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Lab resource: Stem Cell Line

Generation of induced pluripotent stem cells (NIMHi001-A) from a Parkinson's disease patient of East Indian ethnicity carrying LRRK2 I1371V variant

Indrani Datta[a,*], Sowmithra[a], Soham Jagtap[a], Chandrakanta Potdar[a], Ravi Yadav[b], Pramod Pal[b]

[a] Department of Biophysics, National Institute of Mental Health and Neurosciences, Bengaluru 560029, Karnataka, India
[b] Department of Neurology, National Institute of Mental Health and Neurosciences, Bengaluru 560029, Karnataka, India

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ABSTRACT

Mutations in the Leucine Repeat Rich Kinase-2 (LRRK2) gene have been reported in familial Parkinson's disease (PD) cases. We have generated induced pluripotent stem cells (iPSCs) using Sendai-virus reprogramming-method from peripheral blood mononuclear cells of PD-patient of East-Indian ethnicity carrying the I1371V mutation in LRRK2 gene. PD diagnosis was performed using Unified Parkinson's Disease rating scale (UPDRS) score and confirmed by [^{18}F]fluoro-L-dopa [F-DOPA] positron emission tomography (F-DOPA PET). The iPSC line was characterized for self-renewal and pluripotency. This cellular model will provide a valuable resource not only for drug-screening platform but also to understand the pathophysiology of this disease.

Resource table

Unique stem cell lines identifier	NIMHi001-A
Alternative names of stem cell lines	BPPD01
Institution	National Institute of Mental Health and Neurosciences (NIMHANS)
Contact information of distributor	Dr. Indrani Datta, indranidatta.nimhans@gmail.com
Type of cell lines	iPSC
Origin	Human
Cell Source	Blood
Clonality	Clonal
Method of reprogramming	Transgene free (Cytotune™ iPS 2.0 Sendai reprogramming kit)
Multiline rationale	Parkinson's disease, age and gender matched healthy control
Gene modification	Yes
Type of modification	Familial
Associated disease	Parkinson's disease
Gene/locus	Leucine rich repeat kinase (LRRK2)/chr 12q12
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	01-10-2018
Cell line repository/bank	NA
Ethical approval	Institutional Committee for Stem Cell Research (IC-SCR), NIMHANS Institutional Animal Ethics Committee (IAEC), NIMHANS Institutional Ethics Committee (IEC), NIMHANS

* Corresponding author.
E-mail addresses: indranidatta.nimhans@gmail.com, indrani@nimhans.kar.nic.in (I. Datta).

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1. Resource utility

The generated iPSC line (NIMHi001-A) can serve as the master cell-bank of Indian-ethnicity LRRK2 I1371V PD patient for disease modeling of dopaminergic-neurons and cell-types of other lineages which get affected in PD and hence providing a platform for potential therapeutic explorations such as drug development and discovery and toxicology screening.

2. Resource details

Parkinson's disease (PD) is a debilitating neurodegenerative-disorder characterized by muscle-rigidity, bradykinesia, and tremor. Due to overlap of symptoms with other movement-disorders, it is necessary to follow an effective regime of diagnosis. In the present study, the PD patient was clinically evaluated using Unified-Parkinson's Disease rating-scale (UPDRS) and striatal-dopamine was confirmed by [^{18}F] fluoro-L-dopa Positron Emission Tomography (F-DOPA PET), which measures uptake and trapping of dopamine-precursors in nigrostriatal-projections and has been used as a surrogate outcome measure for disease-progression in clinical-trials (Whone et al., 2003). None of the earlier iPSC lines reported in PD have used the confirmatory F-DOPA PET scanning procedure - a lacuna that we address in this line.

Mutations in the leucine-rich repeat-kinase2 gene (LRRK2) are recognized as the most common cause of genetic-PD till date, with great variability in carrier rates in both familial and sporadic forms dependent on geographical-distribution (Spielman et al. 2007). To date, the clinical implications of carrying a LRRK2 mutation are still unknown, although studies report a high risk of developing PD in specific-populations (Ozelius et al., 2006). For LRRK2 sporadic cases, the frequency is around 1% in patients with Caucasian-ancestry.

Indian population has distinct genetic ancestries due to cultural and demographic differences. Recent studies have shown that the peopling of India has a much more complicated history that has also been reshaped by cultural and demographic events. Evidence shows that populations of mainland India have at least four distinct genetic-ancestries (Basu et al., 2016). Sociocultural and geographical differences may influence nutrition and environmental-issues between different ethnicities. Therefore, it is not surprising that the prevalence of PD has been reported to vary between different-ethnicities across the world (). The penetrance of mutations in late-onset Parkinsonism is also dependent on ethnicity and potential environmental-factors. Thus, heterogeneity among mutation-carriers may be an important consideration when identifying modifiers of disease.

