



## Lab Resource: Multiple Cell Lines

## Generation of iPSC lines from hereditary spastic paraplegia 56 (SPG56) patients and family members carrying CYP2U1 mutations

Hannah C. Leeson <sup>a</sup>, Denise Goh <sup>b,c</sup>, David Coman <sup>d,e,f</sup>, Ernst J. Wolvetang <sup>a,\*</sup><sup>a</sup> The University of Queensland, Australian Institute for Bioengineering & Nanotechnology (AIBN), St. Lucia, Brisbane, QLD 4072, Australia<sup>b</sup> National University Hospital, Khoo Teck Puat – National University Children's Medical Institute, Department of Paediatrics, 119228, Singapore<sup>c</sup> National University of Singapore, Yong Loo Lin School of Medicine, 119228, Singapore<sup>d</sup> Queensland Children's Hospital, Department of Metabolic Medicine, South Brisbane, Brisbane, QLD 4001, Australia<sup>e</sup> The University of Queensland, School of Medicine, St. Lucia, Brisbane, QLD 4072, Australia<sup>f</sup> Griffith University, School of Medicine, Gold Coast, QLD 4215, Australia

## ABSTRACT

Hereditary spastic paraplegia 56 (SPG56) is an extremely rare autosomal recessive disorder caused by mutations in the CYP2U1 gene, involved in fatty acid metabolism. SPG56 causes progressive spasticity in upper and lower limbs, though due to the rarity of this subtype of spastic paraplegia, the molecular causes remain unclear and no treatment or cure exists. Here we describe the generation and validation of induced pluripotent stem cell (iPSC) lines from two unrelated patients with SPG56 and two heterozygous family members. These lines can be used to investigate the mechanisms driving progressive spasticity and evaluate the potential for gene replacement therapies.

## 1. Resource Table

(continued)

Unique stem cell lines identifier	<i>AIBNi015-A</i> <i>AIBNi016-A</i> <i>AIBNi017-A</i> <i>AIBNi018-A</i>	Sex: Female Ethnicity if known: N/A
Alternative name(s) of stem cell lines	<i>AIBNi015-A: SPG1-AU01C15</i> <i>AIBNi016-A: SPG2-S376C1</i> <i>AIBNi017-A: SPGh1-S075mC2</i> <i>AIBNi018-A: SPGh2-S964pC6</i>	<i>AIBNi018-A: SPGh2-S964pC6</i> Age: 37 Sex: Male Ethnicity if known: N/A
Institution	<i>Australian Institute for Bioengineering and Nanotechnology</i>	<i>AIBNi015-A: Fibroblasts</i>
Contact information of distributor	<i>Professor Ernst J Wolvetang: e.wolvetang@uq.edu.au</i>	<i>AIBNi016-A, AIBNi017-A, AIBNi018-A: total peripheral blood mononuclear cells</i> Clonal
Type of cell lines	<i>Induced pluripotent stem cells (iPSCs)</i>	<i>Non-integrative Sendai virus delivery of OCT4, SOX2, KLF4 and c-MYC transgenes</i>
Origin	<i>Human</i>	Yes
Additional origin info required for human ESC or iPSC	<i>AIBNi015-A: SPG1-AU01C15</i> Age: 2 Sex: Female Ethnicity if known: N/A	Hereditary
	<i>AIBNi016-A: SPG2-S376C1</i> Age: 1 Sex: Female Ethnicity if known: N/A	PCR
	<i>AIBNi017-A: SPGh1-S075mC2</i> Age: 34	<i>Hereditary spastic paraplegia 56 (SPG56)</i> <i>AIBNi015-A: SPG1-AU01C15</i> CYP2U1 compound heterozygous Exon 4 substitution: Chr4: 107949437; c.1376C > T; p.(Pro459Leu) Exon 5 substitution: Chr4: 107950250; c.1462C > T; p.(Arg488Trp)

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\* Corresponding author.

