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Lab Resource: Multiple Cell Lines

Generation of two isogenic induced pluripotent stem cell lines from a 10-year-old typical nemaline myopathy patient with a heterozygous dominant c.541G>A (p.Asp179Asn) pathogenic variant in the ACTA1 gene

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ABSTRACT

Nemaline myopathy (NM) is a congenital myopathy typically characterized by skeletal muscle weakness and the presence of nemaline bodies in myofibres. Approximately 25% of NM cases are caused by variants in *ACTA1*. We generated two induced pluripotent stem cell lines from lymphoblastoid cells of a 10-year-old female with typical NM harbouring a dominant pathogenic variant in *ACTA1* (c.541C>A). The isogenic lines displayed typical iPSC morphology, expressed pluripotency markers, and could differentiate into each of the three germ layers. Although the lines have partial or complete X chromosome duplication, they may still prove useful as models of human *ACTA1* disease.

Resource Table

Unique stem cell lines identifier	1. HPIi002-A 2. HPIi002-B
Alternative names of stem cell lines	1. iPS-789-C1 2. iPS-789-C2
Institution	Harry Perkins Institute of Medical Research
Contact information of distributor	Dr. Joshua Clayton joshua.clayton@perkins.org.au
Type of cell lines	iPSC
Origin	Human
Cell Source	EBV-immortalized lymphoblastoid cell line (LCL)
Clonality	Clonal
Method of reprogramming	CytoTune™-iPS 2.0 Sendai Reprogramming Kit
Multiline rationale	Isogenic clones
Gene modification	Yes
Type of modification	Spontaneous variant
Associated disease	Nemaline myopathy 3; NEM3 (OMIM#161800), typical form
Gene/locus	Actin Alpha 1, Skeletal Muscle (*ACTA1*), NM_001100: c.541G>A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	September 2020
Cell line repository/bank	1. https://hpscreg.eu/cell-line/HPIi002-A2 2. https://hpscreg.eu/cell-line/HPIi002-B
Ethical approval	Ethics approval was obtained from the Comité de Protection des Personnes (Est IV DC-2012-1693), and national consent forms for genetic testing, banking and research were signed by the patients or their legal guardian. The patient's LCLs were banked by Genethon;

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Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
HPIi002-A (iPS-789-C1)	HPIi002-A	Female	10 years	French	G/A	Nemaline myopathy 3; NEM3 (OMIM#161800), typical form
HPIi002-B (iPS-789-C2)	HPIi002-B	Female	10 years	French	G/A	Nemaline myopathy 3; NEM3 (OMIM#161800), typical form

Resource Table (*continued*)

	activity authorization No. AC-2018-3156, import/export authorization No. IE-2018-994. The study was approved by the University of Western Australia's Human Research Ethics Committee (approval number: RA/4/20/1008).

1. Resource utility

These dominant *ACTA1* iPSC lines will complement other published *ACTA1* lines to enable disease modelling and testing of genomic therapies for nemaline myopathy. We have also performed phased genotyping of this line across the *ACTA1* locus, making it useful for testing of novel allele-specific treatments.

2. Resource details

Nemaline myopathy (NM) is a form of congenital myopathy characterized by skeletal muscle weakness and the presence of abnormal thread- or rod-like structures in myofibres on histologic examination (Sewry et al., 2019). There are six clinical categories of NM; severe congenital, intermediate congenital, typical congenital, mild childhood-onset, adult-onset, and 'other' (Sewry et al., 2019). Dominant variants in the skeletal muscle alpha-actin gene (*ACTA1*) are the second most common cause of NM (Sewry et al., 2019). *ACTA1* disease is rare and often severe; many patients die within the first two years of life. The iPSC lines presented here were generated from a lymphoblastoid cell line (LCL) from a 10-year-old female with typical NM who presented with disease during childhood (Table 1). Genetic testing revealed the patient was heterozygous for a pathogenic variant in *ACTA1*; c.541G>A, p.Asp179Asn (Sparrow et al., 2003). We used LCLs for reprogramming as these were the only available patient material.