In this study, a human iPSC-line (NIMHi001-A) was established from peripheral-blood mononuclear-cells (Fig. 1A) of a 59-year-old male patient carrying a heterozygous missense-mutation in exon 29 of the LRRK2-gene (chr12:40702420A > G; Depth: 69x) that results in the amino-acid substitution of Valine for Isoleucine at codon-1371 (p.Ile1371Val). After clinical diagnosis through UPDRS scale confirmation of disease onset was detected using F-DOPA PET (Table 1, Supplementary Fig. 1A) and mutation analysis was performed through Targeted gene analysis and Sanger sequencing (Table 1; Supplementary Fig. 1B and C). A non-integrating CytoTune-Sendai viral-vector kit (A16517, Thermo Fisher Scientific) containing OCT3/4, KLF4, SOX2 and C-MYC pluripotency transcription factors was employed to transduce the PBMCs (Fig. 1A) using the method described previously (Beers et al., 2012&2015). The resulting iPSC-line (NIMHi001-A) exhibited a classical induced pluripotent stem cell morphology (Fig. 1B), normal karyotype (46, XY) as confirmed by the G-banding karyotype at passage 7 (Fig. 1E), and expressed the major pluripotent protein markers of NANOG, SOX2, OCT4, SSEA4 and TRA-1-60 confirmed by gene expression (Fig. 1H), immunofluorescence staining (Fig. 1C) and flow-cytometry analysis (Fig. 1G). NIMHi001-A was further positive for Blue imaging and alkaline phosphatase staining (Fig. 1D and F). This iPSC-line was not contaminated with mycoplasma (Supplementary Fig. 1D) and short tandem repeat (STR) DNA analysis was performed to confirm the cell identity, both of which were matched 100% with the parental PBMCs (Supplementary Fig. 1F). The viral marker expression in NIMHi001-A was non-detectable from Passage 1 (Supplementary Fig. 1E). Furthermore, pluripotency of this iPS cell line was confirmed by experiments that exhibited its ability to differentiate into cells of all three germ layers (Ectoderm, neural tube and pigmented epithelium; Mesoderm, cartilage; Endoderm, gut) through spontaneous differentiation of the Embryoid bodies generated from the iPSC line (Fig. 1I–K). In conclusion, the NIMHi001-A iPSC line will provide a useful platform for the scientific research of neurodegeneration in Parkinson's disease.

3. Methodology

3.1. Subject selection and clinical assessment

The patient was clinically assessed at the NIMHANS out-patient department. The severity of PD onset was determined using the Unified Parkinson's Disease Rating Scale-III. The clinical diagnosis was confirmed by Fluro-DOPA PET scan at HCG hospital, Bengaluru, India. Targeted gene sequencing for a panel of 13 genes identified as risk factors or mutations associated with PD pathogenesis was performed using the peripheral blood sample, outsourced to Medgenome Labs Ltd., Bengaluru, India.

3.2. PBMC isolation and reprogramming

This study was approved by the Institutional Committee of Stem Cell Research (IC-SCR) NIMHANS and the sanction number is SEC/01/005/BP. Blood was collected aseptically in K2EDTA treated vaccutainer tubes (Cat. No. 367861) and PBMCs were separated using density gradient separation media HiSep LSM density 1073 1.0770 ± 0.0010 g/ml according to the manufacturer's instructions. PBMCs were plated StemPro®34 (Gibco 10639-011) medium containing 100 ng/mL SCF (Gibco PHC2111)), 100 ng/mL FLT3 (Gibco PHC9414), 20 ng/mL IL-3 (Gibco PHC0034) and 10 ng/mL IL-6 (Gibco PHC0065).

3.3. iPSC generation

The isolated PBMCs were reprogrammed using CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific Catalog. No. A16517) with MOI (multiplicity of infection) as hKlf4 MOI = 3, hc-Myc MOI = 5 and KOS MOI = 5. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 for 2 days. On 3rd day the cells were transferred on mitomycin inactivated mouse embryonic fibroblast (MEF) feeder layer. The cells were incubated in complete iPSC medium comprising of DMEM/F12 with 20% Knockout serum replacement (Gibco Cat. No. 10828028), 1% Glutamax(Gibco Cat. No. 35050061), 1% non-essential amino acid solution (Gibco Cat. No. 11140050), 0.1 mM β-mercaptoethanol (Gibco Cat. No. 31350010), 8 ng human basic fibroblast growth factor (Immunotools Cat. No. 11343627) and 1% penicillin streptomycin (Gibco Cat. No. 15070063).

3.4. iPSC maintenance

The iPSC lines were incubated in a 37 °C incubator with a humidified atmosphere of 5% CO_2. Undifferentiated iPSCs colonies were subcultured either by picking manually or with StemPro™ Accutase™ (Gibco Cat. No. A1110501) and transferred onto fresh MEF culture dishes for expansion. MEF cultures were inactivated using Mitomycin-C (Sigma Cat. No. M4287-5X2MG) at a concentration of 10 μg/ml for 2.5 h at 37 °C prior to plating.

