sox2, Klf4, c-Myc, Lin28, and pEB-Tg, 1 × 106 hUCB CD34+ cells were reprogramed [17, 18]. Utilising the 4D-NucleofectorTM System and P3 solution Kit (Lonza, V4XP-3012), these plasmids were inserted into the cells. Following nucleofection, the cells were plated in the priming medium in an incubator with 5% CO2 and 3% O2 at 37 °C and humidity. To improve the reprogramming efficiency, thirty micrograms of Alhydrogel® adjuvant 2% (InvivoGen, vacalu-250) were added right away to the expansion medium in certain experimental settings. After plating, the cells were moved into 6-well plates that had been coated with L7TM hPSC Matrix and supplied with 1 μM TGFβ inhibitor in L7 hPSC medium (Stemgent, 04–0014) two days later. The cells were kept in a humidified incubator with 5% CO2 and 3% O2 at 37 °C. During the reprogramming phase, the cell culture medium was changed every other day until hiPSC colonies emerged and were isolated for additional characterisation and expansion.

Embryoid Body (EB) Differentiation

Using L7TM hPSC Dissociation Solution, confluent cultures of human pluripotent stem cell colonies were separated. Cell aggregates were allowed to settle by gravity in a conical tube after being suspended in EB formation medium made of DMEM/F12 (Life Technologies, 11330–032) and 10 μM Rock Inhibitor Y27632 (Millipore, SCM075). Fresh EB media was used to suspend the cells after the supernatant was removed. After that, cell aggregates were seeded on Ultra Low Attachment (Corning, YO-01835-24) plates with a split ratio of 1:1 and kept in the incubator for 12 to 24 hours. Large cell aggregates were gathered into a conical tube and allowed to settle naturally as they became larger. After that, the medium was taken out and replaced with differentiation medium, which contained the following: 2 mM L-glutamine (Cellgro/Mediatech, 25-005-CI), 80 % DMEM High Glucose (Life Technologies, 11965–092), 20 % defined foetal bovine serum (Hyclone, SH30070.03), 1X non-essential amino acids (Life Technologies, 11140–050), and 55 μM β-Mercaptoethanol (Life Technologies, 21985–023). The cell aggregates were split 1:1 in 0.4 ml of

differentiation medium/cm2 and put on Ultra Low Attachment plates. For the next six days, the culture medium was switched every two days. The seventh day saw the seeding of EBs at a density of 10 EBs/cm2 on gelatin-coated plates using EmbryoMax® ES Cell Qualified Gelatin Solution (Millipore, ES006-B). For two days, the EBs were left in an undisturbed state. After the second day, and then every other day after that, 0.4 ml/cm2 of differentiation medium was added. On day 14, the cultures were ready for immunocytochemistry.

As previously mentioned, 4% PFA was used to fix differentiated hPSCs, and 0.1% Triton X-100 PBS solution was used to permeabilize them. The fixed cells were rinsed with PBS-T solution and then treated for two hours at room temperature in DPBS containing 10% goat serum (Life Technologies, 10000C). Blocking cultures were treated overnight at 2–8 °C with primary antibodies recognising alpha-1 Fetoprotein (Abcam, ab3980; 1:200 or R&D systems, MAB1369, 1:100), beta III tubulin (Millipore, MAB1637; 1:400), and Smooth Muscle Actin (DAKO, M0851; 1:500). Following two PBS-T rinses, the cells were incubated on the secondary antibody for at least two hours at room temperature. The secondary antibody could be either Alexa Fluor 488-conjugated goat anti-mouse IgG(H + L) (Life Technologies, A11001; 1:1000) or Alexa Fluor 494-conjugated goat anti-mouse IgG(H + L) (Life Technologies, A-11032; 1:1000). Before being counterstaining with DAPI, the cultures were rinsed three times (for five minutes each) in 1X DPBS. For analysis, the cells were kept in 50% glycerol.

Flow Cytometry

When the cells in the hPSC media were roughly 70–80% confluent, flow cytometry was carried out on the cells. Using a solution of 0.05 % Trypsin/EDTA (CellGro, 25-052-CI) containing 2% chick serum (Sigma-Aldrich, C5405), the cultures were separated into a single-cell suspension. In accordance with the manufacturer's suggested technique, the cells were fixed and permeabilized for intracellular labelling using the Cytofix/Cytoperm Kit (Becton Dickinson, 554714). PE-conjugated antiOCT3/4 (R&D Systems, IC1759P) or corresponding PE-conjugated anti-IgG isotype control were treated with permeabilized cells. Using PE-conjugated antigen-specific antibodies and corresponding isotypes at the manufacturer's recommended concentration, extracellular antigens were identified on unfixed cells stained with the following antibodies: anti-TRA-1-60 (Becton Dickinson, 560193), anti-TRA-1-81 (Becton Dickinson, 560161), anti-IgG3 isotype (Becton Dickinson, 556659); anti-SSEA4 (Becton Dickinson, 560128) and antiIgM isotype (Becton Dickinson, 555584). After that, the samples were run through a Becton Dickinson FACSCantoTM II flow cytometer. Software called BD FACS Diva was used to collect the data, and Flowjo 7.6 was used to analyse it.

Immunocytochemistry

The medium used for hPSC cell culture was used to cultivate human pluripotent stem cells. The following steps were taken to prepare the hiPSC colonies from days three through five of the cultures for immunocytochemical examination. Cells were twice washed with 1X Dulbecco's Phosphate Buffered Saline (Lonza Biosciences, 17-513 F) after the culture media was aspirated. The cells were then incubated with 10% donkey serum in PBS-T at room temperature for two hours after being fixed in 1X DPBS containing 4% PFA (Electron Microscopy Sciences, 15710) for 20 minutes. The cells were then washed twice with PBS-T (0.2 % Tween-20 in 1X DPBS) for five minutes. The hPSCs were then permeabilized for 20 minutes in 1X DPBS containing 0.1% Triton X-100 (Sigma-Aldrich, T9284) after being treated with primary antibodies that detected the extracellular antigens SSEA4 (Millipore, MAB4304; 1:100), TRA-1- 60 (Millipore, MAB4360; 1:100), and TRA-1-81 (Stem Gent, 09–0011; 1:100) overnight at 2–8 °C. The cells were first blocked using 10% donkey serum solution, and then they were incubated with intracellular primary antibodies for an additional night at 2–8 °C. The secondary antibodies Cy3-conjugated Donkey anti-rabbit IgG (Jackson Immuno Research, 711-165-152; 1:200) and Cy3-conjugated Donkey anti-Goat IgG (H + L) (Jackson ImmunoResearch, 805-165-180; 1:200) were used in combination with primary antibodies raised against pluripotency associated antigens OCT4 (Abcam, ab19857; 1:350) and Nanog (R&D Systems, AF1997; 6.7 µg/ml). Secondary antibodies, Alexa Fluor 488-cojugated donkey anti-mouse IgG (H + L) (Jackson Immunoresearch, 715-545-150; 1:200) and Alexa Fluor 488-cojugated donkey anti-mouse IgM (H + L) (Jackson Immunoresearch, 715-545-140; 1:200), were used in conjunction with primary antibodies specific for SSEA4 and TRA-1-60/TRA-1-81.

After two hours of secondary antibody incubation, all cells were counterstained for 15 to 30 minutes at room temperature using 300 nM DAPI (Life Technologies, D3571) in 1X DPBS. Following permeabilization and in between the primary and secondary antibody incubations, the cells were washed. The wells were immediately filled with 50% glycerol following the last PBS-T wash. A software version 17625-equipped EVOS® FL all-in-one microscope was used to visualise every fluorescence detection. Human pluripotent stem cells that developed into the neural lineage were stained and immunocytoanalyzed according to previously published methods [19]. In summary, the cells were fixed for 20 minutes with 4% paraformaldehyde, blocked for an hour in blocking buffer (10% goat serum, 1% BSA, 0.1 % Triton X-100), and then the primary antibody was incubated for an additional night at 4 °C in 8% goat serum, 1% BSA, and 0.1 % Triton X-100. For single or double labelling, appropriately linked secondary antibodies such as Alexa350, Alexa488, Alexa546, Alexa594-, or Alexa633 (Molecular Probes, and Jackson ImmunoResearch Lab Inc., CA) were utilised. The cross-reactivity and non-specific immunoreactivity of every secondary antibody was examined.

Expression Analysis by Microarray

The microarray core facility at the Burnham Institute for Medical Research performed the hybridization of total RNA to the Illumina Human HT-12 BeadChip (Illumina, Inc., CA) using the RNeasy® Mini kit, following the manufacturer's instructions (Qiagen, CA). The Illumina BeadStudio software's built-in algorithms were used for all data processing and analysis. For normalisation, the background approach was employed. The gene's expression value was determined by taking the maximal expression value for the probe set. Using the full linkage approach and the Euclidian distance, the dendrogram for the processed data was represented by global array clustering of genes across all experimental samples. The R System's measure of Pearson's coefficient was used to express sample correlations.

