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Lab Resource: Single Cell Line

Generation of Brachyury-mCherry knock-in reporter human pluripotent stem cell line (SNUe003-A-2) using CRISPR/CAS9 nuclease

Juwon Jung [a,1], Sunsook Hwang [b,1], Hyewon Seol [a,1], A-Hyeon Kim [c], Ha Myoung Lee [c], Jin Jea Sung [d], Seung Min Jeong [b], Young Min Choi [a], Jong Kwan Jun [a], Dong-wook Kim [d,*], Jiho Jang [c,*]

[a] Institute of Reproductive Medicine and Population, Medical Research Center, Dept. of Obstetrics & Gynecology, Seoul National University College of Medicine, Seoul 03080, Republic of Korea
[b] Department of Biochemistry, Institute for Aging and Metabolic Diseases, College of Medicine, The Catholic University of Korea, 222, Banpo-daero, Seocho-gu, Seoul 06591, Republic of Korea
[c] Department of Biomedical Sciences, College of Medical Convergence, Catholic Kwandong University, Gangneung-si, Gangwon-do, Republic of Korea
[d] Department of Physiology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Republic of Korea

A B S T R A C T

Brachyury is an embryonic nuclear transcription factor required for mesoderm formation and differentiation. Here, we introduced an mCherry reporter into the C-terminus of *Brachyury* in the human pluripotent stem cell line SNUhES3 using the CRISPR/Cas9 nuclease approach. Successful gene editing was verified by DNA sequencing. SNUhES3-Brachyury-mCherry cells expressed pluripotent stem cell markers, exhibited a normal karyotype, and could generate all three germ layers. This cell line expressed the red fluorescence protein mCherry upon the induction of mesoderm differentiation. This reporter cell line could be used to monitor mesodermal population enrichment during mesodermal differentiation.

Resource Table:

Unique stem cell line identifier	SNUe003-A-2
Alternative name(s) of stem cell line	B-mC-KI-SE-3, SNUe003-A-2
Institution	Seoul National University, Medical Research Center (MRC), Institute of Reproductive Medicine and Population (IRMP)
Contact information of distributor	Jong Kwan Jun, jhs0927@snu.ac.kr Jiho Jang, jhjang@cku.ac.kr
Type of cell line	ESC
Origin	Human
Additional origin info	Sex: Male Ethnicity: Korean
Cell Source	SNUhES3 human embryonic stem cell
Method of reprogramming	N/A
Genetic Modification	Yes
Type of Modification	Reporter knock-in
Associated disease	N/A
Gene/locus	Brachyury/6q27
Method of modification	CRISPR/Cas9
Name of transgene or resistance	T2A-mCherry-polyA-PGK-NeoR-polyA
Inducible/constitutive system	Constitutive Neomycin resistant gene
Date archived/stock date	2019/12
Cell line repository/bank	https://hpscreg.eu/cell-line/SNUe003-A-2, Seoul National University, MRC, IRMP http://www.stemcellkorea.org
Ethical approval	All research procedures involving hESCs were approved by the Institutional Review Board (IRB) of Seoul National University, MRC, IRMP (approval no. 219932-201904-04-01-01)

1. Resource utility

The Brachyury-mCherry knock-in (KI) reporter human pluripotetnt stem cell (hPSC) line can be used to monitor the expression of Brachyury and assess mesodermal cell-type enrichment during mesodermal differentiation. Thus, it can be used to develop culture conditions to efficiently induce the enrichment of mesodermal cells.

* Corresponding authors.
E-mail addresses: dwkim2@yuhs.ac (D.-w. Kim), jhjang@cku.ac.kr (J. Jang).
[1] These authors contributed equally to this work.

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2. Resource Details

Brachyury is an embryonic nuclear transcription factor that binds to a specific DNA element, the palindromic T-site called the T-box, and it is necessary for mesoderm formation and differentiation (Knezevic et al., 1997). Variations in this gene are associated with susceptibilities to defective neural tube closure and chordoma (Zhu et al., 2016). Here, we report the generation and characterization of a Brachyury-mCherry reporter hPSC line using a CRISPR/Cas9-mediated knock-in (KI) system (Table 1).

First, the correct clones were chosen based on G418 resistance after electroporation with the Brachyury KI donor vector and Cas9/sgRNA plasmids. The donor construct comprised an mCherry reporter, NeoR marker, and two homology arm sequences (Fig. 1A). A single guide RNA (sgRNA) was designed to target the stop codon region of the *Brachyury* gene. Next, properly targeted clones were validated by a PCR screening strategy using specific primer sets located outside each homology arm, respectively (LA-F/R and RA-F/R), and a KI-F/R primer set located at each homology arm, which showed that the BRACHYURY #2–12 clone was mono-allelically inserted (Fig. S1). Finally, DNA sequencing ensured that the KI gene cassette was incorporated in frame downstream of endogenous *BRACHYURY* (Fig. 1B).

