CHAPTER 2

CHARACTERIZATION OF HUMAN INDUCED PLURIPOTENT STEM CELL LINES
ABSTRACT

This chapter illustrates the detailed characterization of the newly generated hiPSC lines performed to confirm their pluripotency. Several tests were conducted to detect the hallmarks of pluripotency in these hiPSC lines. These tests included the newly reported detection of lipid body or droplet associated BLUE FLUORESCENCE which correlates with expression of pluripotency markers OCT4, SOX2 and NANOG; STANDARD PLURIPOTENCY TESTS like expression of alkaline phosphatase, expression of key pluripotency regulating proteins & genes and up regulated telomerase activity; DIFFERENTIATION TESTS both in vitro and in vivo to prove the functional pluripotency of these hiPSCs by differentiating them into cells of the three embryonic germ lineages; STR PROFILING and KARYOTYPE ANALYSIS of the hiPSC lines to show that they maintain their original genotype and that prolonged culture periods did not cause genetic instability resulting in aneuploidy; Demonstration that the hiPSC lines became TRANSGENE FREE after few passages.
REVIEW OF LITERATURE

With the generation of new hiPSCs it becomes imperative to establish their resemblance to ESCs the ‘gold standards’ of pluripotency. Several parameters have been routinely used to illustrate the pluripotency of new hiPSCs including demonstration of alkaline phosphatase activity, cell surface antigens and transcription factors present in pluripotent stem cells, gene expression profiles of pluripotency related transcripts, increased telomerase activity and differentiation assays.

The use of a characteristic blue fluorescence to identify and isolate human ESCs and iPSCs has been lately described by Muthusamy et al. The blue fluorescence emission (450–500 nm) is readily observed by fluorescence microscopy. It allows easy, manipulation free identification of undifferentiated human pluripotent stem cells. The blue fluorescence appears early during somatic reprogramming. This blue fluorescence arises from the sequestration of retinyl esters in cytoplasmic lipid bodies. The retinoid-sequestering lipid bodies are specific to human pluripotent stem cells and mouse PCSs which are of the primed or epiblast-like state and absent in naive mouse embryonic stem cells. Retinol, commonly present in stem cell culture media, is sequestered as retinyl ester in lipid bodies specifically by pluripotent cells (Muthusamy et al., 2014).

Alkaline phosphatase expression has been traditionally linked with pluripotency (Martí et al., 2013; Republic et al., 2015; Thomson et al., 1998). Pluripotent stem cells characteristically express high levels of the placental isoform of alkaline phosphatase on their cell membranes (Martí et al., 2013). Detection of alkaline phosphatase activity in newly generated hiPSC lines constitutes a basic but reliable method of preliminary pluripotency detection. The colorimetric detection methods are popular, simple and recognize alkaline phosphatase with certainty.

The panel of cell surface antigens routinely used to classify human pluripotent stem cells includes the glycolipid antigens SSEA3, SSEA4 and the keratan sulfate antigens TRA1-60 and TRA1-81 (International and Cell, 2007). These surface antigens were originally identified in terato carcinoma cell lines (Solter and Knowles, 1978). The gene products of OCT4, NANOG and SOX2 are essential to the molecular circuitry of
pluripotency (Jaenisch and Young, 2008; Kim et al., 2008) and are considered as definitive markers for identification of iPSCs. REX1 has a strong transcriptional link with the pluripotency circuitry (Kim et al., 2008) and is hence considered a strong indicator of pluripotency. E-cadherin plays an important role in regulating pluripotency and self-renewal signaling pathways in ESCs and iPSCs (Soncin and Ward, 2011) and is a widely accepted pluripotent stem cell marker. The *de novo* DNA methyltransferases DNMT3a and DNMT3b are activated during the reprogramming process (Jaenisch and Young, 2008). Also DNMT3b has been shown to have high correlation with NANOG expression (International and Cell, 2007) and thus an indicator of pluripotency. Detection of these transcripts and proteins is done by techniques such as immunocytochemistry, flow cytometry, reverse transcriptase polymerase chain reaction and microarrays.

