Stem Cell Research 39 (2019) 101499

Contents lists available at ScienceDirect

Stem Cell Research

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

Lab Resource: Stem Cell Line

Establishment of PITX3-mCherry knock-in reporter human embryonic stem cell line (WAe009-A-23)

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A B S T R A C T

Pituitary homeobox 3 (Pitx3) is a key transcription factor that plays an important role in the development and maintenance of midbrain dopaminergic (mDA) neurons. Here, we established a *PITX3*-mCherry knock-in reporter human embryonic stem cell (hESC) line using the CRISPR/Cas9 system. *PITX3*-mCherry hESCs maintained pluripotency marker expression and exhibited the capacity to generate all 3 germ layers and a normal karyotype. After differentiation into mDA neurons, most PITX3 immunoreactivity overlapped with the red fluorescence of mCherry. This reporter cell line may be used to study the development of mDA neurons or to enrich mDA populations for transplantation.

Resource utility

PITX3-mCherry knock-in (KI) reporter hESC line allows the monitoring and isolation of midbrain dopaminergic (mDA) neurons during neuronal differentiation.

Resource details

Pituitary homeobox 3 (Pitx3) is initially expressed in mature mDA neurons of developing brains and its expression is confined to mDA neurons within the adult central nervous system. It plays an important role in acquiring the DA phenotype by regulating the expression of key genes involved in the metabolism, maintenance, and survival of DA neurons (Smidt et al., 2004; Blaess and Ang, 2015). Here, we generated a reporter human embryonic stem cell (hESC) line that expressed the fluorescent protein, mCherry, which reflected endogenous *PITX3* expression using a CRISPR/Cas9-mediated knock-in (KI) system.

The *PITX3* KI donor vector consisted of the 5′ homology arm-T2A-mCherry-polyA-PGK-Neo-polyA-3′ homology arm (Fig. 1A). The mCherry reporter cassette was placed downstream of the *PITX3* stop codon, thus facilitating mCherry expression regulated by the endogenous *PITX3* promoter. A self-cleaving 2A (T2A) site inserted between the 5′ homology arm and mCherry cassette facilitated the independent production of PITX3 and mCherry polypeptides. To incorporate antibiotic selection in knock-in cells, the PGK-neomycin resistant cassette was placed downstream of the mCherry reporter cassette. Finally, a single guide RNA (sgRNA) binding site was designed to introduce site-specific DNA double-stranded breaks near the stop codon of the *PITX3* locus (Fig. 1A).

After electroporation with the *PITX3* KI donor vector and Cas9/sgRNA plasmids followed by antibiotic selection using G418, resistant colonies were individually picked, expanded, and verified via PCR and sequencing. Correctly targeted clones were identified using targeted PCR analysis for each 5′ and 3’ KI junction with specific primer sets for LA-F/R (LA band) and RA-F/R (RA band), respectively (Fig. 1B, middle/bottom panels). PCR analysis using a KI-F/R primer set confirmed that the *PITX3*-R11 and -R24 clones demonstrated bi-allelic insertion of the targeting vector at the *PITX3* locus, whereas the *PITX3*-R32 clone exhibited mono-allelic insertion (Fig. 1B, upper panel). DNA sequencing of the 5′ and 3′ targeting regions in the PCR products of the *PITX3*-R24 clone revealed that the reporter and antibiotic selection cassettes were incorporated before the *PITX3* gene stop codon at exon 3

