Stem Cell Research 39 (2019) 101485

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Multiple Cell Lines

Generation of three induced pluripotent stem cell lines from postmortem tissue derived following sudden death of a young patient with *STXBP1* mutation

Takuma Yamamoto[a,*,1,2], Makoto Otsu[b,1], Takashi Okumura[b], Yumi Horie[b], Yasuharu Ueno[c], Hideki Taniguchi[b,c], Manami Ohtaka[d,e], Mahito Nakanishi[d,e], Yuki Abe[a], Takehiko Murase[a], Takahiro Umehara[a], Kazuya Ikematsu[a]

[a] *Division of Forensic Pathology and Science, Unit of Social Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan*

[b] *Division of Stem Cell Processing/Stem Cell Bank, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*

[c] *Division of Regenerative Medicine, Center for Stem Cell Biology and Regenerative Medicine, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan*

[d] *Biotechnology Research Institute for Drug Discovery, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, Japan*

[e] *TOKIWA-Bio Inc., G-4 Tsukuba-TCI, 2-1-6 Sengen, Tsukuba, Ibaraki 305-0047, Japan*

ABSTRACT

We established three iPSC lines from postmortem-cultured fibroblasts derived following the sudden unexpected death of an 8-year-old girl with Lennox-Gastaut syndrome, who turned out to have the R551H-mutant *STXBP1* gene. These iPSC clones showed pluripotent characteristics while retaining the genotype and demonstrated trilineage differentiation capability, indicating their utility in disease-modeling studies, *i.e.*, *STXBP1*-encephalopathy. This is the first report on the establishment of iPSCs from a sudden death child, suggesting the possible use of postmortem-iPSC technologies as an epoch-making approach for precise identification of the cause of sudden death.

Resource table.

Unique stem cell lines identifier	IMSUTi001-A IMSUTi001-B IMSUTi001-C
Alternative names of stem cell lines	TkSSD1#AA (IMSUTi001-A) TkSSD1#AB (IMSUTi001-B) TkSSD1#AM (IMSUTi001-C)
Institution	The Institute of Medical Science, The University of Tokyo, Tokyo, Japan Division of Forensic Pathology and Science, Nagasaki University School of Medicine, Nagasaki, Japan
Contact information of distributor	Takuma Yamamoto, tk-yamamoto@hyo-med.ac.jp Takashi Okumura, tokumura@ims.u-tokyo.ac.jp
Type of cell lines	iPSC
Origin	Human
Cell source	Skin fibroblasts
Clonality	Clonal
Method of reprogramming	Transgene free, Sendai virus vector
Multiline rationale	Isogenic clones

* Corresponding author at: Division of Forensic Pathology and Science, Unit of Social Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

E-mail address: tk-yamamoto@hyogo-med.ac.jp (T. Yamamoto).

[1] These authors contributed equally.

[2] Present address: Department of Legal Medicine, Hyogo College of Medicine, 1–1 Mukogawa-cho, Nishinomiya, Hyogo, Japan.

https://doi.org/10.1016/j.scr.2019.101485

Received 10 May 2019; Received in revised form 10 June 2019

Available online 18 June 2019

Gene modification	Yes
Type of modification	Congenital
Associated disease	*STXBP1* encephalopathy
Gene/locus	*STXBP1*/9q34.11 (c.1652G > A)
Method of modification	Not applicable
Name of transgene or resistance	Not applicable
Inducible/constitutive system	Not applicable
Date archived/stock date	2018/6/28
Cell line repository/bank	https://hpscreg.eu/cell-line/IMSUTi001-A https://hpscreg.eu/cell-line/IMSUTi001-B https://hpscreg.eu/cell-line/IMSUTi001-C
Ethical approval	Written informed consent obtained from the family Ethics Committee approval obtained by the Nagasaki University Graduate School of Medicine (15062620 and 150622325) and the Institute of Medical Science, University of Tokyo (#25-3-0701 and #30-8-A0522).

Resource utility

STXBP1 encephalopathy is a hereditary disorder causing various phenotypes of epilepsy, sometimes leading to sudden unexpected death in epilepsy. The established iPSC lines retain the heterozygous R551H-*STXBP1* genotype. Neuronal cells differentiated from them are expected to verify the connection between the genotype and the pathology in *STXBP1* encephalopathy.