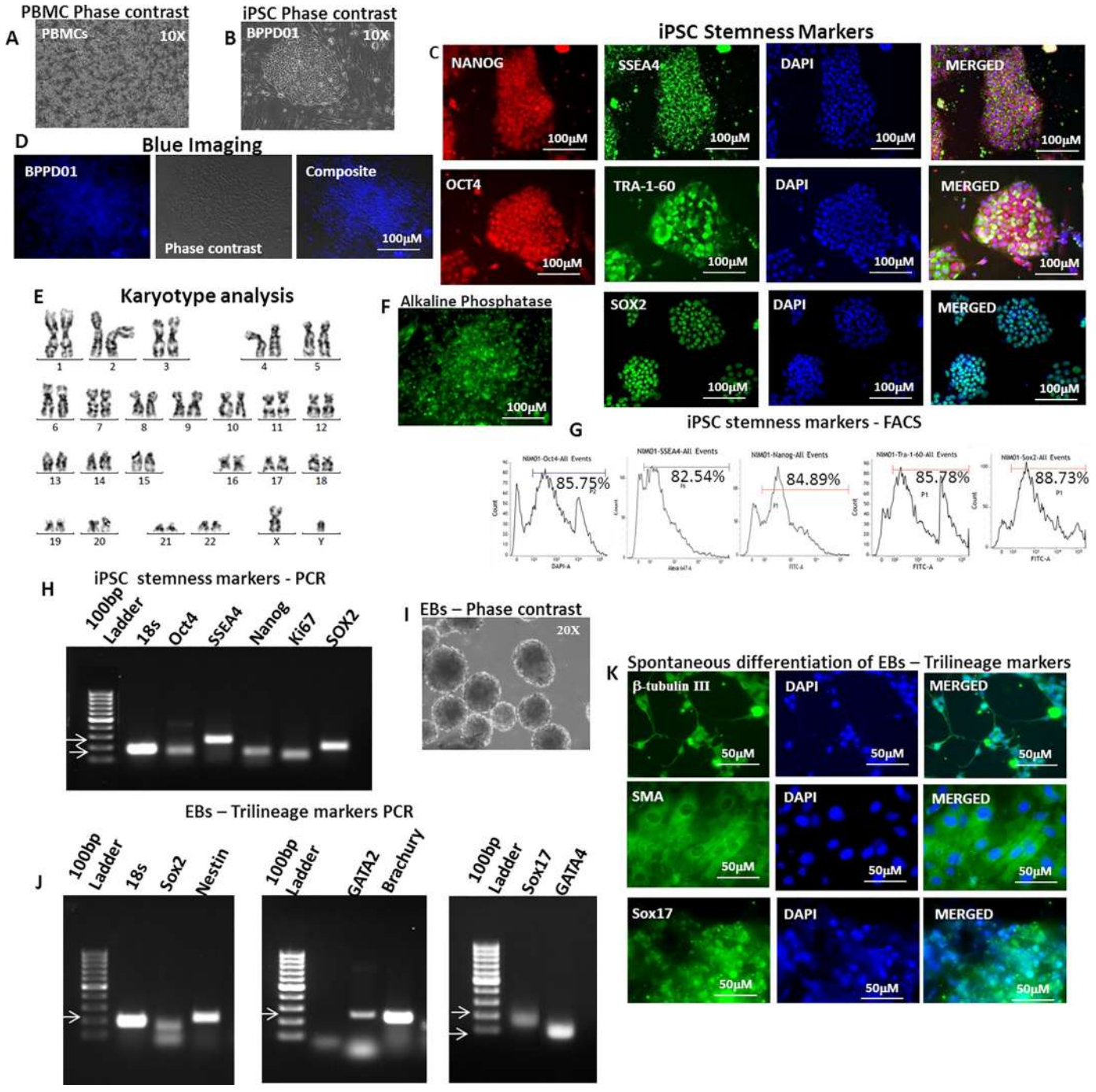

Fig. 1. Characterization of NIMHi001-A.

3.5. RT-PCR

Total RNA was isolated from the cells using TRIzol® reagent (Thermo Fisher Scientific, USA, Cat#15596018) according to the manufacturer's instructions. Reverse complementation was achieved using the Superscript III reverse transcriptase (Thermo Fisher Scientific, USA, Cat#18080093) and PCR was done using the EmeraldAmp® GT PCR Master Mix 2X (TaKaRa Bio Inc., Japan Cat#RR310B) with primers designed against the specific genes mentioned in Table 2. PCR products were analyzed on 1.2% gel in 1X TAE buffer.

3.6. Flow cytometry

The cells were enzymatically detached using StemPro® Accutase (Gibco, Cat#A1110501) and fixed with 2% paraformaldehyde (Sigma, Cat#P6148) in phosphate-buffered saline with 0.01% Sodium azide. The cells were then permeabilized with 0.1% Triton-X and the cells were stained with primary and secondary antibodies for OCT4, SOX2, SSEA4, NANOG, TRA-1-60 as per our previous publication Datta et al., 2013.

3.7. Immunofluorescence

Cells were fixed in 4% paraformaldehyde and then washed twice with PBS with 0.05% Tween-20 (PBST; HiMedia, Cat#MB067). For intracellular antigens cells were permeabilized with 0.1% Triton X-100 (HiMedia, Cat#MB031). Blocking was performed using 3% bovine serum albumin (Sigma–Aldrich, Cat#A3059) in PBST. Primary

Table 1
Characterization and validation.

Classification	Test	Result	Data
Disease onset confirmation	F-DOPA PET scan	Presynaptic striatal dopaminergic deficit in bilateral putamen seen confirming disease onset	Supplementary Fig. 1 panel A
Mutation analysis	Targeted gene analysis Sanger sequencing	Mutation found in LRRK2 p.I1371V, heterozygous	Supplementary Fig. 1 panel B and C
Morphology	Phase contrast microscopy	Normal human embryonic stem cell like morphology of the colonies with well-defined borders	Fig. 1 panel B
Phenotype	Immunostaining Flow cytometry	Expression of pluripotency markers: NANOG, OCT4, SOX2, SSEA-4, TRA-1-60	Fig. 1 panel C and G
Genotype	Karyotype	46XY, no abnormalities seen	Fig. 1 panel E
Identity	Microsatellite PCR STR typing	Matched with the PBMCs from the original donor. 16 locii tested, matched.	Supplementary Fig. 1F
Microbiology and virology	Mycoplasma test	Samples tested negative by PCR	Supplementary Fig. 1D
Differentiation potential	Embryoid body formation	*In vitro* spontaneous differentiation yielded β tubulin III, SMA and SOX17 positive cells	Fig. 1 panel I, J and K

antibody (OCT4, SOX2, SSEA4, NANOG, TRA-1-60, SMA, β tubulin III, SOX17) incubations were done overnight at 4 °C. After PBS wash, cells were incubated with secondary antibody for 45 min at room temperature, and counterstained with 0.5 μg/mL 4′, 6′-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, Cat#D8417) for 15 min. After immunostaining images were captured with a Leica Fluorescence microscope and images were processed with LasX software. The details of antibodies used are given in Table 2.

3.8. Alkaline phosphatase (AP) staining

AP staining was performed using the Molecular Probes® Alkaline Phosphatase Live Stain according to the manufacturer's instructions. Fluorescent-labelled colonies were observed under using a standard FITC filter.