LCLs were reprogrammed into iPSCs using the CytoTune™-iPS 2.0 Sendaivirus reprogramming system. Clones were selected and expanded in mTESR™1 culture medium. Both iPSC clones (HPIi002-A, HPIi002-B) showed typical morphology (Fig. 1A). Pluripotency was confirmed by qRT-PCR (Fig. 1B) and immunocytochemistry (ICC) (Fig. 1C, D). Specifically, iPSCs were enriched for *OCT4*, *SOX2*, *NANOG* and *CRIPTO* by qPCR compared to parental LCLs (Fig. 1B), and stained positively for OCT4, SOX2, SSEA4 and TRA-1–60 by ICC (Fig. 1C, D). Both clones were able to differentiate into each of the three germ layers, as assessed by qRT-PCR (Fig. 1E) and ICC (Fig. S1A, B) following directed differentiation. Mesoderm cultures were enriched for *TBXT* and *BMP4* by qPCR, and Brachyury (TBXT) by ICC. Ectoderm cultures were enriched for *OTX2* and *PAX6* by qPCR, and OTX2 by ICC. Endoderm cultures were enriched for *GATA4* and *SOX17* by qPCR, and GATA4 by ICC. Parental (undifferentiated) iPSC cultures showed no or negligible expression of each germ layer marker (Fig. 1E).

Both iPSC clones were confirmed to be EBV- and SeV-free by PCR and RT-PCR, respectively (Fig. 1F and Fig. 1G). Both lines showed chrX duplication by KaryoStat analysis (Fig. 1H). HPIi002-B had a partial deletion of one of the X chromosomes from Xp21.1 to Xpter (Fig. 1H). Translocations and mosaicism were excluded by G-banding analysis of multiple metaphase spreads (Fig. S1E,F). No other abnormalities were detected in either line. X chromosome duplication is a known recurrent abnormality in human iPSCs (Taapken et al., 2011). This may limit the utility of these lines for some applications, although it is likely that the additional X chromosome would be inactivated by X chromosome inactivation (Bar et al., 2019). Analysis of the parental LCL indicated that despite having a normal karyotype at early passage, this line is prone to X chromosome duplication. Indeed, multiple attempts to generate karyotypically normal clones all yielded the same X chromosome duplication.

Short tandem repeat (STR) typing was used to verify culture identity and purity; both iPSC clones matched the original parental cell line (data archived with journal). The original heterozygous *ACTA1* variant (c.541G>A) was confirmed in both clones by PCR and Sanger sequencing (Fig. 1I). Both lines were free of mycoplasma (Fig. S1D). Characterization of the iPSC clones is summarized in Table 2.

3. Materials and methods

3.1. Generation and maintenance of iPSC lines

Patient lymphoblastoid cell lines (LCLs) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% L-glutamine (R10 medium) at 37 °C and 5% CO_2. LCLs were reprogrammed using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (ThermoFisher). Briefly, $3x10^5$ cells were transduced at recommended multiplicity of infection and plated on growth factor-reduced (GFR) Matrigel® (1:100 in DMEM/F-12; ThermoFisher) in R10 medium. Cells were gradually adapted to mTESR™1 medium (StemCell) and individual clones picked for expansion and validation. iPSCs were cultured on GFR Matrigel and passaged 1:6–1:12 every 3–5 days (at ~80% confluency) using 1X Versene (ThermoFisher) into mTeSR™1 with 1X RevitaCell (ThermoFisher). Cells were cryopreserved in 90% KnockOut™ Serum Replacement (ThermoFisher) with 10% DMSO. Control iPSCs were a gift from Prof. Rhonda Bassel-Duby and were maintained as above.