Resource details

STXBP1 encephalopathy is a hereditary disorder causing various phenotypes such as Lennox-Gastaut syndrome and Ohtahara syndrome, sometimes leading to sudden unexpected death in epilepsy (SUDEP) (Stamberger et al., 2016). The patient in this study is an 8-year-old girl, found dead suddenly and unexpectedly. At postmortem examination the R551H-*STXBP1* gene variant was detected as a heterozygous state (Supplementary Fig. 1). This cell line can offer a useful resource to investigate pathogenic mechanisms in *STXBP1* encephalopathy (Table 1).

Postmortem cultured fibroblasts were reprogrammed by Sendai virus vector containing four Yamanaka factor genes. Written informed consent was obtained from the family. We succeeded in generating three clones (#1, #2, and #3) of induced pluripotent stem cells (iPSCs), each showing a typical pluripotent cell colony-like appearance (Fig. 1A). STR analysis revealed that the original fibroblasts and iPSCs were genetically identical. All clones are confirmed to be mycoplasma-free (Supplementary Fig. 2). The iPSC clones expressed both TRA-1-60 and TRA-1-81 pluripotency-associated markers (Fig. 1B). Flow cytometric analysis revealed expression of pluripotency-associated cell surface antigens SSEA4 (Fig. 1C). By quantitative gene expression analysis, all clones exhibited expression of the *OCT4* and *NANOG* pluripotent marker genes (Fig. 1D). Furthermore, all iPSC clones were shown to be capable of 3 germ layer differentiation, as shown in Fig. 1E. Karyotype analysis showed some abnormalities in clones #1 and #3, but none in clone #2 (Fig. 1F, Table 2). Most importantly, all iPSC clones retained the R511H-heterozygous *STXBP1* variant genotypes, as in the original fibroblasts and patient blood cells (Fig. 1G).

Here, we propose the possibility of new application with the use of iPSCs in a case of sudden unexpected death in childhood. The cause of SUDEP is yet uncovered, for diagnosis of these conditions or diseases requires analysis of living cells, but viable cells generally cannot be derived from postmortem samples obtained at autopsy because of postmortem changes. Applying postmortem iPSC technology would enable recreation of lifetime conditions even in the postmortem period and discovery of inherited functional diseases (Hjelm et al., 2011). To the best of our knowledge, this represents the first “Revival autopsy” of utilizing iPSCs derived from a sudden death patient. As iPSCs derived postmortem retained the heterozygous R551H-*STXBP1* genotype, neuronal cells differentiated from them are expected to show cellular abnormalities, which should allow verification of the connection between the genotype and the pathology, helping the diagnosis of the cause of sudden death in this case.

Materials and methods

Fibroblast culture

Dermis was cut at autopsy of an 8-year-old girl with Lennox-Gastaut syndrome and cultured as previously described (Yamamoto et al., 2012).

Extraction of genomic DNA

Genomic DNA was isolated from blood leukocytes, which were also obtained at autopsy, and frozen fibroblasts using a QIAamp DNA Mini Kit (Qiagen).

Next-generation sequencing

A TruSight One sequencing panel was used and sequencing was performed on a MiSeq System, using Variant Studio version 2.2.3 software (all Illumina).

Establishment of iPSCs and cell culture

The iPSCs were established in a similar manner to that previously described (Nishimura et al., 2017). A newer version of the Sendai virus vector (developed by Mahito Nakanishi, unpublished) was used, that could deliver a single-stranded RNA genome containing four Yamanaka factor genes into somatic cells. Fibroblasts were infected with the Sendai virus vector, seeded on iMatrix-511 (Takara Bio)-coated 6-well plates in StemFit AK02N medium (REPROCELL), followed by expansion culture of picked-up colonies. All iPSC lines were maintained at 37°C, 5% CO_2 on vitronectin (VTN-N, Gibco)-coated 6-well plates in Essential 8™ (E8) medium (Gibco) with daily media changes. For passaging, cells

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
TkSSD1#AA (IMSUTi001-A)	TkSSD1-#1	Female	8	Asian	CGC/CAC	*STXBP1* encephalopathy
TkSSD1#AB (IMSUTi001-B)	TkSSD1-#2	Female	8	Asian	CGC/CAC	*STXBP1* encephalopathy
TkSSD1#AM (IMSUTi001-C)	TkSSD1-#3	Female	8	Asian	CGC/CAC	*STXBP1* encephalopathy