CGH-CHIP Analysis

Cell Line Genetics' aCGH + SNP service was used for the CGH-CHIP study. The contract lab received cryopreserved vials of the iPSCs to prepare the sample and perform the assay in accordance with established protocols, which are outlined below. After being thawed at 37 °C and once again in 1xPBS, the iPSC cryovials were centrifuged. After removing the supernatant, the cell pellet was treated for two minutes at room temperature with proteinase K and RNase. The samples were placed on a DNeasy® mini spin column and attached by centrifugation after the addition of lysis buffer and a 10-minute incubation period at 56 °C. After two rounds of washing in wash buffer, samples were eluted in suspension buffer. A Zymo DNA clean and concentration column was then used to clean the gDNA samples. Centrifugation was used to add the gDNA and ChIP DNA binding buffer to a Zymo-Spin IC-XL column. After twice being cleaned with wash buffer, the tube was eluted in DNA suspension buffer. Using an agarose gel analysis, Qubit TM Fluorometer, and Nano Vue TM UV spectrophotometer, the concentration and purity of the DNA were assessed. The extracted gDNA needs to fulfil three criteria: a concentration of at least 1 µg of dsDNA as determined by the Qubit TM Fluorometer; a 260/280 Ratio between 1.76 and 1.9 as determined by the Nano Vue TM Spectrophotometer; and a 260/230 Ratio of at least 1.9 as determined by the Nano Vue TM Spectrophotometer.

After gDNA isolation, 500–1500 ng of total (RNase treated) DNA input were used to prepare labelling reactions using the Agilent Sure Tag Complete Labelling Protocol for aCGH. The two steps of the Agilent microarray aCGH procedure are hybridization and DNA labelling. Using restriction enzymes Rsa1 and Alu1, equal volumes of the test and reference samples (500–1500 ng) were first enzymatically sheared for aCGH + SNP arrays. Cyanine 5-dUTP was used to label the DNA of the test sample, while the Exo-Klenow fragment used Cyanine 3-dUTP to label the reference DNA. After the labelled DNA was purified, the Nano Vue TM UV spec was used to measure the labelling efficiency and concentration. After that, the test and relevant reference samples were mixed and denatured. For 24 hours at 65 °C, the labelled probes were left to hybridise with the feature on the microarray. Ultimately, the arrays underwent thorough washing before being sent to an Agilent Sure Scan Microarray Scanner with a resolution of 3 µM. Feature data was collected, processed, and mapped using Agilent CytoGenomics' ADM-2 Segmentation Algorithm to the human genome (hg19).

Whole Genome Sequencing

In Rockville, MD, Macrogen Clinical Laboratory carried out whole genome sequencing. A sample preparation guide for Illumina TruSeq Nano DNA was followed in the preparation of the samples. In short, the DNeasy® Blood & Tissue Kit (Qiagen, CA cat#69506) was used to extract the whole genomic DNA in accordance with the manufacturer's instructions. The Illumina TruSeq DNA PCR-Free Library Preparation Kit was then used to prepare one microgram of genomic DNA, yielding a final library with a fragment size of 300–400 bp. Paired-end 2x150bp runs using finished, indexed library pools were conducted on the Illumina HiSeq platform. BAM files were produced by aligning FASTQ files using ISAAC Aligner (version 1.14.08.28) after they were produced using bcl2fastq2 (version 2.15.0.4). The ISAAC Variant Caller version 1.0.6 was used to identify SNPs, Indels, structural variants (SV), and copy number variations (CNV) [20]. Locus readings with genotype quality < 30 were excluded from analysis for both SNPs and Indel. Using the hg19 reference genome and the dbSNP138 build, the vcf file that was thus generated was annotated using SNPEff Version 4.0e (http://snpeff.sourceforge.net/) [21]. For samples of European descent, the ESP6500 and 1000 Genome Project phase1_release_v3 databases provided the alternate allele frequency. Basic statistics including the total number of reads, number of duplicate reads, total number of mapped reads, and total number of unmapped reads were obtained using Sam tools. The mapping quality statistics were reported using SAMSTAT version 1.5.1 (http://samstat.sourceforge.net/) [22]. The Isaac Variant Caller was utilised to calculate the depth of every chromosome.

With the aid of the analytical programme Boogie, the generated variations were utilised to forecast the blood group phenotypes [23]. Predictions of blood groups were produced for the commonly used Rh and ABO systems. In addition, predictions were made for the glycoprotein systems linked with MN and Rh for both cell lines. Gene variations implicated in the blood group systems listed above were inputted with genotype information, which included chromosomal number, genomic position, reference allele, alternate allele, and zygosity. Boogie used the 1-nearest-neighbor approach to validate the pertinent variants in the input genotype with defined phenotypes in the haplotype table that the software supplied by default. The highest score goes to the SNV permutation that most closely resembles the phenotype. Thus, the blood types that were anticipated were compared with available donor data.

Using software called HLAVBseq, created by Nariai and colleagues [24], the HLA class I (HLA-A,-B, and -C) and II (HLA-DQA1, −DQB1 and -DRB1) profiles for the iPSC lines were estimated from the WGS data. BWA-MEM was used to align FASTQ files to the reference genome to create a sam file. The alignment of sequence reads to genomic HLA sequences recorded with the IMGT/HLA database is the foundation of this methodology. The anticipated read counts for HLA alleles are estimated using a statistical framework based on variational Bayesian inference. For paired end data, the hyperparameter alpha zero is set to 0.01. The authors' perl script for 200 bp of data was used to calculate the average depth of coverage for each HLA allele. The HLAssureTM SE SBT kit's HLA typing findings were cross-checked against the expected HLA types.

Only the non-synonymous variants were taken into consideration in order to confirm whether these cell lines displayed any alterations in genes linked to Parkinson's disease. The variants were ranked according to how beneficial or harmful the amino acid substitutions were. While Polyphen predicts these changes based on the impact of the amino substitution on the structure and function of the protein based on physical and comparative considerations [26], the insilico predictions programme, such as SIFT, bases its predictions on the degree of conservation of amino acid residues [25]. dbNSFP was used to calculate the SIFT and Polyphen scores [27]. The prioritised variations were cross-validated against the gene cards (www.genecards.org) list of PD-related genes. The dbNSFP-annotated Clinvar and MIM disease databases were consulted for the variants that made the short list. In order to confirm if these PD-related genes had any alteration in their expression in comparison to the control lines H9, H7, and NCRM6, an integrative analysis of WGS and expression data was carried out [28].

The cell lines were analysed since the Issac variant caller, which was utilised to find structural variants for them, revealed the greatest amount of deletions. Read pairs that support the variant with low confidence, variants with genotype quality <20, and reads with MAPQ of zero around the break-end or uncertain exact breakpoint site were eliminated. With the use of the UCSC table browser (https://genome.ucsc.edu/cgi-bin/hg Tables), the filtered variants were annotated for the genes. Integrated Genomics Viewer (IGV) [29] was

used to manually view deletion events to look for a dip in coverage at the deletion sites. To determine the degree of overlap between two sets of genomic coordinates, Bed Tools v2-2.20.1 was utilised. To find out if there were any difference expression levels because of these deletions, this data was cross-validated against expression data. To confirm the significance of these short listed differentially expressed genes in distinct OMIM_ illnesses and pathways, gene enrichment analysis was conducted [30]. Duplications were handled in a similar manner [31].

To determine whether there was any gene overlap between the two cell lines' SNP CHIP data and the results, this was done. To confirm whether the genes discovered by micro array were overlooked in WGS because of filtering, this cross comparison was performed even with the unfiltered SV data. The published list of imprinted genes was taken from the database (http://www.geneimprint.com/) to confirm the imprinted genes' status. If the alternate allele's allelic depth was less than 10, it was filtered. For these imprinted genes, the number of homozygous and heterozygous SNPs, or INDELS, was computed and the overlap of the genes with expression data was confirmed. Based on the available data, the expression of these genes that is particular to either paternity or mother was reported. Phasing could not be performed on these samples to determine the maternal or paternal specific inheritance pattern of these variations found by the WGS since no parental information was available.

HLA Type Analysis

Texas Biogene, Inc. (Richardson, TX) performed HLA-typing utilising HLAssureTM SE SBT typing kits. With the HLAssureTM SE SBT Kit, HLA alleles can be found by the sequence-based typing (PCRSBT) method of PCR amplification. In summary, the entire genomic DNA was extracted utilising the DNeasy® Blood & Tissue Kit in compliance with Texas Biogene, Inc.'s recommendations and the manufacturer's instructions (Qiagen, CA cat#69506) (i.e., DNA sample with an A260/A280 ratio between 1.65 and 1.8). Following Texas Biogene, Inc. protocol, the genomic DNA was examined using the Accutypes TM (HLADB-3.19.0) programme and the HLAssureTM A, B, C, DRB1, and DQB1 SBT typing kits.

Karyotype and Short Tandem Repeat (STR)

A certified service provider (Cell Line Genetics) carried out the STR and karyotype analyses utilising industry standard procedures. Cell Line Genetics, which was inspected by Lonza Walkersville, Inc., carried out human G-banding karyotyping in compliance with FDA Good Laboratory Practice using clinically trained cytogeneticists skilled in spotting chromosomal aberrations from pluripotent stem cells. Twenty chromosomes from living or fixed cells in metaphase were analysed for each cell line. Leishman stain and G-banding were used in the analysis, and the American College of Medical Genetics' Clinical Cytogenetics Standards and Guidelines [32] were followed in the examination of the cells. In order to find a match of at least 80% of the 16 loci examined, the STR assay used PCR and capillary electrophoresis on a Power Plex 16 multiplex STR platform (Promega). Soft Genetics Genemarker was used to analyse the data. Before reporting, every test was checked for cross contamination, artefacts, and off ladder peaks.