3.2. Immunocytochemistry – pluripotency marker expression and trilineage differentiation potential

For qualitative pluripotency analysis, iPSCs (P11) were plated on Matrigel-coated 96-well Nunc polymer optical bottom plates and stained for OCT4, SSEA4, SOX2, and TRA-1–60 (Table 2). Trilineage differentiation potential was assessed using the STEMdiff™ Trilineage Differentiation Kit (StemCell) using StemPro™ Accutase™ (Gibco) for cell dissociation. Immunocytochemistry was performed as described in the PSC 4-marker Immunocytochemistry Kit (ThermoFisher), except that cells were incubated with primary antibodies (Table 3) overnight at 4 °C in 3% BSA. Nuclei were stained using NucBlue™ Fixed Cell stain (ThermoFisher). Cells were imaged using an Olympus IX71 microscope with a DP74 camera and CellSens software.

3.3. DNA and RNA extraction

Genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN). RNA was extracted using the RNeasy Mini Kit (QIAGEN). Purity was assessed using a NanoDrop One spectrophotometer (ThermoFisher) and integrity assessed by agarose gel electrophoresis.

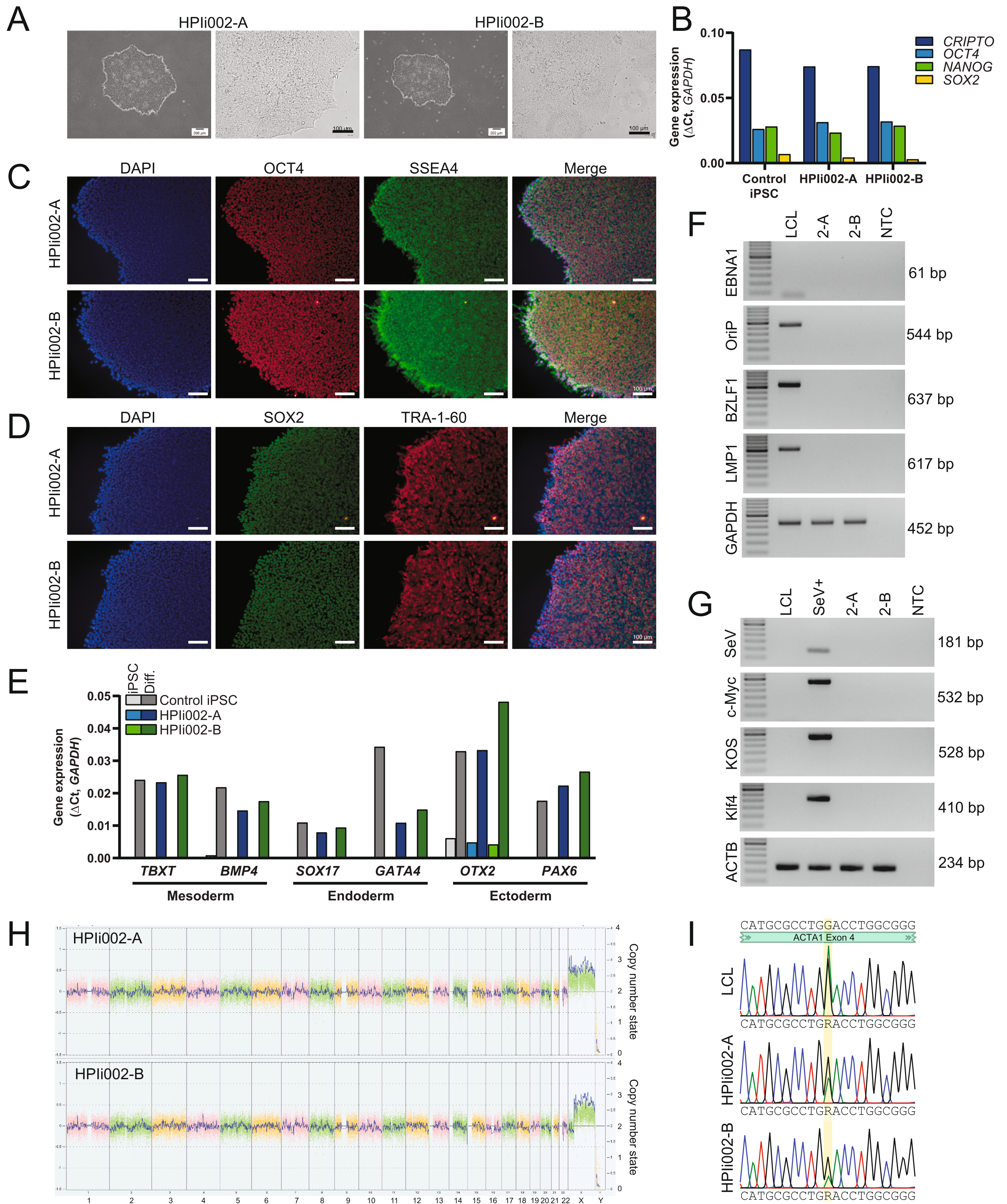

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Fig. 1. Characterisation of HPIi002-A and HPIi002-B iPSC lines. A) Bright-field images of iPSC colonies showing normal, tightly packed morphology. Left scale = 200 µm, right scale = 100 µm. **B)** mRNA expression of pluripotency markers. qPCR data were normalised to *GAPDH*. **C)** Immunofluorescence for OCT4 and SSEA4 pluripotency markers. Scale = 100 µm. **D)** Immunofluorescence for SOX2 and TRA-1-60 pluripotency markers. Scale = 100 µm. **E)** Relative qPCR quantitation of mRNA for markers of mesoderm (*TBXT, BMP4*), endoderm (*SOX17, GATA4*), and ectoderm lineages (*OTX2, PAX6*), from iPSCs differentiated to each germ layer using the STEMdiff™ Trilineage differentiation kit. **F)** Agarose gel of PCRs for four different EBV markers (*EBNA1, OriP, BZLF1, LMP1*) in parental LCL (positive control) and HPIi002 lines (2-A and 2-B). *GAPDH* was run as a loading control. Left lane is 100 bp TrackIT ladder. Expected PCR product sizes are indicated to the right of each image. **G)** Agarose gel of RT-PCRs for CytoTune™ Sendai virus vectors and transgenes (SeV, c-Myc, KOS, Klf4) in parental LCL (negative control), day 3-transduced LCLs (SeV+, positive control), and HPIi002 lines (2-A and 2-B). *ACTB* was run as a loading control. Left lane is 100 bp TrackIT ladder. Expected PCR product sizes are indicated to the right of each image. **H)** KaryoStat copy number state across each chromosome. **I)** Sanger sequencing across *ACTA1* c.541G>A (highlighted). LCL = lymphoblastoid cell line used to generate reprogrammed lines. Control iPSC line was generated from whole blood using the same reprogramming method used for HPIi002-A and HPIi002-B. All data are from iPSC lines around passage 10 (P10). NTC = no template control.

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography (light microscopy)	Normal	Fig. 1, panel A
Phenotype	Qualitative analysis (Immunocytochemistry)	Positive for OCT4, SOX2, SSEA4, TRA-1–60	Fig. 1, panels C/D
	Quantitative analysis (qRT-PCR)	Expression of *OCT4, SOX2, NANOG, CRIPTO*	Fig. 1, panel B
Genotype	KaryoStat™ assay (CytoScan Optima). Confirmation by G-banding.	HPIi002-A. 47, XXX. Complete X chromosome duplication; otherwise normal. HPIi002-B: 47,XX?del(X)(p21); X chromosome duplication, with deletion of part of the short arm at breakpoint Xp21. No other abnormalities detected. Minimum resolution = 1 MB for losses, 2 MB for gains, 5 MB for LOH/AOH	Fig. 1, panel H Supplementary Fig. 1, panel E/F
Identity	Microsatellite PCR (mPCR) OR STR analysis	Microsatellite PCR not performed	
		Matched to parental LCL line at 22/22 STR loci	Archived with journal
Mutation analysis (IF APPLICABLE)	Sanger sequencing	Heterozygous *ACTA1* mutation; NM_001100: c.541G>A	Fig. 1, panel I
	Southern Blot OR WGS	Not performed	
Microbiology and virology	Mycoplasma	Negative by PCR	Supplementary Fig. 1, panel D
Differentiation potential	Directed differentiation, qPCR	Enrichment of *TBXT* and *BMP4* (mesoderm), *GATA4* and *SOX17* (ectoderm), and *OTX2* and *PAX6* (endoderm)	Fig. 1, panel E
	Directed differentiation, immunocytochemistry	Positive for OTX2 (ectoderm), Brachyury (mesoderm), GATA4 (endoderm)	Supplementary Fig. 1, panel A/B
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