After detection of pluripotency markers, the next level of characterization extends to testing the differentiation potential of the new hiPSCs. This is usually demonstrated through *in vitro* and/or *in vivo* differentiation tests. *In vitro* differentiation to embryoid bodies (Kurosawa, 2007) and specialized cells of all three embryonic germ layers (ectoderm, mesoderm and endoderm) is the simplest test for the functional assessment of pluripotency. Usually the cells are treated with combinations of differentiation promoting factors inducing cytokines, morphogens or chemicals provided as supplements in culture medium after which markers of specific target tissues are assessed (Angeles et al., 2015). The more rigorous teratoma formation assay assesses the spontaneous generation of differentiated tissues from all three germ layers following the injection of hiPSCs into immune compromised mice (Angeles et al., 2015).

The identity of the newly generated stem cell lines is established by matching the DNA profiles of the hiPSC lines with that of the original somatic cells. DNA fingerprinting is a common method used to establish cell identity. DNA fingerprinting based on PCR is popular and uses short tandem repeats (STRs). This method uses highly polymorphic regions that have short repeated sequences of DNA. These repeated sequences are unique to each cell line. Thus, analysis of STR will allow cell identification (Martí et al., 2013).
Extensive evaluations of transcriptional profiles, epigenetic landscapes and pluripotential abilities have confirmed substantial similarity between iPSCs and ESCs, but debates about the extent and significance of these subtle differences between the two pluripotent stem cell types remain (Bilic and Belmonte, 2012). Despite these variations iPSCs have been shown to be capable of germline transmission (Okita et al., 2007) and are able to produce iPSC mice in tetraploid complementation test, which are the most stringent assays for determining developmental potency (Kang et al., 2009; Zhao et al., 2009). These reports eliminate uncertainties about the similarity of mouse iPSCs and ESCs in terms of their developmental potential and pluripotency.

RESULTS

Lipid body-associated blue fluorescence expression by hiPSCs

In 2014 Muthusamy et al described the use of a characteristic blue fluorescence to identify and isolate hPSCs. The blue fluorescence emission is readily captured by fluorescence microscopy and allows easy identification of undifferentiated human pluripotent stem cells. The fluorescence is also visible early during somatic reprogramming.

All the three hiPSC lines—ViPSCs, PiPSCs and NiPSCs and generated in this study exhibited the characteristic lipid droplet derived retinyl ester blue fluorescence associated with human pluripotent stem cells (Figure 3B). The blue fluorescence was absent in the somatic cells. This blue fluorescence was visible early during reprogramming. hiPSCs growing in culture media with 20% KnockOut™ serum replacement begin to accumulate these small blue fluorescent lipid bodies. These lipid bodies are efficient reporters of successful reprogramming and permit early identification of reprogramming pluripotent stem cells.

Alkaline phosphatase expression by hiPSCs

Pluripotent stem cells express high amounts of alkaline phosphatase in their cell membranes. Detecting this alkaline phosphatase activity by staining with fast red systems is a routine approach to ascertain pluripotent stem cells. Fast Red systems produce an insoluble intense red end product in presence of alkaline phosphatase. Alkaline phosphatase detection was used as the preliminary identifier of pluripotency in the three hiPSCs. The colonies of ViPSCs, PiPSCs and NiPSCs stained positive for this classic marker of pluripotency as judged by the formation of red end product on the surface of the colonies (Figure 3C) indicating that these hiPSCs have high
alkaline phosphatase activity on their cell membrane. After this preliminary determination the characterization proceeded to identification of other molecular and functional hallmarks of pluripotency.

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**FIGURE 3**
MORPHOLOGY AND PRELIMINARY CHARACTERIZATION OF hiPSCs BY DETECTION OF LIPID DROPLET ASSOCIATED BLUE FLUORESCENCE AND ALKALINE PHOSPHATASE ACTIVITY

A. Phase contrast micrographs of ViPSC, PiPSC and NiPSC colonies generated from CVFs, PMSCs and NFFs.
B. Characteristic lipid droplet associated blue fluorescence exhibited by the three hiPSC lines.
C. Alkaline phosphatase expression by the three hiPSC lines.
D. Embryoid body formation by hiPSC lines.
Scale bars represent 100 microns.