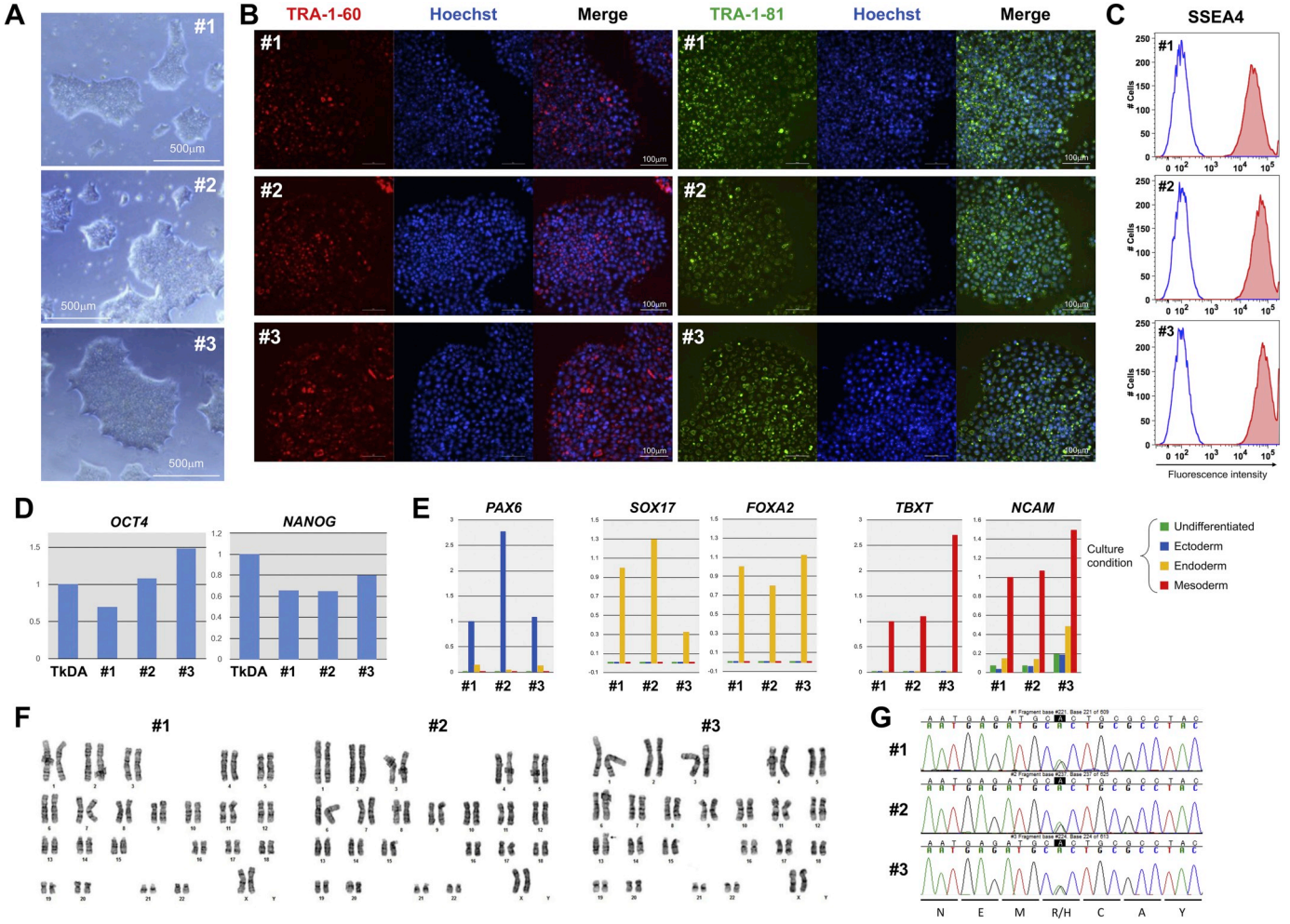

Fig. 1. Characterization of three iPSC clones to confirm their utility in disease-modeling studies. (A) Phase contrast images of three iPSC clones (#1, #2, and #3) showing typical pluripotent cell colony-like appearance. Scale bars = 500 μm. (B) Immunofluorescence images of three iPSC clones demonstrating expression of the TRA-1-60 and TRA-1-81 pluripotency-associated markers (red). Hoechst staining of cell nuclei is shown in blue. Scale bars = 100 μm. (C) Flow cytometric analysis reveals expression of the SSEA4 pluripotency-associated antigen on cell surface of virtually 100% of iPSCs. (D) Quantitative gene expression analysis. All clones in an undifferentiated culture condition exhibit expression of the pluripotent marker genes, OCT4 and NANOG. (E) After cultivation under different conditions, selective upregulation patterns are evident for each marker gene in accordance with the corresponding culture condition (with differentiation for an ectodermal lineage, blue; an endodermal lineage, yellow; a mesodermal lineage, red) in all the iPSC clones. (F) Karyotype analysis shows some abnormalities in clones #1 (1 in 20 analytes) and #3 (all 20 analyses), but none in clone #2. Detailed information is in Table 1. (G) Sanger sequencing results showing that all iPSC clones retain the R511H-heterozygous STXBP1 variant genotype, as in the original fibroblasts and patient blood cells (Supplementary Figure 1).