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	<b>AIBNi016-A: SPG2-S376C1</b> CYP2U1 compound heterozygous Exon 1 deletion: Chr4: 107932113; c.471delC; p.(Ile158fs) Exon 2 substitution: Chr4: 107945564; c.1085 T > C; p.(Leu362Pro)
	<b>AIBNi017-A: SPGh1-S075mC2</b> CYP2U1 heterozygous carrier Exon 1 deletion: Chr4: 107932113; c.471delC; p.(Ile158fs)
	<b>AIBNi018-A: SPGh2-S964pC6</b> CYP2U1 heterozygous carrier Exon 2 substitution: Chr4: 107945564; c.1085 T > C; p.(Leu362Pro)
Date archived/stock date	December 2021
Cell line repository/bank	<a href="https://hpscreg.eu/cell-line/AIBNi015-A">https://hpscreg.eu/cell-line/AIBNi015-A</a> <a href="https://hpscreg.eu/cell-line/AIBNi016-A">https://hpscreg.eu/cell-line/AIBNi016-A</a> <a href="https://hpscreg.eu/cell-line/AIBNi017-A">https://hpscreg.eu/cell-line/AIBNi017-A</a> <a href="https://hpscreg.eu/cell-line/AIBNi018-A">https://hpscreg.eu/cell-line/AIBNi018-A</a>
Ethical approval	UQ HREC 2,019,000,159 - Generation, differentiation and genetic manipulation of human induced pluripotent stem cells

## 2. Resource utility

Human induced pluripotent stem cell (hiPSC) lines were created from two hereditary spastic paraplegia 56 (SPG56) patients and both heterozygous parents of one patient to generate neuronal models, which will be used to uncover the molecular mechanisms underlying disease phenotype and progression, and as a model for drug screening studies.

## 3. Resource details

Hereditary spastic paraplegia 56 (SPG56; OMIM 615030) is a rare form of complicated hereditary spastic paraplegia, presenting during infancy with progressive lower-extremity spastic weakness in combination with other neurological symptoms, including dystonia and developmental delay (Fink, 2013). SPG56 is caused by mutations in CYP2U1 (Fink, 2013; Durand et al., 2018), a cytochrome P450 hydroxylase predominantly localised to the thymus and brain (Chuang, 2004; Karlgren et al., 2004). CYP2U1 is involved in long chain fatty acid metabolism and catalyses the hydroxylation of arachidonic acid to 19- and 20-hydroxy-modified arachidonic acids (19- and 20-HETE), as well as metabolising docosahexaenoic acid (DHA) and other long chain fatty acids (Durand et al., 2018; Chuang, 2004). While the physiological roles of CYP2U1 remain unclear, 19- and 20-HETE have been shown to act as vasoconstrictors and regulate calcium and calcium-dependent potassium channels [outlined in reference 3]. Further, CYP2U1 is present in mitochondrial and microsomal fractions in both HEK293 and human brain samples (Karlgren et al., 2004; Dutheil et al., 2009) and mitochondrial defects have been observed in CYP2U1 mutant fibroblasts (Tesson et al., 2012).

We reprogrammed peripheral blood mononuclear cells (PBMCs) or fibroblast samples from two patients with SPG56, designated SPG1 and SPG2, as well as both heterozygous parents of patient SPG2, which are designated SPGh1 and SPGh2. CytoTune-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher) was used to transduce both PBMCs and fibroblasts according to the manufacturer guidelines, and we successfully generated iPSC lines from all samples. The generated iPSC lines demonstrated typical morphology (Fig. 1A) and we confirmed the loss of the Sendai virus and associated transgenes in all four lines by passage 14 (Fig. 1B),