**Pluripotency marker expression by hiPSCs**
Key developmental events of pluripotency maintenance and initiation of differentiation by pluripotent stem cells depends on a strictly regulated balance between the transcription factors OCT4, NANOG and SOX2. The nuclear presence of all these markers definitively qualifies a stem cell line as pluripotent. Similarly, the cell surface expression of SSEA4, TRA1-60 and TRA1-81 are hallmarks of pluripotent stem cells.

Immunocytochemistry and reverse transcriptase PCR were used to detect the key molecular markers of pluripotency. Markers detected by immunocytochemistry comprised the nuclear transcription factors OCT4, NANOG, SOX2 and cell surface markers TRA1-60, TRA1-81 and SSEA4. E CADHERIN and β CATENIN expression patterns were considered as auxiliary molecular characterization.

The three hiPSC lines generated in this study expressed all the above-mentioned markers validating themselves as genuine pluripotent stem cell lines. All the hiPSC lines showed nuclear localization of OCT4, SOX2, NANOG, cell surface expression of SSEA-4, TRA1-60, TRA1-81, and peripheral localization of E CADHERIN and β CATENIN. Fluorescence photomicrographs of the three hiPSC lines expressing the key pluripotency markers are shown in figures 4, 5 and 6. Overall, the results made it clear that these cells had transformed from a somatic molecular phenotype to a pluripotent molecular phenotype confirming efficacious reprogramming.

Semi-quantitative reverse transcriptase PCR was employed to detect expression of pluripotency-related genes. The embryonic stem cell line HUES7 was used as the positive control pluripotent stem cell line. The original non-reprogrammed somatic cells were included in the test to relate the upregulation of pluripotency markers in the reprogrammed hiPSCs. Mouse embryonic fibroblasts (MEFs) were included as a negative control to detect the species specificity of the primers used in the study. OCT4, NANOG, SOX2, REX1 and E CADHERIN expressions were upregulated in hiPSCs and were comparable to the levels expressed by HUES7 cells. The DNA methyltransferases DNMT1, DNMT3a and DNMT3b were also upregulated. DNMT1, DNMT3a and DNMT3b are highly expressed in pluripotent stem cells and essential for the developmental potential of iPSCs (Liao et al., 2015; Pawlak et al., 2011). In contrast, the original somatic cells showed very low or no expression of these pluripotency-related genes. MEFs were negative for all genes. The overall
transcriptional profiles of hiPSCs generated in this study closely resembled those of hESCs, the “gold standards” of pluripotency. The outcomes of the semi quantitative RT PCR are shown as gel pictures in figure 7.
FIGURE 5
CHARACTERISATION OF PiPSCs BY IMMUNOCYTOCHEMISTRY
Representative Fluorescent Microscopic images of PiPSC colonies stained for pluripotency markers OCT4, SOX2, NANOG, SSEA4, TRA 1-60, TRA 1-81, E CADHERIN and β CATENIN. Nuclei are stained with DAPI. Scale bar represents 100 microns.
FIGURE 6
CHARACTERISATION OF NiPSCs BY IMMUNOCYTOCHEMISTRY
Representative Fluorescent Microscopic images of NiPSC colonies stained for pluripotency markers OCT4, SOX2, NANO, SSEA4, TRA 1-60, TRA 1-81, E CADHERIN and β CATENIN. Nuclei are stained with DAPI. Scale bar represents 100 microns.
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**FIGURE 7**
SEMI QUANTITATIVE RT-PCR ANALYSIS OF PLURIPOTENCY ASSOCIATED GENES
The hiPSCs express genes associated with pluripotency whereas the original non-reprogrammed somatic cells lack expression of these genes.
HUES 7 was used as the positive control.
MEFs were used as negative control to determine species specificity of the primers used for amplification.
Note the comparable expression of pluripotency related genes of hiPSCs and the embryonic stem cell line HUES7.
Karyotype analysis
It is essential to examine the genomic stability of newly generated induced pluripotent stem cells. Karyotyping is routinely used to identify or rule out any chromosomal abnormalities that are inherent or which arise due to prolonged manipulation in culture. Karyotyping assesses the safety of hiPSCs for use in transplantation medicine (Martins-Taylor and Xu, 2012; Taapken et al., 2011). In this study karyotyping of hiPSCs was conducted by the conventional G banding method. PiPSCs and NiPSCs had normal karyotypes with 46 chromosomes. The PiPSC line was female with 2X chromosomes (46, XX). The NiPSCs were obviously male cells (46, XY). Representative karyotypes are shown in figure 8A. The ViPSC line had a 45 XO karyotype of a classic Turner syndrome. This line was generated from the chorionic villi of a spontaneously fetus. Its karyotype matched the karyotype of the original villus fibroblasts linking its identity to the original cells (Figure15). To summarize, in this study three induced pluripotent stem cell lines were generated: one normal female line (PiPSCs); one normal male line (NiPSCs); and one line with 45XO karyotype (ViPSCs).