3.9. Blue imaging

The lipid body associated retinyl ester blue fluorescence was acquired first followed by phase contrast images. The images were merged to obtain the final image. Images were captured with Olympus IX71 with a Retiga monochrome camera and Micromanager software. The blue fluorescence was visualized using DAPI filter Cube Olympus EPI-FL filter.

3.10. STR typing

Genomic DNA was isolated from the cells using TRIzol® reagent (Thermo Fisher Scientific, USA, Cat#15596018) according to the manufacturer's instructions. STR profiling was performed for by Medgenome Labs Ltd., Bengaluru, India.

Table 2
Antibodies and primers used in the study.

	Antibody	Dilution	Company Cat. No. and RRID
Pluripotency Marker	Goat anti-OCT4	1:100	Abcam Cat#AB27985 RRID AB_776898
Pluripotency Marker	Rabbit anti-SOX2	1:100	Abcam Cat#AB97959 RRID AB_2341193
Pluripotency Marker	Mouse anti-SSEA4	1:100	BD Biosciences Cat#560073 RRID AB_1645601
Pluripotency Marker	Rabbit anti-NANOG	1:100	Abcam Cat#AB21624 RRID AB_446437
Pluripotency Marker	Mouse anti TRA-1-60	1:100	Abcam Cat#AB16288 RRID AB_778563
Pluripotency Marker (Flow cytometry)	TRITC goat anti-rabbit	1:200	Abcam Cat#AB6718 RRID AB_955551
Pluripotency Marker (Flow cytometry)	FITC goat anti-rabbit	1:200	Abcam Cat#AB6717 RRID AB_955238
Pluripotency Marker (Flow cytometry)	Alexa fluor 488 goat anti-rabbit	1:200	Abcam Cat#AB150077 RRID AB_955238
Pluripotency Marker (Flow cytometry)	Alexa fluor 488 goat anti-mouse	1:200	Abcam Cat#AB150113 RRID AB_2576208
Pluripotency Marker (Flow cytometry)	Alexa fluor 647 goat anti-mouse	1:200	Abcam Cat#AB150115 RRID AB_2687948
Pluripotency Marker (Flow cytometry)	FITC goat anti-rabbit	1:200	Millipore Cat#AP307F RRID AB_92652
Differentiation marker	Mouse anti SOX17	1:100	BD Biosciences Cat# 561590, RID:AB_10717127
Differentiation marker	Mouse anti β tubulin III	1:100	Sigma-Aldrich Cat# T8578, RRID:AB_1841228
Differentiation marker	Mouse anti SMA	1:100	Millipore Cat# CBL171, RRID:AB_2223166

Primers	Target gene	Forward / Reverse primer (5′–3′)
Pluripotent stem cell marker	OCT4 (165 bp)	F: CCTCACTTCACTGCCACTGTA, R: CAGGTTTTCTTTCTCTAGCT
Pluripotent stem cell marker	SOX2 (151 bp)	F: CCCAGCAGACTTCACATGT; R: CCTCCCATTTCCCTCGTTT
Pluripotent stem cell marker	NANOG (272 bp)	F: CCTCTTAAATTTTTTCCTC; R: AAGTGGGTTGTTTGCCTTT
Pluripotent stem cell marker	Ki67 (198 bp)	F: GACATCACGGATCATATG; R: CTCGCATTGACCATTCAA
Pluripotent stem cell marker	SSEA4 (119 bp)	F: TGGACGGGCACAACTTCA; R: GGGCAGGTTCTTGGCACT
Virus detection marker	cMyc (532 bp)	F: CCCGAAAGAGAAAGCGAACCAG; R:TCCACATACAGTCCTGGATGATGATG
Virus detection marker	SeV (181 bp)	F: GGATCACTAGGTGATATCGAGC; R: ACCAGACAAGAGTTTAACACATATGTA
Virus detection marker	Klf4 (410 bp)	F: TTCCTGCATGCCAGAGGACTCCC; R: AATGTATCGAAGGTGCTCAA
Virus detection marker	KOS (528 bp)	F: ATGCACCGCTACGACGTGAGC; R: ACCTTGACAATCCTGATGTGG
Differentiation marker	Nestin (220 bp)	F: AACAGCGACGGAGGTCTCT; R: TTCTCTTGTCCCGCAGACTT
Differentiation marker	Brachury (219bp)	F: ACCCAGTTCATAGCGGTG; R: ATGAGGATTTGCAGGTGG
Differentiation marker	GATA2 (244 bp)	F: TGACTTCTCCTGCATGCAC; R: AGCCGGCACCTGTTGTGC
Differentiation marker	SOX17 (214 bp)	F: AGTGACGACCAGAGCCAG; R: CCTTAGCCCACACCATGA
Differentiation marker	GATA4 (83 bp)	F: GCTATGCGTCTCCCGTCA; R: GTGACTGTCGGCCAAGAC
Housekeeping gene	18S (180 bp)	F: CGGCTACCACATCCAAGGA; R: GCTGGAATTACCGCGGCT
Mycoplasma detection – Mycoplasma marker	(300bp)	F: GTGGGGAGCAAATAGGATTAGA; R: TATCTCTAGAGTCCTCGACATGACTC

3.11. Karyotype

Karyotyping analysis was carried out to check the presence of any chromosomal abnormalities by Anand Diagnostic Laboratory Services, Bengaluru, India.

3.12. Mycoplasma test

Absence of mycoplasma contamination was confirmed by PCR. The iPSCs were cultured for minimum of 48 h and DNA isolation was carried out from the cell culture supernatant. PCR amplification was set for the positive control, negative control and test samples with specific primers mentioned in Table 2. The products were analyzed on 2% agarose gel in 1X TAE buffer.