(~50% confluent) were dissociated with 0.5 mM EDTA in PBS at 37°C, 5 min (Invitrogen) in PBS, and split to 1:3–1:5 every 3–4 days.

Mycoplasma detection

All clones were confirmed to be mycoplasma-free, using the MycoAlert™ Mycoplasma Detection Kit and MycoAlert™ Assay Control Kit (Lonza). Images of iPSC colonies were captured with a Leica DMI3000B system (Leica).

Immunofluorescence staining

Live iPSC colonies in 48-well culture plates were first incubated with Hoechst 33342 (ThermoFisher Scientific) to counterstain the nuclei. One day later, iPSC colonies were then directly stained for 90 min at 37°C with the antibodies against TRA-1-60 or TRA-1-81 diluted in E8 medium (Table 3). After washing with PBS, fluorescence images were captured with an Eclipse Ti microscope system (Nikon). Images were edited using ImageJ (NIH) or GIMP2.10 (https://www.gimp.org/).

Flow cytometry

Cells harvested with Accutase (STEM CELL Technologies) were incubated for 10 min at 4°C with either anti-SSEA4 or isotype control antibody (Table 3) diluted in PBS containing 1% fetal bovine serum (Nichirei Biosciences). Data were acquired using a fluorescence-activated cell sorting Aria II sorter (BD Biosciences) and analyzed using FlowJo X software (Tree Star).

Trilineage differentiation

To analyze the capability of iPSCs to differentiate into three germ layers, *in vitro* differentiation was performed using the STEMdiff™ Trilineage Differentiation Kit (STEM CELL Technologies) according to the instructions from the manufacturer. Marker genes analyzed were paired box 6 (*PAX6*), SRY-box 17 (*SOX17*), Foxhead box A2 (*FOXA2*), T-box transcription factor T (or Brachyury) (*TBXT*), and neural cell adhesion molecule (*NCAM*).

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1A
Phenotype	Qualitative analysis: immunocytochemistry	Positive staining for pluripotency markers: TRA-1-60 and TRA-1-81	Fig. 1B
	Quantitative analysis: flow cytometry	~100% cells showing high-level expression of SSEA4	Fig. 1C
	Quantitative RT-qPCR	Expression of pluripotency markers: *OCT4* and *NANOG*	Fig. 1D
Genotype	Karyotype (G-banding), resolution 300–500	#1: 46,XX [19]; 46,XX, add(1)(p36.3),add(8)(q11.2) [1] #2: 46,XY [20] #3: 46,XY,add(13)(p11.2) [20]	Fig. 1F
Identity	Microsatellite PCR	N/A	N/A
	STR analysis	10 loci tested, all matched	Available with the authors
Mutation analysis	Sanger sequencing	Heterozygous D551H-*STXBP1* (c.1652G > A)	Fig. 1G, Supplementary Fig. 1
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Negative by MycoAlert™ Mycoplasma detection kit	Supplementary Fig. 2
Differentiation potential	Directed differentiation	Expression of lineage markers confirmed after directed differentiation: ectoderm (*PAX6*), mesoderm (*TBXT, NCAM*), and endoderm (*SOX-17, FOXA2*).	Fig. 1E
Donor screening	HIV 1 + 2, Hepatitis B, Hepatitis C	Tested during autopsy and negative	Available with the authors
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

RT-qPCR

Total RNA was isolated from iPSCs either before or after differentiation, using the RNeasy® Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To quantify the pluripotency markers octamer-binding transcription factor 4 (OCT4) and NANOG expression, multiplex TaqMan real-time PCR analysis was conducted using the Eagle Taq Master Mix with ROX (Roche). Universal probes #34 and #69 (Roche) were used for labeling the *OCT4* and *NANOG* products, respectively. The Human G6PD Assay (Roche) was used for quantifying the reference gene. The relative expression of *OCT4* and *NANOG* was evaluated with reference to their expression levels in our standard hiPSC line TkDA3-4 (TkDA) previously established from a healthy donor (Takayama et al., 2010). Quantification of differentiation markers was conducted using a real-time PCR SYBR Green method with the THUNDERBIRD® SYBR qPCR Mix (Toyobo). Expression of the genes was normalized to *G6PD*. All the real-time PCR analyses were performed using a CFX96 C1000™ thermal cycler (Bio-Rad). Relative expression was calculated by the ΔΔCt method with CFX™ Manager software (Bio-Rad). Primers used are listed in Table 3.