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https://doi.org/10.1016/j.scr.2019.101499
Received 27 May 2019; Received in revised form 27 June 2019; Accepted 9 July 2019
Available online 18 July 2019
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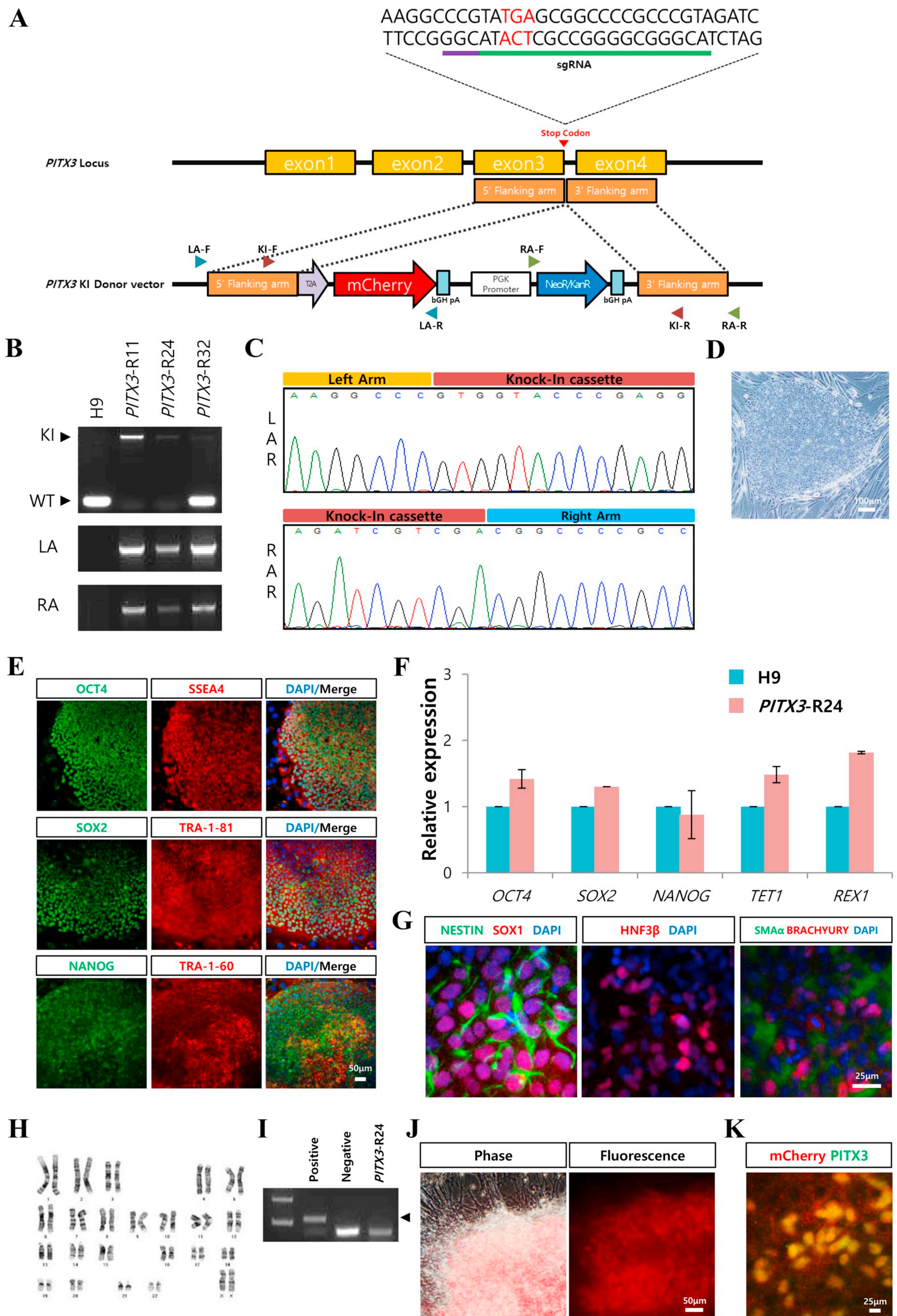

Fig. 1.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel D
Phenotype	Qualitative analysis Immunocytochemistry	Positive for pluripotency markers including OCT4, SOX2, NANOG, SSEA4, TRA-1-81, and TRA-1-60	Fig. 1 panel E
	Quantitative analysis *RT-qPCR*	Positive for pluripotency markers including *OCT4, SOX2, NANOG, TET1, REX1*	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46, XX Resolution 550	Fig. 1 panel H
Identity	Microsatellite PCR (mPCR) OR	N/A	
	STR analysis	15 sites were tested and all matched	Available with the authors
Mutation analysis	Sequencing	Homozygous mutation	Fig. 1 panel C
	Southern Blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	Mycoplasma testing via PCR. Negative	Fig. 1 panel I
Differentiation potential	Spontaneous differentiation	NESTIN and SOX1 (ectoderm); HNF3β (endoderm); SMAα and BRACHYURY (mesoderm)	Fig. 1 panel G
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company cat # and RRID
Pluripotency markers	Rabbit anti-OCT4	1:200	Santa Cruz, cat #sc-9081, RRID:AB_2167703
	Rabbit anti-SOX2	1:200	Millipore, cat # AB5603 RRID:AB_304980
	Goat anti-NANOG	1:50	R&D systems, cat #AF1997 RRID:AB_355097
	Mouse anti-SSEA4	1:200	Millipore, cat #MAB4304 RRID:AB_177629
	Mouse anti-TRA-1-81	1:100	Millipore, cat #MAB4381 RRID:AB_177638
	Mouse anti-TRA-1-60	1:100	Millipore, cat #MAB4360 RRID:AB_2277963
Differentiation markers	Rabbit anti-NESTIN	1:1000	Millipore, cat #ABD69 RRID:AB_2744681
	Goat anti-SOX1	1:100	R&D systems, cat #AF3369 RRID:AB_2239879
	Rabbit anti-HNF3β	1:300	Abcam, cat #AB108422 RRID:AB_11157157
	Mouse anti-SMAα	1:100	Sigma-Aldrich, cat #A5228 RRID:AB_262054
	Goat anti-BRACHYURY	1:100	R&D system, cat #AF2085 RRID:AB_2200235
Secondary antibodies	Donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	1:1000	Invitrogen, cat #A-21206 RRID:AB_2535792
	Donkey anti-goat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 488	1:1000	Invitrogen, cat #A-11055 RRID:AB_2534102
	Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 488	1:1000	Invitrogen, cat #A-21202 RRID:AB_141607
	Donkey anti-mouse IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 594	1:1000	Invitrogen, cat #A-21203 RRID:AB_141633
	Donkey anti-goat IgG (H + L) cross-adsorbed secondary antibody, Alexa Fluor 594	1:1000	Invitrogen, cat #A-11058 RRID:AB_2534105
	Donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor 594	1:1000	Invitrogen, cat #A-21207 RRID:AB_141637