Conflict of Interest

The authors have declared that no competing interests exist.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scr.2020.101768.

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Lab Resource: Multiple Cell Lines

Generation of induced pluripotent stem cells (NIMHi002-A and NIMHi003-A) from two sporadic Parkinson's disease patient of East Indian ethnicity

Indrani Datta[a,*], Soham Jagtap[a], Sowmithra[a], Ravi Yadav[b], Pramod Kumar Pal[b]

[a] Department of Biophysics, National Institute of Mental Health and Neurosciences, Bengaluru 560029, India
[b] Department of Neurology, National Institute of Mental Health and Neurosciences, Bengaluru 560029, Karnataka, India

A B S T R A C T

Epidemiological studies suggest that about 95% of PD have a sporadic component. We have generated induced pluripotent stem cells (iPSCs) using Sendai-virus reprogramming-method from peripheral blood mononuclear cells of two sporadic PD-patient of East-Indian ethnicity carrying no PD-related gene mutations. PD diagnosis was performed using Unified Parkinson's Disease rating scale (UPDRS) score and confirmed by [^{18}F]fluoro-L-dopa [F-DOPA] positron emission tomography (F-DOPA PET). The iPSC lines were characterized for self-renewal and pluripotency. These generated lines will provide a valuable resource to understand the pathophysiology of this disease and a drug-screening platform.

Unique stem cell lines identifier	NIMHi002-A and NIMHi003-A
Alternative names of stem cell lines	BPPD02 and BPPD03
Institution	National Institute of Mental Health and Neurosciences (NIMHANS)
Contact information of distributor	Dr. Indrani Datta, indranidatta.nimhans@gmail.com
Type of cell lines	iPSC
Origin	Human
Cell Source	Blood
Clonality	Clonal
Method of reprogramming	Transgene free (Cytotune™ iPS 2.0 Sendai reprogramming kit)
Multiline rationale	Parkinson's disease, age and gender matched healthy control
Gene modification	Yes
Type of modification	Sporadic
Associated disease	Parkinson's disease
Gene/locus	NA
Method of modification	NA
Name of transgene or resistance	NA
Inducible/constitutive system	NA
Date archived/stock date	01–10-2018
Cell line repository/bank	NIMHi002-A – https://hpscreg.eu/cell-line/NIMHi002-A NIMHi003-A – https://hpscreg.eu/cell-line/NIMHi003-A
Ethical approval	Institutional Committee for Stem Cell Research (IC-SCR), NIMHANS Institutional Animal Ethics Committee (IAEC), NIMHANS Institutional Ethics Committee (IEC), NIMHANS

1. Resource utility

The generated iPSC lines (NIMHi001-B, C and D) can serve as the master cell-bank of Indian-ethnicity sporadic PD patient for *in vitro* disease modelling to study the pathophysiologic mechanism underlying Parkinson's disease onset and progression thus providing a platform for potential therapeutic studies such as drug development and discovery and toxicology screening.

2. Resource details

Parkinson's disease (PD) is the second most age related neurodegenerative disease affecting approximately 1% of the world population above 60 years. The disease is commonly associated with bradykinesia, impaired ability of voluntary movements and resting tremors. These symptoms overlap with other movement-disorders and hence it is necessary to follow an effective regime of diagnosis. In the present study, we have performed clinical evaluation of the patients with Unified-Parkinson's Disease rating-scale (UPDRS) while the PD onset was confirmed by [^{18}F] fluoro-L-dopa Positron Emission Tomography (F-DOPA PET) which measures uptake and trapping of dopamine-precursors in nigrostriatal projections and has been used as a surrogate outcome

* Corresponding author at: Department of Biophysics, National Institute of Mental Health and Neurosciences, P.B. No – 2900, Hosur Road, Bengaluru 560029, Karnataka, India.
E-mail address: indranidatta.nimhans@gmail.com (I. Datta).

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measure for disease-progression in clinical-trials (Whone et al., 2003). Clinical validation of PD-onset for patient-derived hiPSC line has been reported only in the case of NIMHi001-A (Datta et al., 2020).

HiPSC lines from patients with sporadic PD have been reported previously by (Ma et al., 2019, Sanchez et al., 2012). However, there is paucity of hiPSC lines from patients of Indian ethnicity. The Indian population is distinct genetically which is further subdivided compared to other endogamous groups due to underlying cultural and demographic events. These events coupled with geographical differences influence various dietary and environmental factors that can contribute towards PD progression and response to drugs. As gene-environment interactions are more pronounced across ethnicities, patient specific hiPSC lines are valuable resources in studying neurodegeneration in the light of personalized medicine. We have also performed targeted gene analysis for 13 PD related genes for the PD patients.

In this study, two hiPSC lines *viz.* NIMHi002-A and NIMHi003-A were generated from patients with sporadic PD from the peripheral blood mononuclear cells using the CytoTune™-iPS 2.0 Sendai Reprogramming Kit (A16517, Thermo Fisher Scientific). Both the lines exhibited ES-like colony formation with distinct borders. Expression of pluripotency and stemness markers *viz.* NANOG, SOX2, OCT4, TRA-1-60, SSEA4 and SOX2 was confirmed by immunofluorescence (Fig. 1D), flow cytometry (Fig. 1E) and RT-PCR (Fig. 1F). The line is free of mycoplasma contamination and any chromosomal abnormalities. NIMHi002-A was karyotyped at passage 12 and NIMHi003-A at passage 10. For both of them 20 spreads were screened and 10 were karyotyped. The iPSC lines exhibited potency to differentiate into ectoderm, endoderm and mesoderm lineages via spontaneous differentiation of the iPSC-derived embryoid bodies. Short tandem repeat (STR) typing analysis of the genomic DNA from the cell lines at 16 locii confirmed 100% identity with the respective PBMC samples. Expression of viral markers could not be detected after passage 2 (Suppl. Fig. 1D).