MCB Viral Testing

FDA regulations state that before allogeneic MCBs are released for clinical use, a thorough screening process is conducted to check for viral contaminants. Based on the biological properties of pluripotent stem cells, the MCB virus testing protocol for hiPSCs was modified to include both in vitro and in vivo assays [1]. Samples were prepared in accordance with the standard operating procedures advised by the contract laboratory (Bio Reliance), and they were then delivered to Bio Reliance in the proper format and condition. All of Bio Reliance's studies are carried out in accordance with the UK and German GLP Regulations, the US FDA Good Laboratory Practice Regulations (21 CFR 58), the Japanese GLP Standard, and the OECD Principles of Good Laboratory Practice. Bio Reliance is fully accredited for GLP.

Assay Qualification, Characterizations, and in Process Control
Flow Cytometry Assay for Pluripotent Stem Cells

In accordance with the most recent Good Manufacturing Practices and the International Conference on Harmonisation Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation

criteria, the flow cytometry test for evaluating human pluripotent stem cells was qualified [33]. Stage-specific embryonic antigen-4 (SSEA-4), Tra-1-60, and Tra-1-81 were used in the qualifying investigation. Furthermore, the qualifying study included Oct4, a transcription factor that is believed to be essential for preserving the pluripotency and self-renewal of embryonic stem cells [34]. Based on published data as well as data acquired during the process development phase, the release criteria for pluripotency markers were defined based on the positive expression of four distinct markers (SSEA-4, Tra-1-60, Tra-1-81, and Oct3/4). Since the final product, hiPSCs, were generated through reprogramming of cord blood derived CD34 positive cells, negative expression of surface marker CD34 was also included in the qualifying research. During the qualification study, the flow cytometry assay's precision (intra-assay, inter-assay, and intermediate), accuracy, specificity, and sensitivity were assessed. A certified flow cytometry technique with predetermined release parameters was subsequently employed to assess the identity and purity of humaniPSCs.

Quantitative PCR for Evaluation of Residual Plasmid Clearance

Given that pEB-C5 (an EBNA1/OriP episomal plasmid expressing Oct4, Sox2, Klf4, c-Myc, and Lin28) and pEB-Tg (an EBNA1/OriP plasmid for transient expression of SV40 T antigen) were used to generate human induced pluripotent stem cells (iPSCs), a quantitative PCR (qPCR) assay (Residual qPCR^) was created to quantitatively identify leftover EBNA/OriP sequences that could have originated from either pEB-C5 or pEB-Tg. Both pEB-C5 and pEB-Tg are nonintegrating plasmids that are expected to become transparent once hiPSCs are serially passaged [18, 35]. In compliance with the International Conference on Harmonisation Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) validation guidelines, the Residual qPCR assay was qualified with the aim of determining the clinical safety of the hiPSC clones produced by episomal plasmids. During the PCR qualification experiments, the following parameters were ascertained: accuracy, specificity, limit of detection (LOD), and limit of quantification (LOQ). This qualification study was carried out utilising a validated qPCR machine and nine assays in all, carried out by three analysts on three different days. In the qualification investigation, appropriate reference, control positive, and control negative materials were used.

Characterization Assays

Assessment of the morphology of hiPSC colonies, the effectiveness of plating hiPSCs after thawing, and the production of embryoid bodies (EBs) were categorised as FIO tests because of the difficulties in validating these tests, particularly the subjective interpretation of the outcomes. By examining spontaneous differentiation into the three germ layers—ectoderm, mesoderm, and endoderm—and assessing the outcomes using qPCR analysis at the transcript level or immunofluorescence at the protein level, EB formation was utilised to prove the identification and potency of hiPSCs. Alkaline phosphatase (AP) staining was utilised to assess the effectiveness of post-thaw plating. Undifferentiated pluripotent stem cells, comprising both embryonic stem cells and iPSCs, have been extensively evaluated using AP, a hydrolase enzyme that dephosphorylates compounds under alkaline conditions, including nucleotides, proteins, and alkaloids [3, 36–38]. Staining causes the differentiated cells to look white, while the undifferentiated cells appear red or purple. However, it was challenging to set specifications and cut-off values for the cells positively stained with AP marker due to variations in the quality of AP reagents provided by various suppliers and the ensuing intensity of AP staining. As a result, this assay was unable to be qualified.

Pluritest

To confirm pluripotency, Pluritest is an online bioinformatic assay that uses gene expression data from Illumina microarrays [3, 39]. Based on 450 genome-wide transcriptional profiles, Pluritest was created. These samples are from many laboratories and range from differentiating stem cell samples to mature and developing human tissue samples. 41 samples come from iPSCs, and the remaining 223 are human embryonic stem cells. To determine pluripotency and non-pluripotency, a pluripotency score and a novelty score were created. The 450-genome wide transcriptional profiles are used to calculate the pluripotency score, which is based on the expression levels of known pluripotent and non-pluripotent genes. The pluripotency of unknown samples is inferred via a comparison of their gene expression levels with those of the 450 samples. The novelty score compares samples to well-known PSCs in the collection to determine the technical and biological variation [40].

Result and Discussion

Basic Cell Line Characterization

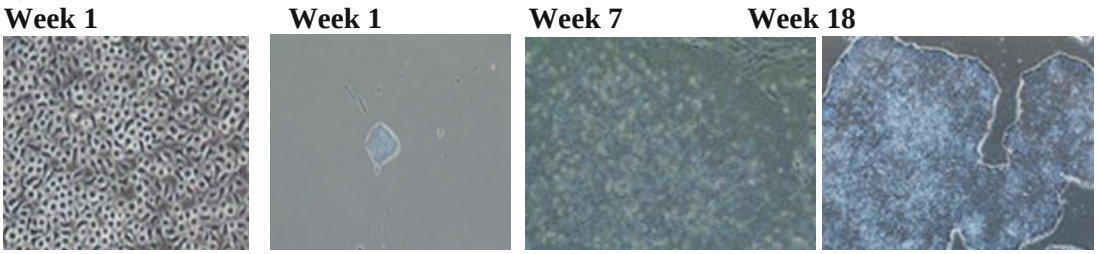
As we attempted to build a cGMP manufacturing process for hiPSCs, we produced more than fifty lines utilising a variety of techniques and many donors to assess the best approach that produced consistent, repeatable results in our hands. A crucial set of necessary tests that were like those needed for most of the cell lines were discovered during the process development (Table 1). To further enhance the release assay, we included karyotype analysis. This is because karyotype abnormalities, such as trisomies of chromosomes 12 and 17, have been reported in vitro cultures of human ESCs and iPSCs , and these abnormalities have been proposed as hallmarks of malignant germ cell tumours. Since regulatory bodies have not established any necessary tests for assessing the quality of ESC or iPSC lines, we reasoned that assessing the lines' performance according to their intended application would be a reasonable place to start. We discovered that pluripotency could be ascertained by performing functional assays on the cells' capacity to differentiate into ectoderm, endoderm, and mesoderm (i.e., EB formation) as well as by the presence of pluripotency markers, which were evaluated by immunocytochemistry using well-characterized and widely accepted markers (Fig. 1).

Table 1: Assays used to characterize the iPSC lines

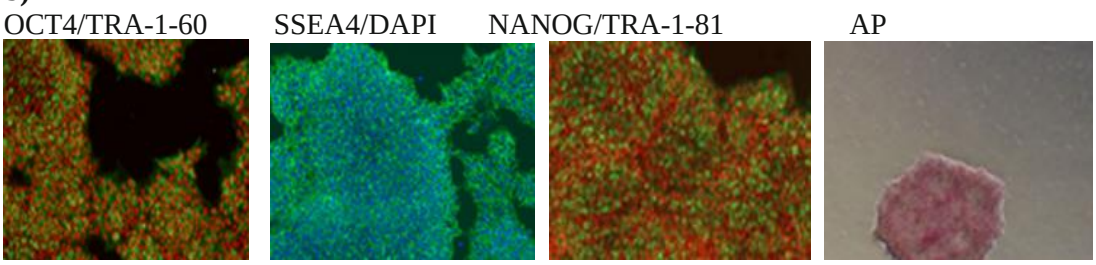
Assay	Objective	Evaluation Criteria	Category	Tested iPSC Line
Assay Release				
Pluripotency Markers	Identity & Purity	SSEA-4 > 70 %, Tra-1-60 > 70 %, Tra-1- 81 > 70 %, Oct3/4 > 70 %; Purity: CD34 < 5 %	Release Assay	All lines
Karyotype Analysis	Safety	46, XX or 46, XY	Release Assay	All lines
Mycoplasma Testing	Safety	Negative	Release Assay	All lines
Sterility Testing	Safety	Negative	Release Assay	All lines
Endotoxin Testing	Safety	Standard QC release (<0.5 EU/ml)	Release Assay	All lines
Vector Clearance	Safety	No trace of episomal plasmid DNA detected	Release Assay	All lines
STR Genotyping	Purity & Identity	STR Profile of starting population and iPSC line are identical	Release Assay	All lines
Cell Count & Viability	Viability	% viability >50; minimum cell number/vial	Release Assay	All lines
Viral Panel Testing	Safety	Standard MCB Release Panel	Release Assay	LiPSC-GR1.1
Characterization Assays				
EB Formation	Identity & Potency	Detection of at least one marker per germ layer	FIO*	All lines
Gene Array Analysis	Identity	Clustering with established hPSCs	FIO*	All lines
Colony morphology	Identity & Purity	Characteristic morphology of culture/colonies; lack of spontaneously differentiated cells	FIO*	All lines
Post-thaw Plating	Thawing efficiency and Viability	20+ colonies / vial (after 7 days or 50 % confluency)	FIO*	All lines
HLA Typing	Identity	HLA-A, B, C, DRB1 and DQB1 type	FIO*	All lines
CGH + SNP microarray	Identity	Amplifications and/ or deletions of specific genes	FIO*	LiPSC-GR1.1 and ER2.2
Whole Genome Sequencing	Identity	HiSeq X Human Whole Genome sequence	FIO*	LiPSC-GR1.1 and ER2.2