Transgene free status of hiPSC lines
No Ori P or EBNA 1 DNA was detected in ViPSCs, PiPSCs and NiPSCs after 15 passages as shown by genomic DNA PCR (Figure 8B). This confirmed that the three hiPSC cell lines become transgene free.

Up regulated telomerase activity of hiPSCs
Induced pluripotent stem cell up regulate their telomerase activity upon reprogramming (Allsopp, 2012; Marion et al., 2009) a trait of pluripotent stem cells. A schematic diagram showing the steps of the TRAP assay is given in figure 9A. The human embryonic stem cell line HUES7 was used as the positive control pluripotent stem cell line to compare the telomerase activity of the hiPSC lines. As expected the process of reprogramming had indeed up regulated the telomerase activity of the hiPSCs. The presence of active telomerase in the cell extracts of the hiPSC lines elongated the substrate oligonucleotides by addition of telomeric repeats which were amplified by PCR to generate a series of products. The products were analyzed both by PAGE analysis (Figure 9B) and fluorometric quantification (Figure 9C).
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A

ViPSCs

PiPSC

NiPSCs

FIGURE 8
KARYOTYPE ANALYSIS AND DETERMINATION OF TRANSGENE FREE STATUS OF hiPSC LINES
A. Representative images of karyotype (G Banding) of ViPSCs, PiPSCs and NiPSCs. Note the normal Karyotypes of PiPSC and NiPSC lines. The ViPSC line is a turner syndrome 45XO karyotype. Resolution of karyotyping is 300Mb.
B. Genomic DNA PCRs of ViPSCs, PiPSCs and NiPSCs showing the absence of episomal lasmid sequences EBNA1 and Ori P in the genome of hiPSCs indicating that the lines are transgene free. hiPSCs of greater than passage 15 were used for PCR. The episomal plasmid vector pCXLE-hSK was used as the positive control.
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A

**TELOMERIC REPEAT AMPLIFICATION PROTOCOL (TRAP)**

<table>
<thead>
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<th>POLYMERASE CHAIN REACTION</th>
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<td>4°C</td>
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<td>PAGE analysis</td>
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<td>3 holds</td>
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TTAGGG

Concentration of amplified product is directly proportional to Telomerase Content of cell.

B

**FIGURE 9**

**TELOMerase ACTIVITY ASSESSMENT**

A. A schematic representation of TRAP Assay used to analyse telomerase activity.
B. Quantitative analysis of TRAP Assay products.
C. PAGE analysis of TRAP Assay products.
In vitro and in vivo differentiation potentials of hiPSCs