3. Methodology

3.1. Subject selection and clinical evaluation

The patients − 63 (NIMHi002-A) and 62 (NIMHi003-A) years old were diagnosed for the motor symptoms at the NIMHANS Out Patient Department. Severity of PD onset was determined by the UPDRS-III. Striatal dopamine deficit, a hallmark of PD onset was confirmed by F-DOPA PET (Suppl. Fig. 1A) at HCG hospital. Peripheral blood samples collected from the patients were sent to Medgenome Labs Ltd., Bengaluru, India for targeted gene sequencing for a panel of 13 genes which are commonly identified as risk factors with PD progression.

3.2. PBMC isolation and maintenance

Ethical approval for the study was obtained from the Institutional Committee of Stem Cell Research (IC-SCR) NIMHANS with the sanction number is SEC/01/005/BP. Peripheral blood was collected aseptically into K2EDTA treated vaccutainer tubes (Cat. No. 367861). Density gradient separation of the PBMCs was achieved using HiSep LSM density 1073 1.0770 ± 0.0010 g/ml according to the manufacturer's instructions. PBMCs were incubated using StemPro®34 (Gibco 10639-011) medium containing 100 ng/mL SCF (Gibco PHC2111)), 100 ng/mL FLT3 (Gibco PHC9414), 20 ng/mL IL-3 (Gibco PHC0034) and 10 ng/mL IL-6 (Gibco PHC0065) in humidified atmosphere with 5% CO_2 at 37 °C.

3.3. PBMC reprogramming and iPSC generation

After 3 days incubation, PBMCs were reprogrammed using CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Thermo Fisher Scientific Catalog. No. A16517) with multiplicity of infection as hc-Myc = 5, KOS = 5 and hKlf4 = 3. After 24 h incubation, the virus was removed by centrifugation at 200g and the cells were maintained at 37 °C with 5% CO_2 in PBMC media without cytokines. After 72 h, the reprogrammed cells were transferred on feeder layer of mitotically inactivated mouse embryonic fibroblasts (MEF) with alternate day media changes. MEFs were inactivated by treatment with 10 μg/ml Mitomycin-C (Sigma Cat. No. M4287-5X2MG) On the 7th day, the cells were transitioned to complete iPSC medium comprising of DMEM/F12 with 20% Knockout serum replacement (Gibco Cat. No. 10828028), 1% Glutamax (Gibco Cat. No. 35050061), 1% non-essential amino acid solution (Gibco Cat. No. 11140050), 0.1 mM β-mercaptoethanol (Gibco Cat. No. 31350010), 20 ng human basic fibroblast growth factor (Immunotools Cat. No. 11343627) and 1% penicillin streptomycin (Gibco Cat. No. 15070063).

3.4. iPSC maintenance

The iPSCs (Fig. 1A) were maintained in complete iPSC media with daily media changes. Colonies with absence of differentiated cells were subcultured either by manual picking or with StemPro™ Accutase™ (Gibco Cat. No. A1110501) on fresh MEF feeder dishes.

3.5. Alkaline phosphatase (AP) staining

Presence of AP was determined using Molecular Probes® Alkaline Phosphatase Live Stain according to the manufacturer's instructions. Colonies expressing AP were visualised using a standard FITC filter (Fig. 1C)

3.6. Blue imaging

Sequestration of retinyl esters into cytoplasmic lipid bodies, leading to blue fluorescence was visualized using DAPI filter Cube Olympus EPI-FL filter. The corresponding phase contrast images were acquired using Olympus IX71 with a Retiga monochrome camera and Micromanager software. Both the images were merged into the final image (Fig. 1B).

3.7. Embryoid body (EB) formation

iPSC colonies were enzymatically dissociated using accutase (Thermo Fisher Scientific Cat#A11105-01) and made into single cell suspension by pipetting gently. The cell suspension was plated in non-adherent 35 mm dishes and incubated with iPSC media without bFGF at 37 °C in humidified atmosphere with 5% CO_2. Media change was performed every alternate day. EB formation was observed 48 h post-plating. After 96 h, the EBs were plated on 0.1% matrigel coated dishes for spontaneous differentiation (See Table 1) (Fig. 1H-J).

3.8. rT-PCR

RNA isolation was achieved using TRIzol® reagent (Thermo Fisher Scientific, USA, Cat#15596018) according to the manufacturer's instructions. Isolated RNA was reverse compemented using Superscript III reverse transcriptase (Thermo Fisher Scientific, USA, Cat#18080093). Gene expression was assessed using EmeraldAmp® GT PCR Master Mix 2X (TaKaRa Bio Inc., Japan Cat#RR310B) with the respective primers as mentioned in Table 2. The amplicons were visualized by gel electrophoresis in 1.2% gel in 1X TAE buffer (Fig. 1F, I, Suppl. Fig. 1C).