The tests conducted on the two cGMP lines and the three engineering run lines are summarised in the table (all). It should be noted that the two cGMP lines were generated from a different donor (Male), whilst the three engineering lines were generated at various times from the same donor sample (Female). Only for Information (FIO)

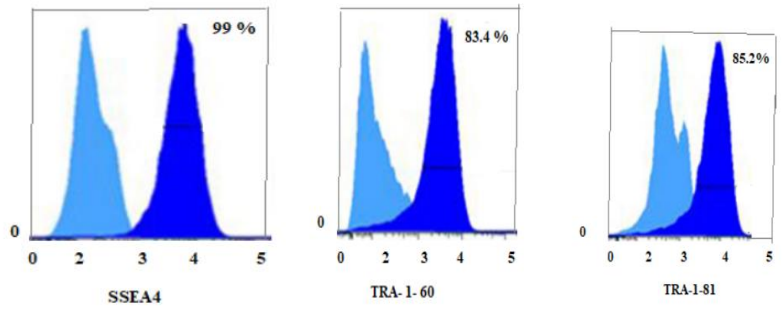
a)



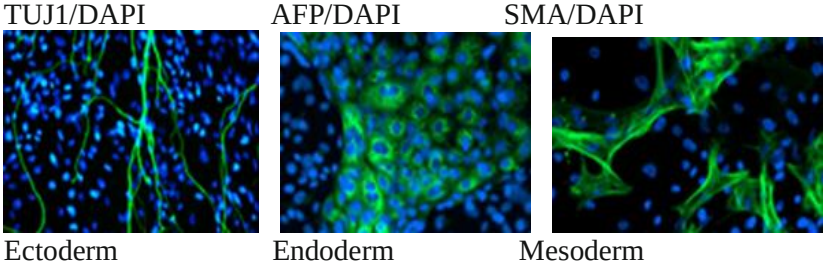
b)



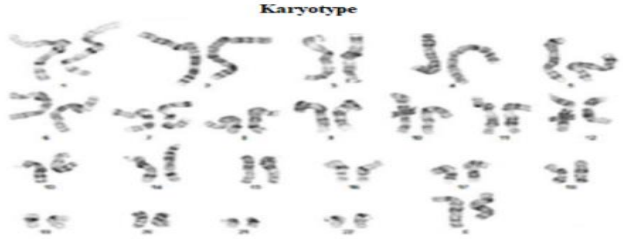
c)



d)



e)



f)

Cord blood- CD34 + cells			**STR Analysis**			**Matched hiPSC ER2.1**					
Amelogenin	X		D18S52	13		Amelogenin	X		D18S52	13	
Vwa	17		Penta E	11		Vwa	17		Penta E	10	
D8s1180	15	16	D5S819	12	14	D8S1180	15	16	D5S819	12	14
TPOX	9	10	D13S318	12		TPOX	9	10	D13S318	12	
FGA	23	24	D7S821	9	11	FGA	23	24	D7S821	9	11
D3S1359	17	19	D16S540	12	13	D3S1359	17	19	D16S540	12	13
THO1	9	10.4	CSF1PO	11	14	THO2	9	10.4	CSF1PO	11	14
D21S11	25.3	31.3	Penta D	10	15	D21S12	25.2	31.3	Penta D	10	15

Fig. 1: Engineering runs for the creation, proliferation, and characterisation of human iPSCs (LiPSC-ER2.1). Panel a show how CD34+ cells that were isolated from a cord blood unit were primed and grown in culture on day three before the nucleofection (Priming); on day nine after the nucleofection (D9 Post-Nucleofection); on day six (P6 Colonies); and on day eighteen (P18 Culture). Panel B shows iPSCs that have been positively labelled with alkaline phosphatase (AP), OCT4, TRA-1-60, SSEA4, NANOG, and TRA-1-81. The pluripotent stem cell surface markers SSEA4, TRA-1-60, and TRA-1-81 are expressed by the iPSCs in Panel c (dark blue). Light blue displays the control of isotype. In panel d, it is demonstrated that iPSCs underwent differentiation into embryoid bodies and that they displayed the markers for mesoderm (Smooth Muscle Actin; SMA), endoderm (Alpha-Feto Protein; AFP), and early ectoderm (TUJ1). The nuclei stain blue when seen with DAPI. After 17 passes, the iPSCs showed a normal karyotype (e). The iPSCs matched the initial CD34+ donor sample, according to STR analysis (f). Except for the Priming image, which has a scale bar of 250 microns, every image in Panel A has a 500-micron scale bar. The AP image has a 500-micron scale bar, but the other photographs in Panel B have a 250-micron scale bar. Panel D's photos all have a 125-micron scale bar.

Furthermore, we believed that it would be critical to be able to track the identity of the cells as they move through the manufacturing process and are dispersed globally, considering that iPSCs have the potential to be immortal. Although there are several approaches, we chose to use STR (single tandem based repeat) monitoring since it is quickly available with findings and is regularly performed by CLIA approved laboratories. After analysing our procedure, we concluded that STR typing ought to be carried out on the donor sample before the reprogramming phase begins, and it ought to be compared to the last iPSC sample obtained following the cryopreservation phase at the conclusion of the production process. Quantitative PCR was used for additional characterisation, including the examination of residual plasmid removal. Additionally, the final generated cell product (see procedures) and the beginning materials (cord blood derived CD34+ cells) were sterile as per standard procedure. As a normal procedure, testing for endotoxins and mycoplasma were also performed on the finished iPSC products.

The process flow diagram, in-process samples, and related testing are shown in Figure 2. It was crucial to test CD34+ cells for karyotype, gather samples for STR analysis and matching with the final iPSCs, and assess the purity of CD34+ cells at the end of the priming stage (in-process QC2). At the beginning of the process, it was also important to test the sterility and purity of these cells using flow cytometry analysis (in-process QC1). To make sure the beginning materials going through the reprogramming procedure were normal, the karyotype study was essential. An RT-PCR based plasmid clearance test and in-process QC3 were used to assess the quality of iPSCs chosen during reprogramming based on their morphology. Before final expansion and banking, in-process QC4 was performed at many passages to verify plasmid clearance. Prior to conducting a thorough characterisation of iPSCs by a variety of QC tests, these numerous in-process experiments were essential for assessing the quality of iPSCs.

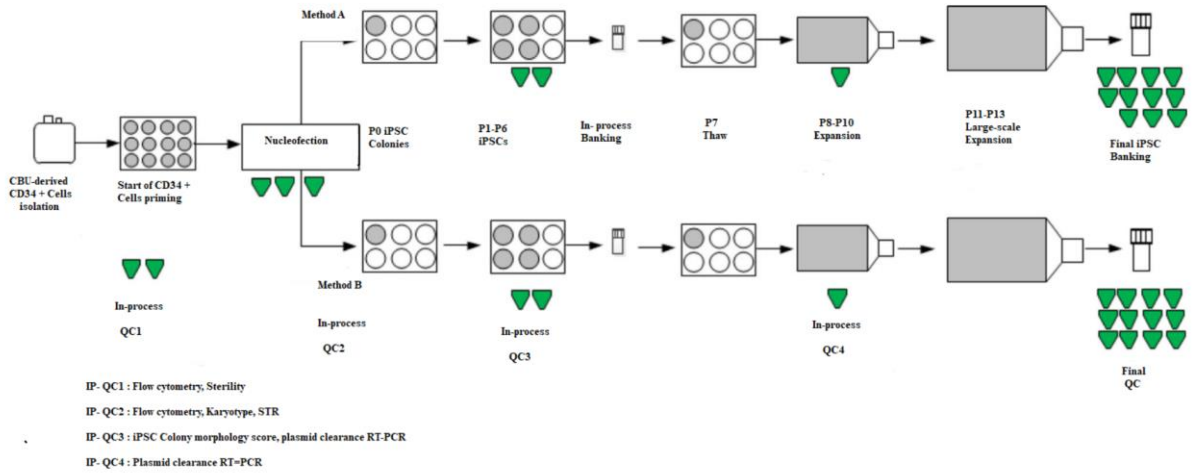


Fig. 2: Flow diagram for the human iPSC manufacturing process including sample testing during production. The steps involved in producing human induced pluripotent stem cells (iPSCs) under controlled and cGMP settings are as follows: (1) isolating CD34+ cells from a fresh cord blood unit; (2) priming CD34+ cells for four days; and (3) reprogramming CD34+ cells into iPSCs using an episomal-based technique and a 4D Nucleofector system. About nine iPSC colonies were isolated, iPSCs were serially cultured for up to six passages, and all iPSC colonies were cryopreserved in process so that the two best iPSC colonies could be chosen based on the outcomes of in process control IP-QC3. (6) two chosen iPSC colonies are expanded, and the plasmid clearance is confirmed; (7) additional expansion is carried out into large tissue culture flasks; (8) banking is done; and (9) final characterizations and QC are performed. Several in-process samples (highlighted in green) were obtained at various points during the procedure and submitted for pertinent testing. In the picture, each in-process test has been explained. The quantity of in-process samples demonstrates how many tests are run at each stage.