prior to further characterisations. Positive controls for transgenes were harvested on day 7 of reprogramming. Immunohistochemistry confirmed expression of pluripotency markers NANOG, OCT-4, SOX2, TRA 1-60 and TRA 1-81 (Fig. 1C) in at least 80 % of all cells imaged (Fig. 1D). Directed differentiations to ectoderm, mesoderm and endoderm confirmed germ layer potential and validated pluripotency. When assessed by qPCR (Fig. 1E), all four iPSC lines showed upregulation of markers for endoderm (FOXA2, SOX17, CXCR4), mesoderm (PDGFRA, HAND1, BMP4, RUNX1), and ectoderm (OTX2, PAX6, NR2F2, VIMENTIN) along with a downregulation of pluripotency markers NANOG, OCT4 and SOX2 (data not shown). Sanger sequencing was used to confirm the presence of compound heterozygous mutations in SPG1 and SPG2, and heterozygous carrier status of SPGh1 and SPGh2 (Fig. 1F). Patient SPG1 has two missense amino acid changes in exon 4 [c.1376C > T; p.Pro459Leu] and exon 5 [c.1462C > T; p.Arg488Trp]. Patient SPG2 and heterozygous carrier SPGh1 have a deletion in exon 1 generating a frameshift (seen by dual base calling in sequence) and premature stop codon [c.471delC; p.Ile158fs]. Additionally, patient SPG2 has an amino acid change at a highly conserved position in exon 2 [c.1085 T > C; p.Leu362Pro], which is carried by heterozygous SPGh2. The SPG heterozygous lines were both used as controls for sequencing the mutations they did not carry. Short tandem repeat (STR) analysis confirmed that the iPSC profiles matched those of the donor PBMCs and fibroblasts, and single nucleotide polymorphism (SNP; Illumina HumanCytoSNP-12) array analysis conducted at passage 16 showed normal karyotypes across all four lines (supplementary data). SNP array detects unbalanced chromosomal aberrations at a higher resolution compared to conventional g-banding karyotype, including duplications and amplifications, deletions, copy-neutral loss of heterozygosity, and low-level mosaicism, however does not detect balanced rearrangements such as reciprocal translocation. Additional information about the characterisation and validation of SPG1, SPG2, SPGh1 and SPGh2 iPSC lines is provided in Tables 1 and 2. Collectively, this data demonstrates the successful generation and quality of a pluripotent hiPSC line from SPG patients and family members.

## 4. Materials and methods

### 4.1. Reprogramming of PBMCs and fibroblasts

SPG56 patients were diagnosed following trio analysis of genomic sequencing, which identified compound heterozygous mutations in the CYP2U1 gene in both SPG1 and SPG2. Blood samples and skin biopsies were collected for the purpose of iPSC generation with informed consent and ethical approval. PBMCs were isolated using a Ficoll-Paque separation technique, and fibroblasts were cultured for 2 passages before stocks were banked. PBMCs were expanded in StemSpan SFEM II medium supplemented with the Erythroid Expansion Supplement (Stemcell Technologies) for 7 days, while fibroblasts were cultured in DMEM/F12 with 10% FBS for 2 days. Expanded cells were transduced with a non-integrating Sendai virus carrying the 4 transgenes: OCT4, SOX2, KLF4 and c-MYC (CytoTune-iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher), according to the manufacturer's instructions. Cells were maintained daily in ReproTeSR until colony formation. Colonies displaying appropriate morphology were picked manually, replated on hESC qualified Matrigel (Corning) and medium was transitioned to mTeSR Plus (Stemcell Technologies). Subsequent passages were performed using 0.5 mM EDTA at a split ratio of 1:5 – 1:8 approximately every 5 days, and clumps were plated in mTeSR Plus without ROCK inhibitor on Matrigel coated plates and were maintained at 37°C in a 5% CO<sub>2</sub> incubator. All analysis was conducted on cells passage 5 onward.

### 4.2. Immunofluorescence staining

The iPSCs were fixed with 4% paraformaldehyde for 10 min at 4°C before blocking and permeabilisation for 1 h with 3% Bovine Serum