3.9. Flow cytometry

The cells were treated with StemPro® Accutase (Gibco, Cat#A1110501) and the single cell suspension was fixed using 2% paraformaldehyde (Sigma, Cat#P6148) in phosphate-buffered saline with 0.01% Sodium azide. Cells were permeabilized using 0.1% Triton-X-100 (HiMedia, Cat#MB031). followed by blocking with 3% Bovine

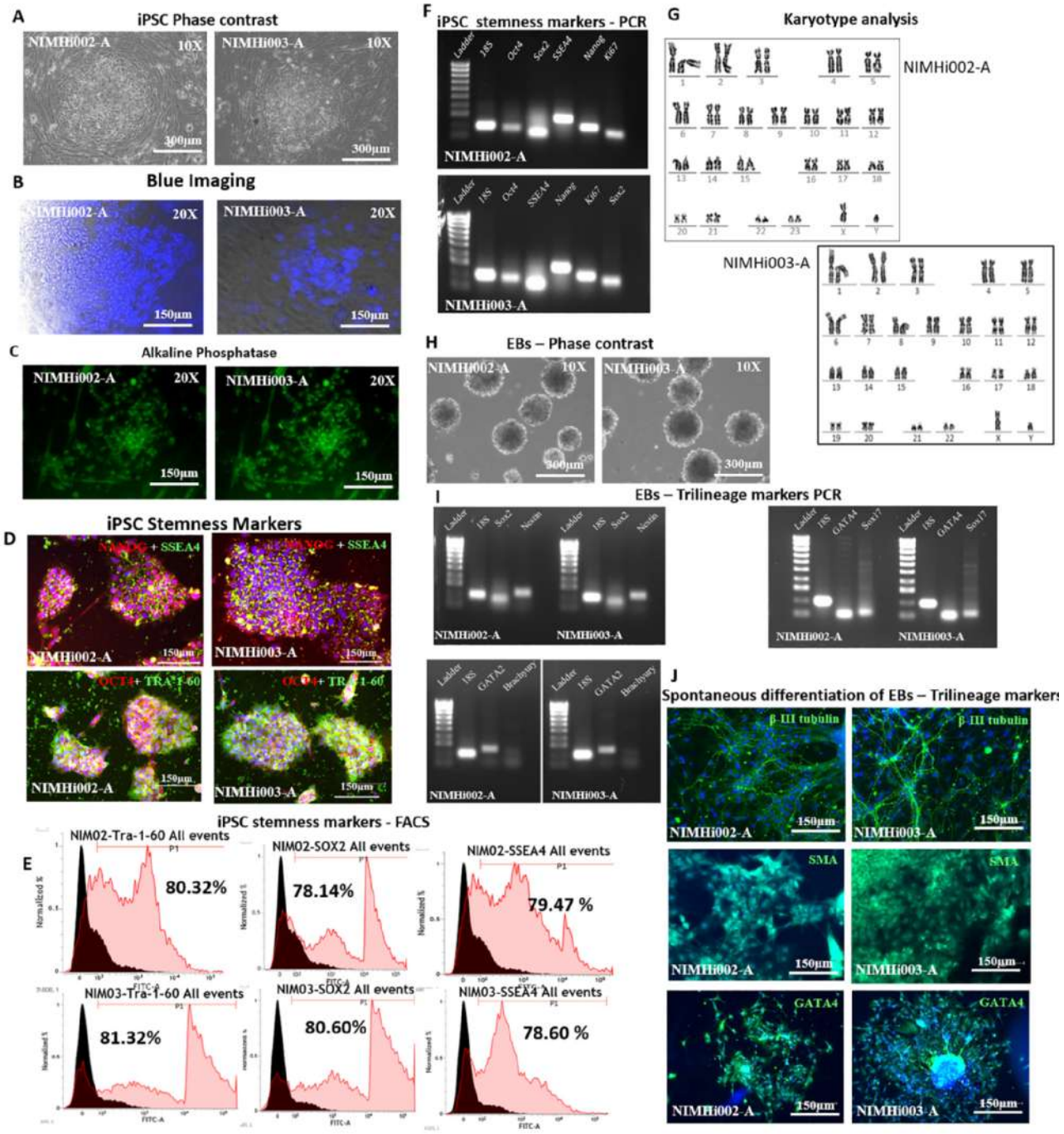

Fig. 1. Characterization of NIMHi002-A and NIMHi003-A.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Disease onset confirmation	F-DOPA PET scan	Presynaptic striatal dopaminergic deficit in bilateral putamen seen confirming disease onset	Supplementary Fig. 1 panel A
Morphology	Phase contrast microscopy	Normal human embryonic stem cell like morphology of the colonies with well-defined borders	Fig. 1 panel A
Phenotype	Immunostaining Flow cytometry	Expression of pluripotency markers: NANOG, OCT4, SSEA-4, TRA-1-60	Fig. 1 panel D and E
Genotype	Karyotype	46XY, no abnormalities seen	Fig. 1 panel G
Identity	Microsatellite PCR STR typing	Matched with the PBMCs from the original donor. 16 locii tested, matched.	Supplementary Fig. 1panel D
Microbiology and virology	Mycoplasma test	Samples tested negative by MycoAlert Lonza kit	Supplementary Fig. 1 panel B
Differentiation potential	Embryoid body formation	*In vitro* spontaneous differentiation yielded β tubulin III, SMA and GATA4 positive cells	Fig. 1 panel H, I and J

Table 2
Antibodies and primers used in the study.