A comprehensive characterization of one of the iPSC lines (LiPSC-ER2.1) generated during the previously mentioned engineering runs is exemplified in Fig. 1, and Tables 1 and 2 offer additional specific information on the tests performed on the remaining lines. While this thorough characterisation might be considered satisfactory for these iPSC lines, we believed that more crucial data needed to be gathered to fully utilise iPSC lines in the future. This featured high-resolution HLA typing and blood group (ABO and Rh). We saw that this was not a standard procedure for collecting donor samples, and we were unable to visit the donor again to collect blood type information (Table 2) from one of the locations where tissue was acquired.

Table 2: iPSC lines generated by Lonza: STR, HLA, and donor information

Line	M/F	STR	HLA Type	ABO/RH +/–	Ethnicity
LiPSC-GR1.1 LiPSC-GR1.2	M	Amelogenin (X, Y); vWA (16); D8S1179 (13, 14); TPOX (8); FGA (18, 25): D3S1358 (15); THO1 (6, 9.3); D21S11 (31.2, 33.2); D18S51 (14); Penta E (7, 11); D5S818 (11); D13S317 (9, 11); D7S820 (10, 11); D16S539 (11, 13); CSF1PO (12), Penta D (11, 13)	A*02/*03, B*07/*39, C*07/*15 DRB1*04/*08,DQB1*03/*04	O+	Hispanic
LiPSC-ER2.1 LiPSC-ER2.2 LiPSC-ER2.3	F	Amelogenin (X); vWA (16); D8S1179 (14, 15); TPOX (8, 9); FGA (22, 23): D3S1358 (16, 18); THO1 (8, 9.3); D21S11 (24.2, 30.2); D18S51 (12); Penta E (10); D5S818 (11, 13); D13S317 (11); D7S820 (8, 10); D16S539 (11, 12); CSF1PO (10, 13), Penta D (9, 14)	A*03/*24, B*27/*27, C*01/*05 DRB1*01/*11,DQB1*03/*05	Inferred A	Caucasian

The information gathered for identity, HLA typing, and ethnic background is summarised in the table. The data does not allow for the distinction between clones that belong to the same individual, and the problem of tracing and tracking clones will need to be addressed in addition to the physical tracking techniques and barcoding that we advise.

In conclusion, we were able to monitor every line, identify its fundamental traits, and offer a fair degree of predictability regarding the line's quality and propensity to differentiate into the intended cell types using this limited set of assays. In addition to revealing the sample's purity, immunocytochemistry evaluated the extent of contaminating cell populations and offered a neutral means of comparing cells to predetermined comparability standards in the future. Unfortunately, it was uncertain if these tests would be adequate to exclude all undesirable cell types, even though they could be required to assess the cells' quality at a minimum. It will not be possible to detect minor karyotypic anomalies or abnormalities in a tiny fraction of the cells. Changes or mutations in genes that are not functionally significant at the pluripotent stage will also go unnoticed, as will integration of genes employed in the iPSC creation process. We wanted to see if adding more tests would make the quality more predictable, and more significantly, if adding more tests now would make them necessary later. In addition to karyotyping, we have suggested three more tests: a transcriptome analysis, a whole genome sequencing (WGS) assay, and a CGH array based on hybridization, which would supplement the basic analysis and provide information on critical parameters that need to be evaluated for new lines.

CGH + SNP Microarray Analysis

An experiment based on SNP hybridization was chosen to produce a map of the cell state at a higher resolution. To assess the usefulness of the methodology, we used the three engineering lines produced from the same human using the same cGMP compliant process, as well as both clones of the cGMP line. Table 3 shows the outcomes, which we obtained from a CLIA-certified provider (for GMP lines).

Table 3: aCGH-SNP analysis of the iPSCs Manufactured during cGMP runs

Chromosome	Start	Stop	Genes	Cytoband	LiPSC GR-1.1	LiPSC GR-1.2
List A: Common changes observed in GR-1.1 and GR-1.2						
chr2	45,168,836	45,171,902	SIX3	P21	Present	Present
chr15	34,695,166	34,841,446	GOLGA8B, GOLGAA8A,MIR1233-1, MIR1233-2	q14	Present	Present
chr16	31,959,074	33,773,134	HERC2P4, LOC390705, TP53TG3, TP53TG3B, LOC653550, SLC6A10P	P11.2	Present	Present
chr20	57,463,534	57,464,754	GNAS	q13.32	Present	Present
chrX	130,813,232	131,201,564	LOC286467, MST4	q26.2	Present	Present
chrY	17,130,014	17,630,471		q11.221	Present	Present
Total No. of common aCGH						
List B. Non overlapping changes observed GR-1.1 and GR-1.2						
chr2	81,631,218	84,380,876	LOC1720	p12-		Present
chr2	166,180,491	166,815,270	SCN2A, CSRNP3, GALNT3, TTC21B	p11.2	Present	
chr3	46,620,840	46,622,617	TDGF1	q24.3	Present	
chr5	124,645,064	128,776,611	GRAMD3, ALDH7A1, PHAX, C5orf48, LMNB1, MARCH3, FLJ44606, MEGF10, PRRC1, CTXN3,	p21.31 q23.2 -		Present
chr6	47,311,409	54,276,380	FLJ33630, SLC12A2, FBN2, SLC27A6, ISOC1 CD2AP, GPR111, GPR115, OPN5, C6orf138, MUT, CENPQ, GLYATL3, C6orf141, RHAG, CRISP2, CRISP3, PGK2, CRISP1, DEFB133, DEFB114, DEFB113, DEFB110, DEFB112, TFAP2D, TFAP2B, PKHD1, MIR206, MIR133B, IL17A, IL17F, MCM3, PAQR8, EFHC1, TRAM2, LOC730101, TMEM14A, GSTA7P, GSTA2, GSTA1, GSTA5, GSTA3, GSTA4, ICK, FBXO9, GCM1, ELOVL5, GCLC, KLHL31, LRRC1, C6orf142, TINAG	q23.3 p12.3 - p12.1		Present
chr7	155,596,206	155,601,974	SHH			Present
chr10	114,549,196	117,896,129	VTI1A, LOC143188, TCF7L2, HABP2, NRAP, CASP7, C10orf81, DCLRE1A, NHLRC2, ADRB1, C10orf118, MIR2110, TDRD1, VWA2, AFAP1L2, ABLIM1, FAM160B1, TRUB1, ATRNL1, GFRA1	q36.3 q25.2 - q25.3		Present
chr11	19,664,494	20,906,873	NAV2, LOC100126784, DBX1, HTATIP2, PRMT3, SLC6A5, NELL1	p15.1	Present	
chr12	19,001,106	19,925,941			Present	
chr19	50,816,568	51,294,521	PLEKHA5, AEBP2 KCNC3, NAPSB, NAPSA, NR1H2, POLD1, SPIB, MYBPC2, FAM71E1, C19orf63, JOSD2, ASPDH,	p12.3 q13.33		Present
chrX	306,955	329,692	LRRC4B, SNAR-F, SYT3, LOC342918, SHANK1, CLEC11A, GPR32, ACPT PPP2R3B	p22.33	Present 5	6

aCGH-SNP analysis of the cloned iPSCs GR1.1 and GR1.2. Common modifications found are shown in Panel A, and non-overlapping changes are listed in Panel B. The genes are colour coded: Red indicates a known disease-related alteration. Black: Not stated to be impacted within a database. Teal in a disease-related pathway Be aware that the analysis was completed on the same run at the same time. There were two distinct runs of clones produced.

Table 3 shows that GR1.1 has a few minor duplications and deletions despite having a normal karyotype and phenotype. Refer to Panel A and B of Table 3. A comparison between GR1.1 and GR1.2 reveals that several of the duplications and deletions were shared, indicating that they existed before the iPSC process was started. The two samples differed in a modest but noteworthy number that was probably produced during the cell line derivation or culture propagation phase.

Microarray Analysis

For iPSC lines produced during process development, training runs, engineering runs, and GMP manufacturing runs, we reported a whole genome expression study. In this instance, we examined the gene expression of ten iPSC lines produced using the same production method. Cell samples were obtained to perform whole genome expression analysis and RNA extraction on the Illumina Bead Array platform (Human HT-12 v4 Expression BeadChip). As demonstrated earlier, this platform may be used to detect differential gene expression in a wide range of samples with robustness and reliability. We used a CD34+ sample, from which one iPSC line was produced and the NSC separated from the iPSC, along with extra iPSC and ESC lines as a control. Supplemental Table 1 contains the list of samples that were analysed, and Supplemental Table 2, which is accessible upon request, contains the complete gene expression profile.
As previously mentioned, Genome Studio software was used for the initial data processing. Gene expression accession number GSE72078 provides access to normalised and non-normalized whole genome expression raw data. Table 4 provides a summary of the quality control tests and the different analyses we conducted.