The value of induced pluripotent stem cells lies in the fact that they can differentiate into cells of the three embryonic germ layers. Pluripotent stem cell differentiation can be used to understand molecular and cellular events that occur during development of humans. More importantly the differentiated progeny cells can be used in disease modelling and cellular therapies. Any new iPSC line generated should be able to differentiate into cells of three germ lineages. To explore the differentiation potentials of hiPSC lines, in vitro (EB formation followed by directed differentiation) and in vivo (teratoma formation) differentiation assays were carried out. Figure 10 outlines the strategies used to differentiate the hiPSCs into cell of three embryonic germ lineages. ViPSCs, PiPSCs and NiPSCs readily formed embryoid bodies (Figure 3D) and differentiated into derivatives of the three embryonic germ layers in vitro upon induction with specific factors. The differentiated cells of three germ lineages were
judged by their morphologies and expression of lineage specific markers. Specialized cells such as neurons, cardiomyocytes and hepatocytes formed after directed differentiation with growth factors specifying definite germ lineages.

The in vivo differentiation potential of the PiPSC line was examined by teratoma formation assay. Injection of PiPSCs in the testis and thigh muscle of immune deficient SCID mice resulted in the formation of tumors within three months. The tumors consisted of both cystic and solid parts typical of mixed teratomas. Histological evaluation confirmed that these tumors were indeed teratomas containing tissues of the three germ layers including glandular tissue, cartilage and pigmented epithelium.

Endoderm differentiation resulted in the formation of cuboidal cells typical of hepatocyte morphology (Figure11A). The differentiated cells stained positive for the endoderm markers SRY BOX 17 (SOX17) and α FETO PROTEIN (AFP) indicating maturity of the hepatocytes (Figure 11B & C). The testis teratoma formed by PiPSC line showed presence of distinct glandular structures as detected by hematoxylin and eosin staining of teratoma sections (Figure11D).

Mesoderm differentiation was directed towards cardiomyocytes. Beating cardiomyocytes differentiated from the PiPSC line. The morphologies of the mesoderm differentiated cells were typical of cardiomyocytes (Figure12A). The ViPSCs, PiPSCs and NiPSCs differentiated into cells expressing markers of the cardiac lineage. These cells expressed the early mesoderm marker SMOOTH MUSCLE ACTIN (SMA) (Figure12B) and the mature cardiomyocyte marker VENTRICULAR MYOSIN HEAVY CHAIN α/β (Figure 12C). Histological examination of the testicular teratoma formed by PiPSCs revealed cartilage, a derivative of the mesoderm (Figure 12D).

The three hiPSC lines were differentiated into neurons (Figure 13A). Immunocytochemistry detected cells positive for SRY BOX 2 (SOX2) and β III TUBULIN (Figure 13B). The differentiated neuronal cells were also positive for MICROTUBULE-ASSOCIATED PROTEIN 2 (MAP2) (Figure 13C) indicating the maturity of the neurons. Histological sections of the testicular teratoma formed by PiPSCs showed pigment containing epithelial tissues representing ectoderm differentiation (Figure 13D).


**FIGURE 11**  
ENDODERM DIFFERENTIATION OF THE hiPSC LINES

A. Phase contrast images of cell differentiated into hepatocytes showing cuboidal morphology.

B. SRY Box 17 (SOX17) expression by the endoderm differentiated cells. Nuclei are stained with DAPI.

C. α FETO PROTEIN (AFP) expression by the endoderm cells. Nuclei are stained with DAPI.

D. Glandular tissue formed in teratoma by PiPSCs.  
Scale bars represent 100 microns.
**FIGURE 12**

**MESODERM DIFFERENTIATION OF hiPSC LINES**

A. Phase contrast images of cells differentiating into cardiomyocytes.

B. Immunocytochemistry of cells expressing smooth muscle actin (SMA). The nuclei are stained with DAPI.

C. Cells expressing ventricular myosin heavy chain α/β. Nuclei are stained with DAPI.

D. The histological section of the testicular teratoma showing cartilage tissue. Scale bars represent 100 microns.
### FIGURE 13
ECTODERM DIFFERENTIATION OF hiPSC LINES

A. Phase contrast images of cells differentiated into neurons.

B. Staining of neurons with SRY Box2 (SOX2) and β III Tubulin. Nuclei are stained with DAPI.

C. Staining of cells with Microtubule Associated Protein 2 (MAP2). Nuclei are stained with DAPI.

D. Presence of pigmented tissue in testis teratoma formed by PiPSCs. Scale bars represent 100 microns.
STR analysis of somatic cells and hiPSCs