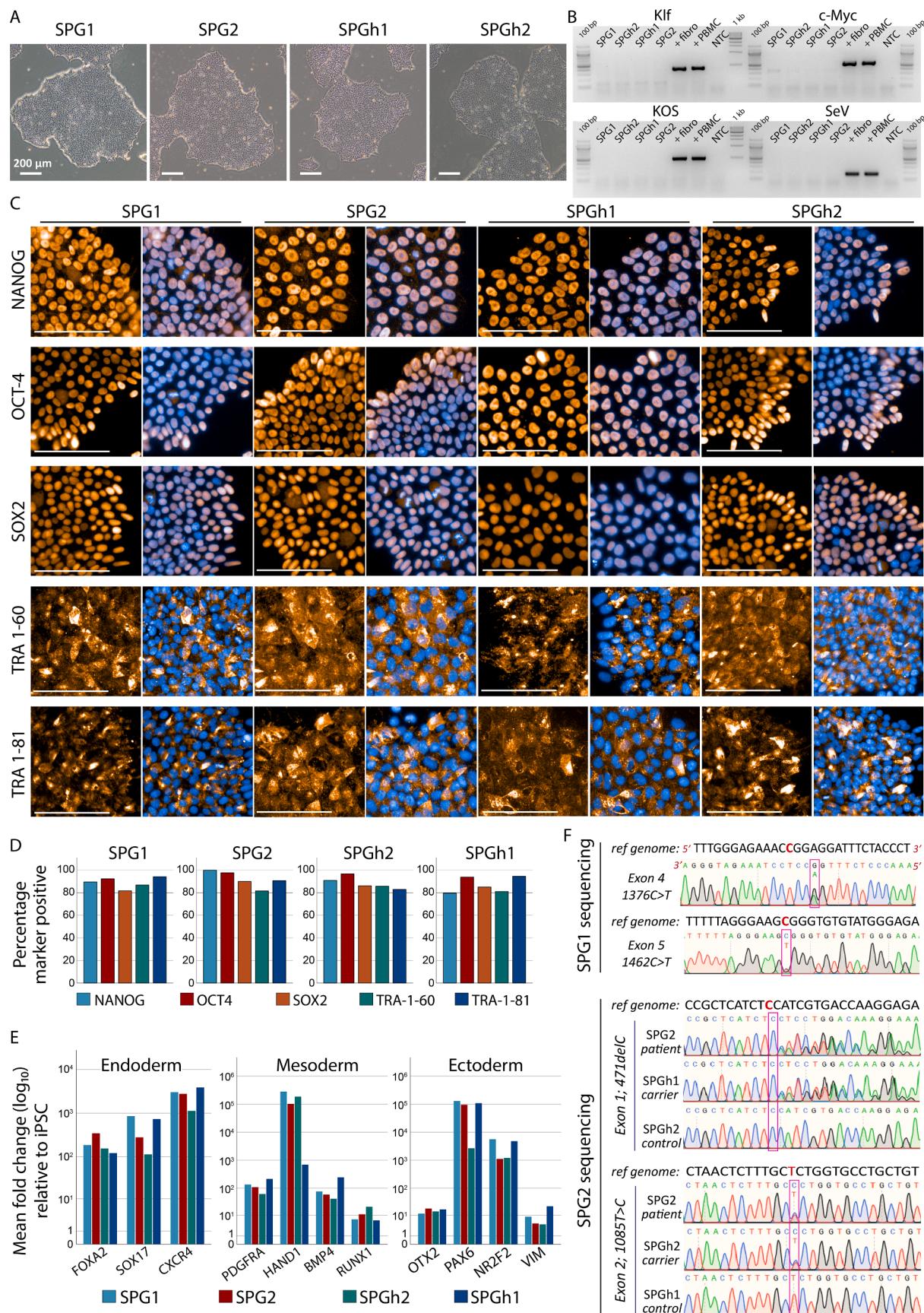


Fig. 1. Characterisation of SPG1, SPG2, SPGh1, and SPGh2 iPSC lines.