Table 4: Microarray Analysis for pluripotent cells

Data Set analysis
QC Tests Total reads Intensity range Distribution No detected > 100 RFU Normalization
Other Results Overall profile (relatedness) Chromosomal Bias M/F X chr inactivation Pluritest Pluripotent marker set Contaminating cell detection Incomplete programming
Gene Sets MicroRNA Mitochondrial profiling Pathway analysis Positional markers Imprinted genes

The regular QC tests and their outcomes are compiled in the table with GR1.1. Note that tests like the Pluritest, correlation coefficient, and unbiased hierarchical clustering can be used to infer both gene subsets and an assessment of overall quality. Comparing the data to a database improves its quality, and if there are enough lines, ranges for a comparability assay can be established.

We started by checking the quality of our data set. There were no significant differences in the hybridization signal intensity distributions (not shown), and the average number of detected genes for all samples was very similar: 12,799.5 ± 702.7 (detection p-value < 0.01; mean ± standard deviation; non-normalized data) and 15,662 ± 711.2 (detection p-value < 0.05; mean ± standard deviation; non-normalized data). By submitting the samples for the Pluritest assay, the quality of the iPSC was confirmed; the outcomes are displayed in Fig. 3. PR1.0, TR1.1, TR1.2, GR1.1, GR1.2, ER2.1, ER2.2, ER2.3, and XCL1iPSC samples, as well as our added positive controls H14 ESC, fall within the same pluripotency range (red lines) of ESC samples and iPSC samples used to construct Pluritest. Our iPSC lines correspond with known iPSC and ESC, as shown by the CD34++ cord blood line and ER2.2 NSC, which fall into the non-pluripotent range (blue lines) (Fig. 3a).

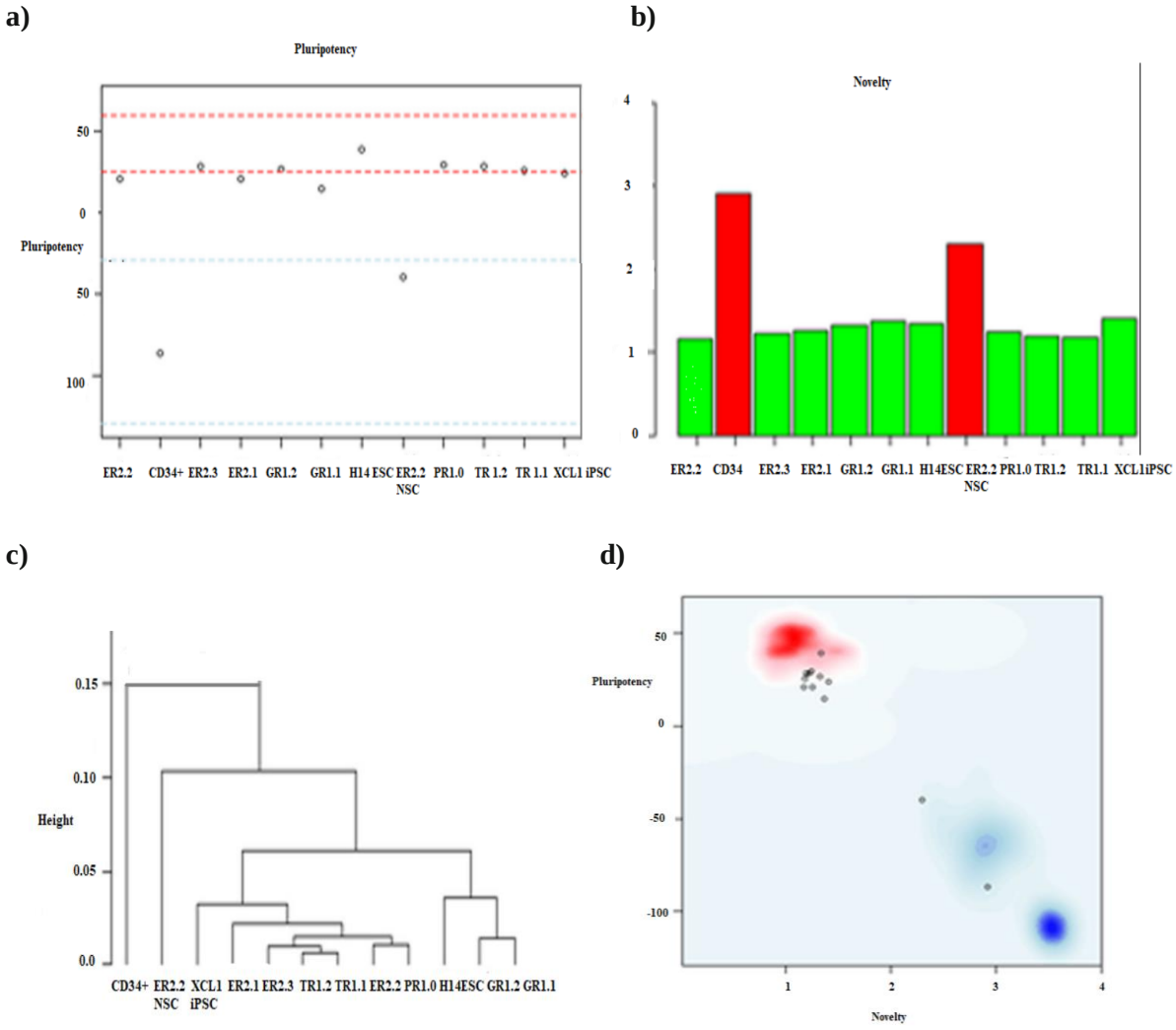


Fig. 3: Analysis of test and control samples using Pluritest. A 95% pluripotency signature is indicated by a pluripotency score between red lines in a model-based multi-class pluripotency score. Samples within the blue line represent 95% of samples that are not pluripotent. Because they are concentrated between or near the red lines, line PR1.0, TR1.1, TR1.2, GR1.1, GR1.2, ER2.1, ER2.2, ER2.3, XCL1iPSC, and H14 ESC are all pluripotent. Between the blue lines, the CD34+ cord blood line and the ER2.2 NSC (negative controls) show a non-pluripotent signature. b. Novelty Score: Well-characterized pluripotent samples (coloured green) and non-pluripotent samples (coloured red) provide the basis for this score. While CD34+ cord blood line and ER2.2 NSC demonstrate non-pluripotent color-coded red, all iPSC and ES samples are in green, which indicates pluripotent samples. C. Samples were changed using variance stabilising transformation (VST) and robust spline normalisation (_ENREF_35) to enable hierarchical clustering of vst-transformed data. Pearson correlations are used to determine distance on the x-axis. d. Synopsis: This combines the X-axis novelty score and the Y-axis pluripotency score. The iPSC and ESC samples, which are situated against a red background, indicate the empirical distribution of pluripotent cells. The ER2.2 NSC and CD34+ cord blood line is situated nearer the blue non-pluripotent background.

Using non-normalized data, we showed the signal-to-noise ratios and high-end intensity variation (95th percentile of signal intensity, P95) (Fig. 3b) to visualise the overall strength of observed signal across samples and highlight the presence of probable outliers. Every analysed sample had a similar signal strength, and no outliers were found, indicating that the microarray samples' quality was consistent. All our iPSC and ESC samples come within the novelty score (green) based on technical and biological variation, however the ER2.2 NSC and CD34++ cord blood cells fall outside the novelty score (red) (Fig. 3b). The overall relatedness of the samples was next ascertained by computing the pairwise correlation coefficients (r 2) (data now provided). The cGMP compliant process produced iPSC samples with correlation coefficients greater than 0.9. Additionally, no discernible global differences in the gene expression profiles of these iPSC lines were found when compared to ESC or iPSC lines that had been previously generated using either the same or a different reprogramming methodology. After that, we divided averaged samples into groups based on the similarity in gene expression using an unsupervised one-way hierarchical clustering approach (Fig. 3c). Three distribution aspects were seen in the results: developed neuronal cells, pluripotent stem cells (iPSC and ESC), and starting material (CD34+ cells used to make iPSC). When taken as a whole, these results showed that the microarray data had good overall quality. An empirical distribution of pluripotent cells is suggested by the combination of novelty score and pluripotency score (Fig. 3d), which shows that ESC and iPSC samples are grouped together (red background). Near the non-pluripotent (blue background), CD34++ cord blood cells and ER2.2 NSC are found (Fig. 3d). These outcomes further demonstrate that Pluritest is a useful additional tool for confirming the pluripotency of freshly generated iPSCs.

We reasoned that in addition to using Pluritest, we might also find subsets of genes that have been identified as exclusive to PSC cells and compare their expression levels with those of other well-characterized PSC, including XL1. Furthermore, to evaluate full transformation and the absence of contaminating cells, markers of CD34+ cells, trophoblast, and early differentiating cells can be analysed (see supplementary materials). We observe that the Illumina chips contain microRNA, and our isolation method maintains small RNA species, so we can evaluate the cell microRNA expression patterns using the same array technology. The two lines and the other lines in this investigation were compared, and the results indicated that the lines had similar expression profiles and lacked differentiated cell markers (data not shown). Specifically, there was no expression of positional markers like HOX genes.

The expression of imprinted genes is another element that could change PSC behaviour and is not likely to be measured by standard testing procedures. Thus, we took the list of imprinted genes that have been published and analysed how these genes expressed throughout the whole sample set (Supplemental Table 3, available upon request). While we observed a substantial differential in the expression of NNAT in XCL1 iPSC and GR1.2, overall, the expression patterns were similar. Although the significance of this finding is uncertain, considering the links between these genes and illness, we think monitoring their levels may be crucial.