Primers

	Target	Forward/Reverse primer (5′-3′)
Genotyping	KI (2854 bp)	CCAAACAGCACGCCTCCTTCAGCTACC / CCCAGTCCGCGGAGGCTGTGAATCG
	LA (1705 bp)	CGTGCGGGTATGCTCTCCAGACC / GGCCTTGTAGGTGGTCTTGACC
	RA (2127 bp)	CCCATTCGACCACCAAGCG / GAGCCATCGTCAGCATCCTCACG
Pluripotency markers (qPCR)	*OCT4* (164 bp)	CCTCACTTCACTGCACTGTA / CAGGTTTTCTTTCCCTAGCT
	SOX2 (80 bp)	TTCACATGTCCCAGCACTACCAGA / TCACATGTGTGAGAGGGGCAGTGTGC
	NANOG (154 bp)	TGAACCTCAGCTACAAACAG / TGGTGGTAGGAAGAGTAAAG
	TET1 (165 bp)	CTGCAGCTGTCTTGATCGAGTTAT / CCTTCTTTACCGGTGTACACTACT
	REX1 (145 bp)	TCACAGTCCAGCAGGTGTTTG / TCTTGTCTTTGCCCGTTTCT
House-keeping gene (qPCR)	*GAPDH* (159 bp)	CAATGACCCCTTCATTGACC / TTGATTTTGGAGGGATCTCG

(Fig. 1C).

PITX3-mCherry hESCs (*PITX3*-R24 clone) maintained a typical hESC morphology (Fig. 1D). These colonies highly expressed pluripotency genes (OCT4, SOX2, and NANOG) and surface markers (SSEA4, TRA-1-81, and TRA-1-60) (Fig. 1E). Pluripotency marker expression levels in *PITX3*-mCherry hESCs were similar to those in the parental hESCs (Fig. 1F). Upon differentiation, *PITX3*-mCherry hESCs spontaneously differentiated into cells of all 3 germ layers as shown by immunocytochemistry with markers for the ectoderm (NESTIN/SOX1), endoderm (HNF3β), and mesoderm (SMAα/BRACHYURY) (Fig. 1G). Furthermore, *PITX3*-mCherry hESCs exhibited a normal karyotype at passage 52 (Fig. 1H) and no mycoplasma contamination (Fig. 1I). STR analysis also revealed that *PITX3*-mCherry hESCs and parental hESCs were genetically identical.

For mDA neuronal differentiation from *PITX3*-mCherry hESCs, previously reported protocols were used with slight modifications (Kirkeby et al., 2012). At differentiation day 40, mCherry expression was visually detected using a fluorescence microscope (Fig. 1J). Immunocytochemical analysis confirmed that mCherry positive cells expressed PITX3 (Fig. 1K), indicating that mCherry reflected endogenous *PITX3* expression in mDA neurons differentiated from *PITX3*-mCherry hESCs.

Materials and methods

Maintenance of human embryonic stem cells

Undifferentiated hESCs (H9 [WA09]; WiCell Inc.) and hESC reporter lines were cultured on a layer of mitotically-arrested mouse fibroblasts (STO, CRL-1503, American Type Culture Collection) in hESC medium composed of DMEM/F12 medium (Gibco) supplemented with 20% Knockout-Serum Replacement (Gibco), 1% non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/mL of basic FGF (PeproTech) at 37 °C in 5% CO_2. Colonies of hESC were transferred weekly onto fresh feeder cells by mechanical dissection with a split ratio of 1 to 25.