3.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was reverse transcribed into cDNA using the SuperScript™ III First-Strand Synthesis System (ThermoFisher). qRT-PCR was performed using the Rotor-Gene SYBR Green RT-PCR Kit (QIAGEN) on a Rotor-Gene Q thermocycler. Cycling conditions were as follows: 95 °C for 5 min, 45 cycles of 95 °C for 10 sec and 60 °C for 15 sec (acquiring), followed by melt curve analysis (60 °C to 95 °C, 1°C/step). *GAPDH* was used for normalization, using the ΔC_T method. Primers are listed in Table 3.

3.5. Polymerase chain reaction (PCR)

EBV genes (*OriP*, *EBNA1*, *LMP1* and *BLZF1*) and a reference gene (*GAPDH*) were detected by PCR using GoTaq® G2 Hot Start Master Mix (Promega) with primers from Barrett et al., 2014. Presence of Sendai virus genome and transgenes were assessed by RT-PCR at passage 10 as per the CytoTune™ manufacturer's protocol. Transduced LCLs (day 3) were used as a positive control. Primers are listed in Table 3.

3.6. Karyotyping

iPSCs were screened for the 8 most common karyotypic abnormalities at P10 using the hPSC Genetic Analysis kit (StemCell) on a ViiA 7 system (Applied Biosystems). Karyostat analysis (P10) was performed by the Ramaciotti Centre for Genomics (Sydney, NSW, Australia). Data were analyzed using Chromosome Analysis Suite 4.2 (ThermoFisher). G-banding (P14) was performed at PathWest Diagnostic Genomics (Perth, WA, Australia). At least 8 metaphase spreads were analyzed per cell line.

3.7. Confirmation of pathogenic ACTA1 variant

ACTA1 exon 4 was amplified by PCR using GoTaq® G2 Hot Start Master Mix (Promega) on a BioRad C1000™ Thermal Cycler. Sanger sequencing was performed by the Australian Genome Research Facility (Perth, WA, Australia). Chromatograms were analyzed using Benchling (benchling.com). Primers are listed in Table 3.

3.8. Mycoplasma testing

Lines were screened for mycoplasma (P10) using the ATCC Universal Mycoplasma PCR test kit.

3.9. Short tandem repeat typing

STR typing was performed by PathWest Diagnostic Genomics (Perth, WA, Australia) using the QSTR Plus assay (Elucigene).

3.10. Graphs and statistics

Graphs and statistics were generated using GraphPad Prism v8.0.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Table 3
Reagent details.