An analysis of gene expression using a panel of about 325 markers (Supplemental Table 3), which included markers for pluripotency, gender, imprint, endoderm, mesoderm, and ectoderm, showed that there was (1) no difference in the expression of imprinted genes between the male and female lines, and (2) several pluripotency markers, such as Oct4, Nanog, and Sox2, were highly expressed in all iPSC samples (1). Gene expression profiling of the lines produced by the cGMP compliance procedure was generally consistent with that of iPSC and ESC lines that have been previously reported.

We also reasoned that Y chromosome gene expression could be used to determine an individual's sex, and that XIST expression and X chromosome gene expression levels could be compared to cell lines that have shown X chromosome activation or inactivation to determine whether X chromosome inactivation had occurred in female samples. This test can be used to indicate areas that need more testing, but it is by no means conclusive. By using Whole Genome Sequencing (WGS) to compare the sequences of the two imprinted alleles of the genes and detecting whether the expression originated from the paternal or maternal allele, further details on imprinting can be discovered. While this analysis was not conducted by us for this work, it can be readily performed if considered necessary.

Making a list of the deletions and duplications in GR1.1 and GR1.2 and looking at the expression of the genes that are known to be expressed at the PSC stage would be a simple way to conduct an additional analysis that would determine whether the deletions and duplications reported in the WGS and SNP/CGH array tests affected gene expression. There was no discernible change in expression (Sup Table 3). As

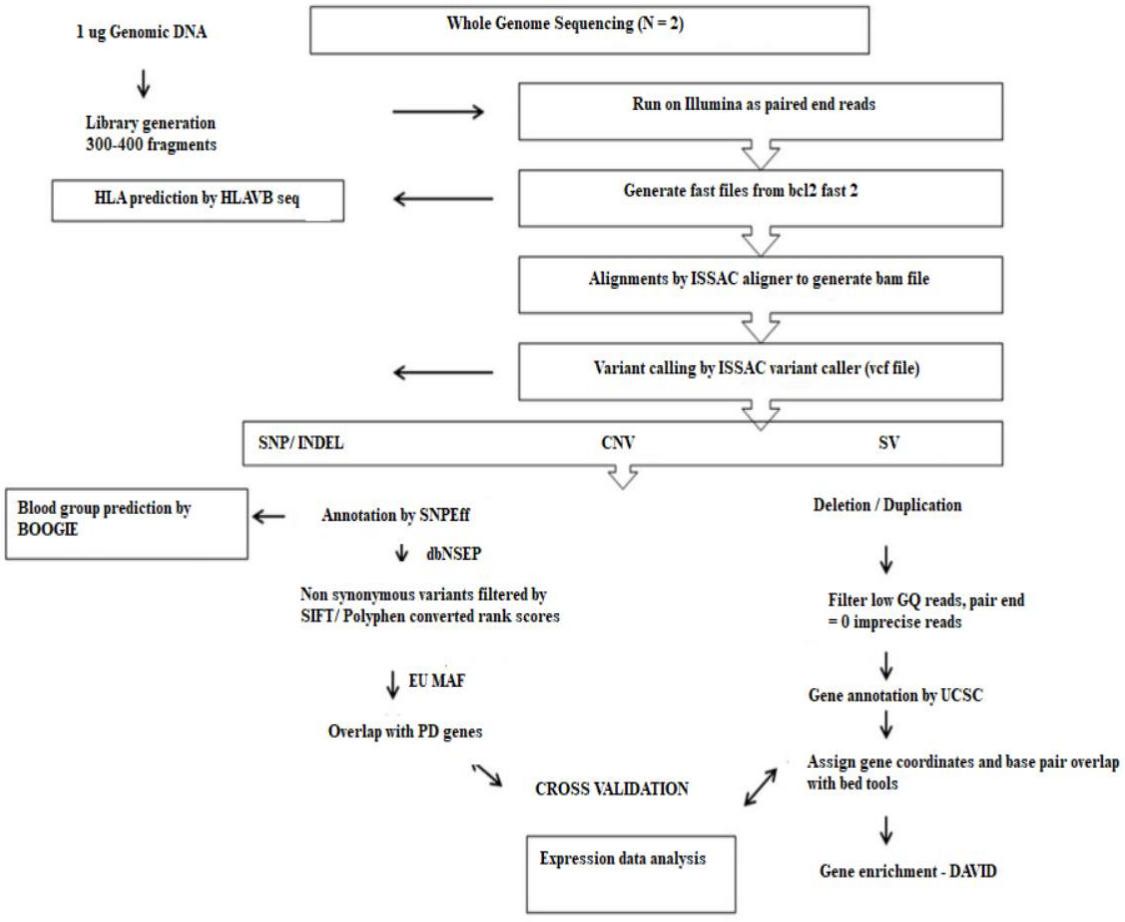
demonstrated before, one can look at the overall expression of a specific signalling pathway or a group of genes linked to a given disease and see if there are any clear differences in expression instead of comparing the expression of individual genes. As data on lines is gathered, we hypothesised that, in the case of iPSC, the expression of genes linked to cell death and mitochondria may be sensitive indicators of growth rate and proliferation. Furthermore, a wide range of inherited illnesses are caused by abnormalities in the mitochondria. As a result, we looked at the subset of mitochondrial genes to see if there were any notable variations from other iPCS and ESC lines. The results of these tests did not demonstrate a significant alteration in the expression of genes related to mitochondria.

Overall, our findings imply that microarray analysis is a reasonably priced technique that enables quick assessment of pluripotency and the existence of contaminated cells through a range of techniques. Importantly, a reference database is provided to examine the continuous evolution of the cells as they are kept in culture. It is possible to compare the expression of gene subsets that may be essential for the usage of these cells for a particular purpose. Establishing a database of this kind could help us create acceptance and release assay cut-offs for cell processing, as well as criteria for comparability.

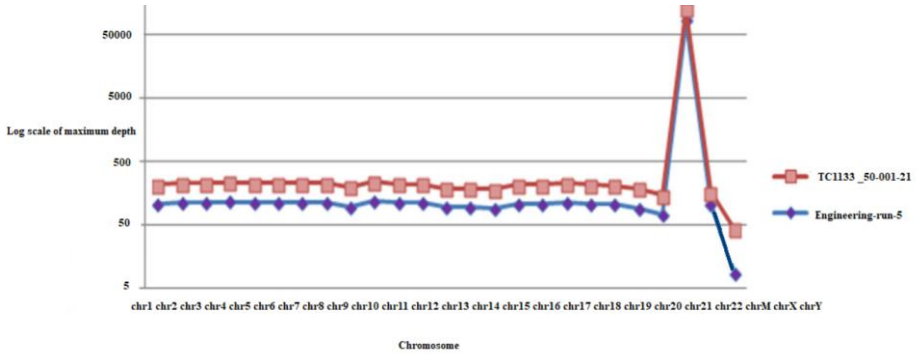
Whole Genome Analysis

To determine the general state of two iPSC lines—one male and one female—we produced genomic DNA and used it for the WGS. The pipeline that is summed up in Fig. 4a was used to analyse the data. Table 5 provides a summary of the tests we ran utilising these data sets. The evaluated QC parameters are summarised in Table 6. The two lines satisfied the required QC standards.

a)



b)



c)

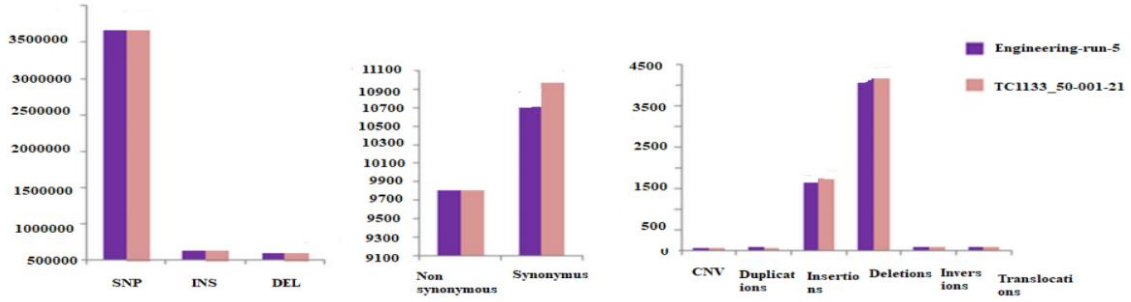


Fig. 4: Whole genome study carried out on two iPSC lines produced under manufacturing procedures complying with cGMP. a. The pipeline for characterising WGS data. The work flow used in this investigation is depicted in this image. The techniques section contains a list of the filters that were used at different stages. To create a bam file, the fastq files were aligned using the Isaac aligner. Samstat was used to assess the mapping quality of the resulting bam file. HLAVBseq was utilised to predict HLA types based on the fastq files. Using SnpEff, the variations called by the Isaac variant caller were annotated, and BOOGIE was used to predict blood types based on this data. Only non-synonymous variations were examined for their potential relationship to Parkinson's disease (PD) and cross-validated using expression data. Filtered structural variation included duplications and deletions. Microarray and expression data were used for cross-validation of this data. Through DAVID, the gene enrichment relevant to illness and pathway was confirmed for the differentially expressed genes. b. For the two cell lines under investigation, the x-axis displays the different chromosomes, and the Y-axis shows the max-depth calculated by Isaac Variant Caller across each chromosome on a log scale. c. A bar graph showing the total number of variants found for synonymous, non-synonymous, CNVs, small insertions, small deletions, duplications, large deletions (length > 50), large insertions (length > 50), inversions, and translocations. The quantity of deletions was greater than that of other SV kinds.