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker (flow cytometry)	Mouse anti-SSEA4, APC-conjugated	1:200	Biolegend cat. # 330418, RRID:AB_2616819
Isotype control (flow cytometry)	Mouse IgG3, kappa, Alexa Fluor 647-conjugated	1:2000	Biolegend cat. # 401321, RRID:AB_10683445
Pluripotency markers	Mouse anti-TRA-1-60, NL(NorthernLights™)557-conjugated (MAB4770)	1:50	R&D Systems cat. # NLLC4770R, RRID:AB_2773722
	Mouse anti-TRA-1-81, NL(NorthernLights™)493-conjugated (MAB8495)	1:50	R&D Systems cat. # NLLC16581G, RRID:AB_2773721

Primers

	Target	Forward/Reverse primer (5′-3′)
Pluripotency markers (qRT-PCR)	*OCT4*	TGCAGAAAGAACTCGAGCAA / AGATGGTCGTTTGGCTGAAT
	NANOG	ATGCCTCACACGGAGACTGT / CAGGGCTGTCCTGAATAAGC
House-keeping gene (qRT-PCR)	*G6PD*	Primers from the Universal ProbeLibrary Human G6PD Assay (Roche)
Trilineage markers (qRT-PCR)	*PAX6*	GTCCATCTTTGCTTGGGAAA / TAGCCAGGTTGCGAAGAACT
	TBXT	AATTGGTCCAGCCTTGGAAT / CGTTGCTCACAGACCACA
	NCAM	ATGGAAACTCTATTAAAGTGAACCTG / TAGACCTCATACTCAGCATTCCAGT
	SOX17	CAGCAGAATCCAGACCTGCA / GTCAGCGCCTTCCACGACT
	FOXA2	GGAGCGGTGAAGATGGAA / TACGTGTTCATGCCGTTCAT
Targeted mutation sequencing	*STXBP1* c.1652G > A (691 bp)	GAACCAATCCACTTGAAAAT / CAAGGCTGTGAATGAGTATG

Karyotype analysis

Standard G-banded karyotyping was conducted by the NIHON Gene Research Laboratories (passage 8 for clones #1 and #2, passage 10 for clone #3).

Identification and verification of iPSCs

The identities of iPSCs and fibroblasts were confirmed by STR analysis and Sanger sequencing. Genomic DNA was isolated from iPSCs and fibroblasts using QIAamp DNA Mini Kit (Qiagen) in accordance with the manufacturer's standard methods. STR analysis was performed at TAKARA Bio using the GenePrint® 10 System (Promega).

Declaration of Competing Interest

None.

Acknowledgements

We wish to thank Hiroko Ohkura, Chen-Yi Lai, Akiko Kikuchi, Huan-Ting Lin, and Chisako Tsujita for their technical assistances. This work was supported in part by a grant from The Ministry of Education, Culture, Sports, Science and Technology of Japan (18K19712).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101485.

References

Hjelm, B.E., Rosenberg, J.B., Szelinger, S., et al., 2011. Induction of pluripotent stem cells from autopsy donor-derived somatic cells. Neurosci. Lett. 502, 219–224.

Nishimura, K., Ohtaka, M., Takada, H., et al., 2017. Simple and effective generation of transgene-free induced pluripotent stem cells using an auto-erasable Sendai virus vector responding to microRNA-302. Stem Cell Res. 23, 13–19.

Stamberger, H., Nikanorova, M., Willemsen, M.H., et al., 2016. STXBP1 encephalopathy: a neurodevelopmental disorder including epilepsy. Neurology 86, 954–962.

Takayama, N., Nishimura, S., Nakamura, S., et al., 2010. Transient activation of *c-MYC* expression is critical for efficient platelet generation from human induced pluripotent stem cells. J. Exp. Med. 207, 2817–2830.

Yamamoto, T., Emoto, Y., Murayama, K., et al., 2012. Metabolic autopsy with post-mortem cultured fibroblasts in sudden unexpected death in infancy: diagnosis of mitochondrial respiratory chain disorders. Mol. Genet. Metab. 106, 474–477.