	Antibody	Dilution	Company Cat. No. and RRID
Pluripotency Marker	Goat anti-OCT4	1:100	Abcam Cat#AB27985 RRID AB_776898
Pluripotency Marker	Rabbit anti-SOX2	1:100	Abcam Cat#AB97959 RRID AB_2341193
Pluripotency Marker	Mouse anti-SSEA4	1:100	BD Biosciences Cat#560073 RRID AB_1645601
Pluripotency Marker	Rabbit anti-NANOG	1:100	Abcam Cat#AB21624 RRID AB_446437
Pluripotency Marker	Mouse anti TRA-1-60	1:100	Abcam Cat#AB16288 RRID AB_778563
Pluripotency Marker (Flow cytometry)	TRITC goat anti-rabbit	1:200	Abcam Cat#AB6718 RRID AB_955551
Pluripotency Marker (Flow cytometry)	FITC goat anti-rabbit	1:200	Abcam Cat#AB6717 RRID AB_955238
Pluripotency Marker (Flow cytometry)	Alexa fluor 488 goat anti-rabbit	1:200	Abcam Cat#AB150077 RRID AB_955238
Pluripotency Marker (Flow cytometry)	Alexa fluor 488 goat anti-mouse	1:200	Abcam Cat#AB150113 RRID AB_2576208
Pluripotency Marker (Flow cytometry)	Alexa fluor 647 goat anti-mouse	1:200	Abcam Cat#AB150115 RRID AB_2687948
Pluripotency Marker (Flow cytometry)	FITC goat anti-rabbit	1:200	Millipore Cat#AP307F RRID AB_92652
Differentiation marker	Rabbit anti GATA4	1:100	Abcam Cat# ab134057, RID: AB_2725747
Differentiation marker	Mouse anti β tubulin III	1:100	Sigma-Aldrich Cat# T8578, RRID:AB_1841228
Differentiation marker	Mouse anti SMA	1:100	Millipore Cat# CBL171, RRID:AB_2223166
Primers			
	Target gene	Forward/Reverse primer (5′–3′)	
Pluripotent stem cell marker	OCT4 (165 bp)	F: CCTCACTTCACTGCCACTGTA, R: CAGGTTTTCTTTCTCTAGCT	
Pluripotent stem cell marker	SOX2 (151 bp)	F: CCCAGCAGACTTCACATGT; R: CCTCCCATTTCCCTCGTTT	
Pluripotent stem cell marker	NANOG (272 bp)	F: CCTCTTAAATTTTTTCCTC; R: AAGTGGGTTGTTTGCCTTT	
Pluripotent stem cell marker	Ki67 (198 bp)	F: GACATCACGGATCATATG; R: CTCGCATTGACCATTCAA	
Pluripotent stem cell marker	SSEA4 (119 bp)	F: TGGACGGGCACAACTTCA; R: GGGCAGGTTCTTGGCACT	
Virus detection marker	cMyc (532 bp)	F: CCCGAAAGAGAAAGCGAACCAG; R:TCCACATACAGTCCTGGATGATGATG	
Virus detection marker	SeV (181 bp)	F: GGATCACTAGGTGATATCGAGC; R: ACCAGACAAGAGTTTAACACATATGTA	
Virus detection marker	Klf4 (410 bp)	F: TTCCTGCATGCCAGAGGACTCCC; R: AATGTATCGAAGGTGCTCAA	
Virus detection marker	KOS (528 bp)	F: ATGCACCGCTACGACGTGAGC; R: ACCTTGACAATCCTGATGTGG	
Differentiation marker	Nestin (220 bp)	F: AACAGCGACGGAGGTCTCT; R: TTCTCTTGTCCCGCAGACTT	
Differentiation marker	Brachury (219 bp)	F: ACCCAGTTCATAGCGGTG; R: ATGAGGATTTGCAGGTGG	
Differentiation marker	GATA2 (244 bp)	F: TGACTTCTCCTGCATGCAC; R: AGCCGGCACCTGTTGTGC	
Differentiation marker	SOX17 (214 bp)	F: AGTGACGACCAGAGCCAG; R: CCTTAGCCCACACCATGA	
Differentiation marker	GATA4 (83 bp)	F: GCTATGCGTCTCCCGTCA; R: GTGACTGTCGGCCAAGAC	
Housekeeping gene	18S (180 bp)	F: CGGCTACCACATCCAAGGA; R: GCTGGAATTACCGCGGCT	

Serum Albumin (BSA) (Sigma–Aldrich, Cat#A3059). Further the cells were stained with primary antibodies for TRA-1-60, SOX2 and SSEA4 and anti-mouse Alexa Fluor488 was used as secondary antibody (Datta et al., 2020). iPSCs stained with only anti-mouse Alexa Fluor488 represented the cell-population showing non-specific staining (black peak) in the histograms and was used for gating (as represented by the red line) the immunopositive population for the pluripotent markers (Fig. 1E). Flow cytometry was performed using FACSVerse (BD Biosciences). Cells were identified by light scatter for 10,000 events and analysed using FACS Suite software (BD Biosciences).

3.10. Immunofluorescence

Cells grown on 16 mm coverslips were fixed using 4% paraformaldehyde for 45 min PBS followed by 2 washes with 0.05% Tween-20 (PBST; HiMedia, Cat#MB067). Cytoplasmic protein detection was achieved by permeabilizing the cell surface with 0.1% Triton-X-100. Blocking was carried out using 3% BSA followed by overnight incubation of primary antibodies for NANOG, SSEA4, OCT-4, TRA-1-60, SMA, GATA4, β-III tubulin and the corresponding secondary antibodies for 45 min at room temperature. The cells were counterstained with 0.5 μg/mL 4′, 6′-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, Cat#D8417) for 15 min. Images were acquired using Leica Fluorescence microscope and EVOS M5000 and processed using LasX and Image J software respectively (Fig. 1D,J).

3.11. STR analysis

Genomic DNA was isolated from cell suspension using TRIzol® reagent (Thermo Fisher Scientific, USA, Cat#15596018) according to the manufacturer's instructions. The isolated DNA was subjected to STR typing performed by Medgenome Labs Ltd., Bengaluru, India (Suppl. Fig. 1D).

3.12. Karyotype analysis

Karyotype analysis by G-banding (Fig. 1G) was carried out to check for chromsomal aneuploidy performed by Anand Diagnostic Laboratory Services, Bengaluru, India.

3.13. Mycoplasma detection

Absence of mycoplasma was confirmed using the MycoAlertTM Mycoplasma Detection Kit (Lonza, Cat# LT07-218) according to the manufacturer's instructions (Suppl. Fig. 1C).

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2020.101995.

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