Table 5: Whole Genome Analysis for pluripotent cells

Data Set analysis
QC Tests
Total reads
Unmapped reads
Mapped reads
Chr. coverage
Error Rate
Other Results
Mutations detected
Mitochondrial Seq
Comparison with CGH
STR verification
Distinguishing alleles
HLA
Minor Blood groups
Other transplant antigens
Other gene subsets
Plasmid/Viral Insertion sites
Utilizing unmapped data
Mycoplasma
Other infectious agents
Foreign DNA

The regular QC tests and their outcomes are compiled in the table with GR1.1. Note that tests like the Pluritest, correlation coefficient, and unbiased hierarchical clustering can be used to infer both gene subsets and an assessment of overall quality. Comparing the data to a database improves its quality, and if there are enough lines, ranges for a comparability test can be established.

Table 6: Basic QC analysis of WGS data. Basic QC and mapping quality summary statistics for both the cell lines computed using samtools and Samstat

QC analysis Summary	LiPSC-ER2.2	LiPSC-GR1.1
Total reads	840146878	898394686
Total reads mapped	33656253	768473456
Mean depth	34.49	30.83
deduplicated reads	90375272	157504614
Total reads after removing duplicates (%)	749771606 (89.2 %)	740890072 (82.5 %)
Reads mapped to human genome	693727158	613291081
MAPQ > =30	86.9 %	79.6 %
MAPQ <30	0.1 %	0.0 %
MAPQ <20	0.5 %	0.5 %
MAPQ <10	0.7 %	0.7 %
MAPQ <3	0.3 %	0.3 %

The average number of variants found in the two cell lines was comparable and had consistent patterns in terms of synonymous and non-synonymous variants as well as SNP, INS, and DEL variant counts. Since there were more deletions than insertions or translocations, they were examined in more detail (Fig. 4c). Figure 4b displays the maximum depth of coverage for both cell lines across each chromosome. As might be predicted, both cell lines showed higher depths of mtDNA coverage. LiPSC-ER2.2's Y chromosome dipped in the female line as predicted. The X and Y chromosomes from the LiPSC-GR1.1 (male line) overlapped in a total of 36 different ways. This emphasises the problems associated with sequence data assignment.

Our goal was to investigate Parkinson's disease (PD) and see whether a targeted data analysis could be carried out. To demonstrate how a report might be created for particular gene sets associated with a disease, we created a list of PD-related genes and evaluated their status. Following screening for the deleterious consequences of the amino acid change, predicted in silico using SIFT and Polyphen converted rank scores calculated by dbNSFP, both iPSC lines revealed 23 distinct genes associated with Parkinson's disease. The expression data was used to validate these filtered variations. When compared to the MIM disease database, the LiPSC-ER2.2 genes SYNJ1 and E1F4G1 have been linked to Parkinson's disease. However, line LiPSC-GR1.1 displayed one variant (rs2254562) with the SYNJ1 gene and two variants (rs63750417) with the MAPT gene, both of which indicated a relationship with Parkinson's disease (Table 7).

Table 7: Identification of PD implicated genes from WGS. The table shows the genes that are identified by WGS, having damaging SIFT and polyphen scores that are implicated in PD. These genes were cross verified for the changes in their RNA expression

SAMPLE	SYMBOL	CHR	POS	SNP ID	SIFT	POLYPHEN 2	1000Gp3_EUR_AF	ER1.2 (F)	GR1.1 (M)	H9(F)	H7(F)	NCRM6(F)	MIM_disease
LiPSC-ER2.2	SYNJ1 EIF4G1	21 3	34059352 184039151	rs2254562 rs367754765	0.43 0.912	0.629 0.647	0.29	56 1483		69 1740	65 1426	61 1404	[MIM:615530]Parkinson disease [MIM:614251]Parkinson disease
LiPSC-GR1.1	MAPT SYNJ1 MAPT	17 21 17	44060775 34059352 44061278	rs63750417 rs2254562 rs17651549	0.91 0.43 0.91	0.72 0.63 0.72	0.24 0.29 0.24		4 84 4	37 69 37	26 65 26	10 61 10	[MIM:600274]Parkinson Disease [MIM:615530]Parkinson disease [MIM:260540]Parkinson-dementia syndrome

PPP2R2B gene overlap was detected by LiPSC-ER2.2 on each of these systems. The unprocessed WGS data displayed overlap between CSRNP3 and PPP2R3B according to LiPSC-GR1.1. However, the genes were eliminated since the filtered set of the WGS had poor quality. Microarray data for the CSRNP3 gene revealed a disparity that was interpreted as GAIN; however, WGS data reveals that it was a deletion event. Table 8

provides a summary of this data as well as the log fold change with respect to the control lines H9_H7 and NCRM6. We used DAVID to test for gene enrichment to confirm whether the genes overlapping with structural variants found in the WGS-filtered data set had any implications with established diseases or pathways. LiPSC-ER2.2 did not exhibit any disease-associated signals or deletion event pathways. HTR6, GRIK3, and OPRD1 were discovered to be linked to chemdependency in the duplication event and to be enriched for the Eicosanoid Metabolism pathway, although only with two genes (CYP2J2, PTGER3).

Table 8: Cross-validation of the structural variants identified across various platforms: WGS, Microarray, and SNP analyses

	Microarry platform	**EVENT**	**overlap with WGS-unfiltered**	**overlap with expression data**	**H9_H7**	**NCRM6**
LiPSC-ER2.2	IRF4	DEL	No	Yes	0.27	0
	DUSP22	DEL	No	Yes	0.64	0
	LOC283914	AMP	No	Yes	-	−1
	LOC146481	AMP	No	Yes	−4	0
	LOC10013070	AMP	No	Yes	2.85	-
	PPP2R3B	AMP	No	Yes	1.3	0
LiPSC-GR1.1	SIX3	GAIN	yes (but as del)	Yes	1	4
	SCN2A	GAIN		Yes	2	-
	GALNT3	GAIN		Yes	0	3
	TTC21B	GAIN		Yes	−13	1
	CSRNP3	GAIN		Yes	0	3
	TDGF1	GAIN		Yes	0	−1
	NAV2	GAIN		Yes	0	1
	HTATIP2	GAIN		Yes	0	0
	SLC6A5	GAIN		Yes	-	-
	NELL1	GAIN		Yes	1	0
	LOC100126784	GAIN		Yes	1	0
	DBX1	GAIN		Yes	−1	-
	PRMT3	GAIN		Yes	0	0
	PLEKHA5	GAIN		Yes	0	0
	AEB2	GAIN				
	GOLGA8B	LOSS		Yes	−2	0
	GOLGA8A	LOSS		Yes	-	-
	MIR1233-1	LOSS				
	MIR1233-2	LOSS		Yes	1	2
	HERC2P4	LOSS		Yes	0	1
	LOC390705	LOSS	yes	Yes	0	2
	TP53TG3	LOSS				
	TP53TG3B	LOSS				
	LOC653550	LOSS		Yes	-	−1
	SLC6A10P	LOSS		Yes	−1	−1
	GNAS	GAIN		Yes	0	0
	PPP2R3B	GAIN		Yes	0	1
	LOC286767	GAIN				
	MST4	GAIN		Yes	−1	0

Table 9 displays that the LiPSC-GR1.1 line did not exhibit any disease-specific enrichment, but it did show enrichment for the deletion event pathways for T Cytotoxic Cell Surface Molecules and T Helper Cell Surface Molecules, and no enrichment was seen for the duplication event. The list of published imprinted genes was retrieved from the database (http://www.geneimprint.com) to verify the imprinted genes' status. For the analysis, genes with the expected and imprinted statuses were considered. A sufficient depth of coverage was found for 148 out of 203 genes in the LiPSC-ER2.2 cell line. Similarly, 147 out of 203 genes were found for the LiPSC-GR1.1 cell line.

Table 9: Structural variants identified by WGS and its enrichment in any disease related pathways (if any)

	Event	No. after filter	Max size	Min size	no of genes log2 ratio >1.5 with H7_H9	no of genes log2 ratio >1.5 with NCRM6	overlap if any*	DAVID-OMIM disease GE	DAVID-pathway GE
LiPSC-ER2.2	DEL DUP	65358 387	16797153 22	89 1283753	11 28	18 53	No 6	No Chemdependency (Genetic Association_DB_Disease_Class): HTR6, GRIK3, OPRD1	No Eicosanoid Metabolism (BIOCARTA)-CYP2J2,PTGER3
LiPSC GR1.1	DEL DUP	555 58	16797153 17376	122 462	24 0	20 0	4 no	No NA	T Cytotoxic Cell Surface Molecules, T Helper Cell Surface Molecules (BIOCARTA)-CD3D, THY1 NA

Conclusion

In conclusion, we have demonstrated that useful additional information can be obtained for a relatively small investment. If this information is widely disseminated and collected in a database alongside data from calibration material, it will enable us to evaluate the safety of therapy and give regulators a plan for evaluating Haplobanks and other models for administering cell-based therapy.

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