Generation of PITX3-mCherry KI reporter hESC line

Plasmids encoding Cas9 and sgRNA were designed by and purchased from ToolGen, Inc. Korea. *PITX3* targeting vector were constructed using pUC19 as a backbone. To introduce targeting vector and plasmids into hESCs, Y-27632-pretreated hESCs were harvested using Accutase (Merck) and resuspended in 100 µL of R buffer (Neon transfection kit; Thermo Fisher Scientific) at a final concentration of 1.0×10^7 cells/mL. Resuspended cells were mixed with 6 µg of *PITX3* targeting vector and plasmids (3 µg of each plasmid) then pulsed with a voltage of 850 for 30 ms using the Neon transfection system. After electroporation, the cells were plated on a new plate containing fresh feeder cells in hESC media. After 4 days, the cells were treated with 100 µg/mL of G418 for 2 weeks. Following antibiotic selection, G418-resistant colonies were individually picked and expanded (Table 1).

To screen for correctly targeted colonies, genomic DNA was prepared using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. PCR was performed via a PCR thermal cycler (Applied Biosystems Geneamp 2720) using EmeraldAmp® GT PCR Master Mix (TAKARA Bio Inc.) and primers listed in Table 2. The reaction was performed according to the following parameters: initial denaturation at 98 °C for 1 m, 30 cycles of denaturation at 98 °C for 7 s, annealing at 65 °C for 30s and extension at 72 °C for 2 m 20s, and final extension at 72 °C for 10 m.

RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA was extracted using the Easy-Spin® Total RNA Extraction kit (iNtRON Biotechnology) according to the manufacturer's instructions. cDNA was then synthesized from 1 µg of total RNA using PrimeScript™ RT Master Mix (TAKARA Bio Inc.). Transcript levels were quantified via real-time RT-PCR (RT-qPCR) using FastFire qPCR PreMix (TIANGEN) in a CFX96 Real-Time System (Bio-Rad). Transcription levels for each targeted gene were normalized to *GAPDH* expression. Primer sequences are listed in Table 2.

Immunocytochemistry

Cells were fixed using 4% paraformaldehyde for 15 min at room temperature. To visualize intracellular markers, cells were permeabilized with 0.1% Triton X-100 in PBS, blocked with 2% BSA-PBS for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies diluted in 2% BSA-PBS (Table 2). After washing with PBS, cells were exposed to fluorescence-tagged secondary antibodies (Thermo Fisher Scientific) in 2% BSA-PBS for 30 min at room temperature and mounted in DAPI mounting medium (Vector Laboratories). Images were obtained using an Olympus IX71 microscope equipped with a DP71 digital camera (Olympus FSX100 system).

Differentiation into three germ layer cell types

In vitro differentiation of *PITX3*-mCherry hESCs was performed using Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems) according to the manufacturer's instructions.

Karyotyping and mycoplasma testing

G-banding karyotype analysis was performed at passage 52 at GenDix, Inc. using standard protocols for GTG banding. Total 20 metaphases were analyzed at 550 band resolution. The absence of mycoplasma contamination was assessed using an e-Myco™ Mycoplasma PCR Detection kit (iNtRON Biotechnology).

STR analysis

PITX3-mCherry hESCs (*PITX3*-R24 clone) and parental hESCs (H9) were sent to the HUMANPASS Inc. (Korea) for STR analysis. Briefly, the target loci were amplified using AmpFLSTR™ Identifiler™ PCR Amplification Kit (Applied Biosystems) and then analyzed with ABI3130xl (Applied Biosystems) genetic analyzer using the software program GeneMapper v.5.0 (Applied Biosystems).

Key resources table

Unique stem cell line identifier	WAe009-A-23
Alternative name(s) of stem cell line	*PITX3*-mCherry hESC, WAe009-A-PITX3
Institution	Yonsei University, College of Medicine, Seoul, Korea
Contact information of distributor	Dong-Wook Kim, dwkim2@yuhs.ac
Type of cell line	ESC
Origin	Human
Additional origin info	Sex: Female
Cell source	H9 human embryonic stem cell
Clonality	Clonal
Method of reprogramming	N/A
Genetic modification	Yes
Type of modification	Reporter knock-in
Associated disease	N/A
Gene/locus	*PITX3*/10q24.32
Method of modification	CRISPR/Cas9
Name of transgene or resistance	T2A-mCherry-polyA-PGK-NeoR-polyA
Inducible/constitutive system	N/A
Date archived/stock date	2016/1
Cell line repository/bank	https://hpscreg.eu/cell-line/WAe009-A-23
Ethical approval	Ethical committee: Yonsei university health system, Severance Hospital, Institutional review board approval number: 2–116,224-ABCDGH-N-01

Acknowledgements

This work was supported by 1) the Bio & Medical Technology Development Program of the National Research Foundation (NRF) (2017M3A9B4042580), 2) the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI18C0829), 3) a Korea University Research Grant, and 4) BK21 PLUS Program for Biotechnology, Korea University.

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