DNA finger printing by STR profiling showed the unique identity of the hiPSC lines matching with the original somatic cells. The ViPSC line generated in this study was confirmed to be identical with the original CVFs by STR analysis of 16 genetic loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, Amelogenin, D5S818, FGA) PiPSCs and NiPSCs were matched with PMSCs and NFFs respectively by PCR amplification of DNA 21 autosomal STR loci (D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, D2S441, D19S433, TH01, FGA, D22S1045, D5S818, D13S317, D7S820, SE33, D10S1248, D1S1656, D12S391, D2S1338), 1 Y-STR (DYS391), 1 insertion/deletion polymorphic marker on the Y chromosome (Y indel) and Amelogenin (sex determining marker). (Figure 14).

FIGURE 14
STR ANALYSIS OF SOMATIC CELLS AND hiPSCs.
STR analysis showing genetic identity of ViPSCs, PiPSCs and NiPSCs with CVFs, PMSCs and NFFs respectively
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FIGURE 14 continued

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DISCUSSION

Pluripotent stem cells are defined by the two distinguishing features of self renewal and differentiation potential. PSCs maintain sustained self renewal by suppressing differentiation. Pluripotent stem cells can differentiate into specialized cells of three embryonic germ layers but do not readily form cells of trophoblast or placenta (Angeles et al., 2015). They maintain differentiation inducing genes in a quiescent paused state reflecting their emergent developmental potential. A combined demonstration of diagnostic molecular signatures and functional characteristics of pluripotency provides robust validation of newly derived stem cells. I have shown the expression of OCT4, SOX2 and NANOG, the core transcription factors which demonstrate pluripotency of the newly generated lines without doubt. Along with core transcription factors I have shown the expression of surface markers SSEA4, TRA1-60 and TRA1-81 by the new lines. These markers are considered definite indicators of pluripotency.

Cardinal assays employed to test the developmental potential of PSC include in vitro differentiation, teratoma formation, chimaera formation, germ line transmission, tetraploid complementation and single-cell chimaera formation. Of these I used in vitro differentiation to derivatives of all three embryonic germ layers and the teratoma formation assay to demonstrate the developmental potential of the new lines. For in vitro differentiation to cells of ectoderm, mesoderm and endoderm cells were grown in specific culture medium supplemented with cocktails of differentiation inducing chemicals, cytokines and growth factors for a specified periods. This was followed by surveying tissue specific markers in the differentiated cells (Angeles et al., 2015). All the three cell lines readily differentiated into cells of all three germ layers. Teratoma assay was used to assess if the PiPSCs could spontaneously differentiate into tissues from the three germ layers after injecting PiPSC into immune-compromised mice. The PiPSCs could spontaneously form teratomas in the testis and thigh muscle of SCID mice. These data validate the developmental potential of the newly generated cell lines.

The three hiPSCs generated in this study were subjected to extensive characterization to define their pluripotency. The expression of key molecular signatures and their ability to undergo tri-lineage differentiation in vitro and in vivo confirmed their
pluripotent nature. Additional data regarding up regulated telomerase activity further strengthened the characterization. The karyotype gave insight into the gender of the lines and confirmed the 45XO genotype of the ViPSCs. ViPSCs, PiPSCs and NiPSCs differentiated into cells of endodermal, mesodermal and ectodermal lineages as judged by the expression of lineage specific markers by the differentiated progeny cells. The ability of PiPSCs to form teratomas, a stringent test of pluripotency further emphasized the pluripotential nature of the line. Put together the above results confirm the molecular and functional pluripotency of the three hiPSCs generated in this study.

The three cell lines had DNA profiles matching with the DNA profiles of the original cell from which they were derived indicating their identity. The STR profiles of these cell lines did not match with previously existing STR profiles in data bases. They had DNA profiles which were unique confirming the cell line authenticity.