**Table 1**  
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography Bright field	Compact flat colonies with a well-defined smooth edge, containing cells with a high nucleus to cytoplasm ratio and prominent nucleoli	<a href="#">Fig. 1</a> , panel A
Phenotype	Qualitative analysis: Immunocytochemistry staining	Positive staining of pluripotency markers: OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81	<a href="#">Fig. 1</a> , panel C
	Quantitative analysis	Robust endogenous expression of OCT4, NANOG, SOX2, TRA 1-60 and TRA 1-81 in 80 to 95 % of cells	<a href="#">Fig. 1</a> , panel D
Genotype	SNP array (~300000 markers)	normal	Provided in <a href="#">supplementary</a>
Identity	STR analysis	10 loci tested and matched to original cell line	Provided in <a href="#">supplementary</a>
Mutation analysis (IF APPLICABLE)	Sanger sequencing	<i>SPG1: CYP2U1 compound heterozygous: c.1376C &gt; T; p. (Pro459Leu) c.1462C &gt; T; p. (Arg488Trp)</i>  <i>SPG2: CYP2U1 compound heterozygous c.471delC; p. (Ile158fs) c.1085 T &gt; C; p. (Leu362Pro)</i>  <i>SPGh1: CYP2U1 heterozygous carrier c.471delC; p. (Ile158fs)</i>  <i>SPGh2: CYP2U1 heterozygous carrier c.1085 T &gt; C; p. (Leu362Pro)</i>	<a href="#">Fig. 1</a> , panel F
Microbiology and virology	Mycoplasma	Mycoplasma testing by Myco Alert Assay returned negative results with testing monthly	Not shown, available with author
Differentiation potential	Directed differentiation	Expression of markers for endoderm (FOXA2, SOX17, CXCR4), mesoderm (PDGFRA, HAND1, BMP4,	<a href="#">Fig. 1</a> , panel E

**Table 1 (continued)**

Classification	Test	Result	Data
		RUNX1), and ectoderm (OTX2, PAX6, NR2F2, VIMENTIN)	
List of recommended germ layer markers	Germ layer expression validated by qPCR	Expression of markers for endoderm (FOXA2, SOX17, CXCR4), mesoderm (PDGFRA, HAND1, BMP4, RUNX1), and ectoderm (OTX2, PAX6, NR2F2, VIMENTIN)	<a href="#">Fig. 1</a> , panel E
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A	N/A

Albumin (BSA) and 0.1% TritonX-100 in PBS. Pluripotency markers ([Table 2](#)) were incubated overnight at 4°C, and secondary antibodies were incubated for 1hr at room temperature. Nuclei were counterstained with 1 µg/ml DAPI or Hoechst.

#### 4.3. Germ lineage differentiations

Directed differentiation was used to confirm potential for generation of three germ layers. Directed differentiations for each germ layer involved culturing the iPSCs in respective differentiation medium. For ectoderm differentiation, neural induction medium (1:1 DMEM/F12 and Neurobasal medium, 0.5X N2, 0.5X B-27, 1X GlutaMax, 0.5X NEAA, 2.5 µg/mL insulin and 50 µM β-mercaptoethanol) with dual SMAD inhibition (10 µM SB431542 and 0.1 µM LDN-193189) was fed for 10 days. Endoderm differentiation used STEMdiff Definitive Endoderm Kit (Stemcell Technologies) for 5 days and mesoderm was fed with RPMI supplemented with 1X B-27 and 5 µM CHIR for 5 days. Differentiations were harvested for RNA and analysed by qPCR.

#### 4.4. Quantitative PCR (qPCR)

RNA was extracted using Nucleospin RNA extraction kit (Macherey-Nagel) and cDNA was synthesised using BioRad iScript cDNA Synthesis kit. All qPCR reactions were performed in triplicate using PowerUp SYBR Green Master Mix (Thermo Fisher), and results were expressed as ΔΔCT. Primers are listed in [Table 2](#).

#### 4.5. DNA isolation, Short tandem repeat (STR) analysis and karyotyping

DNA was extracted from iPSCs and the corresponding patient PBMC or fibroblast lines and underwent STR analysis (GenePrint-10) and SNP array (Illumina HumanCytoSNP-12, ~300,000 SNPs) by the Australian Genome Research Facility in Melbourne.