Antibodies used for immunocytochemistry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	Rabbit anti-OCT4	1:200	Thermo Fisher Scientific Cat# A24867, RRID: AB_2650999
Pluripotency marker	Mouse anti-SSEA4	1:100	Thermo Fisher Scientific Cat# A24866, RRID: AB_2651001
Pluripotency marker	Rat anti-SOX2	1:100	Thermo Fisher Scientific Cat# A24759, RRID: AB_2651000
Pluripotency marker	Mouse anti-TRA-1–60	1:100	Thermo Fisher Scientific Cat# A24868, RRID: AB_2651002
Secondary antibody	Alexa Fluor™ 594 donkey anti-rabbit	1:250	Thermo Fisher Scientific Cat# A21207, RRID: AB_141637
Secondary antibody	Alexa Fluor™ 488 goat anti-mouse IgG3	1:250	Thermo Fisher Scientific Cat# A24877, RRID: AB_2651008
Secondary antibody	Alexa Fluor™ 488 donkey anti-rat	1:250	Thermo Fisher Scientific Cat# A24876, RRID: AB_2651007
Secondary antibody	Alexa Fluor™ 594 goat anti-mouse IgM	1:250	Thermo Fisher Scientific Cat# A21044, RRID: AB_2535713
Differentiation marker (ectoderm)	Anti-human Otx-2 NL557-conjugated goat IgG	1:10	R&D systems Cat# SC022, Part# 967389, RRID: Not in database
Differentiation marker (mesoderm)	Anti-human Brachyury NL557-conjugated goat IgG	1:10	R&D systems Cat# SC022, Part# 967388, RRID: Not in database
Differentiation marker (endoderm)	Anti-human GATA-4 NL493-conjugated goat IgG	1:10	R&D systems Cat# SC022, Part# 967391, RRID: Not in database

Primers

	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers (qPCR)	*OCT4*	F: GGGTTTTTGGGATTAAGTTCTTCA R: GCCCCCACCCTTTGTGTT
	SOX2	F: CAAAAATGGCCATGCAGGTT R: AGTTGGGATCGAACAAAAGCTATT
	NANOG	F: ACAACTGGCCGAAGAATAGCA R: GGTTCCCAGTCGGGTTCAC
	CRIPTO	F: CGGAACTGTGAGCACGATGT R: GGGCAGCCAGGTGTCATG
Mesoderm markers (qPCR)	*TBXT*	F: GGTCCAGCCTTGGAATGCCT R: CCGTTGCTCACAGACCACAG
	BMP4	F: GCACTGGTCTTGAGTATCCTG R: TGCTGAGGTTAAAGAGGAAACG
Endoderm markers (qPCR)	*SOX17*	F: GTGGACCGCACGGAATTTGA R: GCTGTCGGGGAGATTCACAC
	GATA4	F: CAGCGAGGAGATGCGTCC R: AGACATCGCACTGACTGAGAA
Ectoderm markers (qPCR)	*OTX2*	F: GACCCGGTACCCAGACATCTT R: GCGGCACTTAGCTCTTCGATT
	PAX6	F: AACGATAACATACCAAGCGTGT R: GGTCTGCCCGTTCAACATC
House-keeping Genes (qPCR)	*GAPDH*	F: TCGGAGTCAACGGATTTGGT R: TTGCCATGGGTGGAATCATA
Sendai virus vectors (RT-PCR)	*SeV genome*	F: GGATCACTAGGTGATATCGAGC R: ACCAGACAAGAGTTTAAGAGATATGTATC
	KOS transgene	F: ATGCACCGCTACGACGTGAGCGC R: ACCTTGACAATCCTGATGTGG
	Klf4 transgene	F: TTCCTGCATGCCAGAGGAGCCC R: AATGTATCGAAGGTGCTCAA
	c-Myc transgene	F: TAACTGACTAGCAGGCTTGTCG R: TCCACATACAGTCCTGGATGATGATG

Table 3 (*continued*)

Antibodies used for immunocytochemistry

	Antibody	Dilution Company Cat # and RRID
EBNA testing (PCR)	*BZLF-1*	F: CACCTCAACCTGGAGACAAT R: TGAAGCAGGCGTGGTTTCAA
	LMP1	F: ATGGAACACGACCTTGAGA R: TGAGCAGGATGAGGTCTAGG
	EBNA1	F: ATCAGGGCCAAGACATAGAGA R: GCCAATGCAACTTGGACGTT
	OriP	F: TCGGGGGTGTTAGAGACAAC R: TTCCACGAGGGTAGTGAACC
House-keeping Genes (PCR)	*GAPDH*	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA
Targeted mutation analysis (PCR/sequencing)	*ACTA1* exon 4 (F primer used for sequencing)	F: TAGCGCTGAGAGCCTAGCC R: CTGTGGTCACGAAGGAGTAGC

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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.2021.102482.

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