#### 4.6. Mycoplasma testing

Culture medium routinely underwent mycoplasma testing using MycoAlert Assay.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial

**Table 2**  
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry				
	Antibody	Dilution	Company Cat #	RRID
Pluripotency Markers	Mouse Anti OCT4 IgG	1:100	Millipore Cat# MAB4419	RRID:AB_1977399
	Rabbit Anti SOX2 IgG	1:400	Cell Signaling Technology Cat# 23064	RRID:AB_2714146
	Mouse Anti NANOG IgG	1:2000	Cell Signaling Technology Cat# 4893	RRID:AB_10548762
	Mouse Anti Tra-1-60 IgG	1:200	Millipore Cat# MAB4360	RRID:AB_2119183
Secondary Antibodies	Mouse Anti Tra-1-81 IgG	1:100	Millipore Cat# MAB4381	RRID:AB_177638
	Goat Anti Mouse IgG H + L Alexa Fluor 647	1:500	Thermo Fisher Scientific Cat# A-21235	RRID:AB_2535804
	Goat Anti Mouse IgG H + L Alexa Fluor 488	1:500	Thermo Fisher Scientific Cat# A-11029	RRID:AB_2534088
	Goat Anti Rabbit IgG H + L Alexa Fluor 647	1:500	Thermo Fisher Scientific Cat# A-21245	RRID:AB_2535813
Sendai transgenes (PCR)	Donkey Anti Mouse IgG H + L Alexa Fluor 568	1:500	Thermo Fisher Scientific Cat# A-10037	RRID:AB_2534013
	<b>Primers</b>			
	<b>Target</b>	<b>Size of band</b>	<b>Forward/Reverse primer (5'-3')</b>	
	Sendai virus (SeV) genome	181	GGATCACTAGGTGATATCGAGC/ ACACAGACAAGAGTTAACAGAGATATGTATC	
Endogenous pluripotency markers (qPCR)	KOS	528	ATGCACCGCTACCGACGCTGAGCGC/ ACCTTGACAACTCTGATGTGG	
	KLF-4	410	TTCCTGCATGCCAGAGGAGCC/AATGTATCGAAGGTGCTCAA	
	c-MYC	532	TAACTGACTAGCAGGTTGTCG/ TCCACACATACTCTGGATGATGATG	
	OCT-4	113	AGTTTGTGCCAGGGTTTTG/ACTTCACCTTCCCTCCAAC	
Differentiation primers (qPCR)	Nanog	194	TTTGGAGCTGGGGAG/GATGGGAGGGAGAGGA	
	SOX2	234	CATGTCGGCAGCTACAGA/GTCATTGCTGGGTGATG	
	SOX17 (endoderm)	94	GTGGACCGCACGGAATTG/GGAGATTACACCCGGAGTC	
	FOXA2 (endoderm)	83	GGAGCAGCTACTATGCAGAGC/CCTGTTCATGCCGTTATCC	
House-Keeping Gene (qPCR)	CXCR4 (endoderm)	61	GCCTTACTACATTGGGATCAG/CCCTTGCTGATGATTTC	
	PDGFRA (mesoderm)	92	GTCCTCTCACAGGGCTGAG/TGAATTCTGACACAACC	
	HAND1 (mesoderm)	170	CCATGCTCACGAACCCCTTC/CCTGGCGTCAGGACCATAG	
	BMP4 (mesoderm)	80	CCACCAACAGAAGAACATCTG/ ATGCTGCTGAGGTTAAAGAG	
	RUNX1 (mesoderm)	104	CATGGCTTCAAGGTGGTG/ GTAGCATTTCTCAGCTCAGC	
	Otx2 (ectoderm)	81	CCAGACATCTCATGGCAG/TCGATTCTAAACCATACCTG	
	Pax6 (ectoderm)	320	ACACACTTGAGCCATCAGCA/TTCACACAAACAGCTCGCTC	
	NR2F2 (ectoderm)	151	TCATGGGTATCGAGAACATTG/TTCAACACAAACAGCTCGCTC	
	Vimentin (ectoderm)	81	TCCACGAAGAGGAATCCA/CAGGCTTGAAACATCCAC	
	GAPDH	66	AGCCACATCGCTCAGACAC/GCCCAATACGACCAAATCC	

interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scr.2022.102917>.

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