Brachyury-mCherry hPSCs showed a normal karyotype (46, XY) at passage 46 (Fig. 1C) and no mycoplasma contamination (Fig. S2). SNUe003-A-2 cells exhibited typical human pluripotent stem cell-like morphology and alkaline phosphatase (AP) activity (Fig. 1D). The pluripotency of SNUe003-A-2 hPSCs was confirmed by immunostaining using pluripotency markers (OCT4, SSEA-4, TRA-1–60, and TRA-1–81) (Fig. 1E) and also by RT-PCR for pluripotency genes (*NANOG*, *SOX2*, *REX1*, *OCT4*, and *TERT*) (Fig. 1F). This cell line had the potential to differentiate into all three germ layers, the endoderm (Alpha-Fetoprotein (AFP) and HNF3β), mesoderm (Enolase and Brachyury), and ectoderm (Nestin, Vimentin, and MAP2) (Fig. 1G).

The expression of endogenous mCherry was observed from day 2 of mesoderm differentiation (Fig. 1H). To augment the mCherry specificity, we confirmed the clear co-localization of BRACHYURY and mCherry by immunostaining (Fig. 1I). To enable independent production of Brachyury and mCherry polypeptides, a self-cleaving 2A (T2A) site was placed between the 5′ homology arm and the mCherry cassette, and mCherry expression was regulated by the endogenous *Brachyury* promoter. We thus checked the mesodermal sublineage differentiation potential of the parental and SNUe003-A-2 cell line by inducing the differentiation of two cell lines into cardiomyocytes and endothelial cells (ECs). The beating characteristics and the expression of the cardiac-specific marker cardiac troponin T (cTnT) in cardiac clusters derived from SNUe003-A-2 hPSCs were observed comparably to those in the parental hPSCs after day 8 ~ 12 of cardiac differentiation (Fig. S3and 1J). Furthermore, SNUe003-A-2 hPSCs displayed the expression of CD31 and Von Willebrand factor (vWF), endothelial lineage markers and a mature EC marker, similarly to those in the parental hPSCs at day 8 ~ 10 of differentiation (Fig. 1K). Together, these data suggest that the fusion of a 2A non-joining peptide barely affected the mesodermal sublineage differentiation potential.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel DF
Phenotype	Qualitative analysis Immunocytochemistry	Positive for pluripotency markers including Oct4, SSEA4, Tra-1–60, Tra-1–81, AP	Fig. 1 panel D & E
	Quantitative analysis RT-PCR	Positive for pluripotency markers including Nanog, SOX2, REX1, OCT4, TERT	Fig. 1 panel F
Genotype	Karyotype (G-banding) and resolution	46,XY	Fig. 1 panel C
Identity	Microsatellite PCR (mPCR) OR	Not performed	N/A
	STR analysis	16 STR loci were tested, all matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	heterozygous	Fig. 1 panel B
	Southern Blot OR WGS	Not performed	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing via PCR Negative	Fig. S2
Differentiation potential	Spontaneous differentiation	AFP, HNF3ß (endoderm), Enolase, Brachyury (mesoderm), Nestin, Vimentin, MAP2 (ectoderm)	Fig. 1 panel G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	N/A
	HLA tissue typing	Not performed	N/A

3. Materials and methods

3.1. Cell culture

Undifferentiated hPSCs (SNUhES3) were maintained on vitronectin-coated plates in Essential 8 media (Gibco) without feeders. For single cell culture, the cells were passaged weekly in Essential 8 medium containing Y-27632 using accutase, following a split ratio of 1 to 40.

3.2. Generation of Brachyury-mCherry KI reporter cell line

The SNUhES3 hESC line was used to generate the Brachyury-mCherry reporter hESC line. First, 2×10^5 cells/ml were transfected with a donor construct, Cas9 (Type II-A Streptococcus pyogenes Cas9, ToolGen), and sgRNA plasmid (ToolGen) using the Neon transfection system (Thermo Fisher Scientific). Following electroporation, the transfected cells were plated on vitronectin-coated plates in E8 medium containing Y-27632. After day 4, the cells were treated with G418 (Calbiochem) increasing sequentially from 50 μg/ml to 100 μg/ml. After drug selection, G418-resistant clones were individually picked and expanded. To confirm Brachyury-mCherry KI colonies, genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen), and PCR was performed using the EmeraldAmp® GT PCR Master Mix (TAKARA Bio Inc.) and TaKaRa LA Taq® DNA polymerase (TAKARA Bio Inc.). The primers used are listed in Table 2.

3.3. In vitro differentiation into three germ layers

For the formation of embryoid bodies, hPSC colonies were harvested after 2 mg/ml collagenase IV (Gibco) treatment for 3 min and grown in suspension culture on low attachment Petri dishes with differentiation medium (TeSR™-E6™ medium) for 2 weeks. The culture medium was changed every other day.

3.4. Cardiomyocyte and EC differentiation

For cardiomyocyte differentiation, we employed a protocol using the PSC Cardiomyocyte Differentiation kit (Gibco) with slight modifications. Brachyury expression was observed using a fluorescence microscope from day 2 to day 12. For EC differentiation from hPSCs, we used a previously described protocol with slight modifications (Harding et al., 2017). On day 8 ~ 10 of differentiation, CD31 and vWF were observed

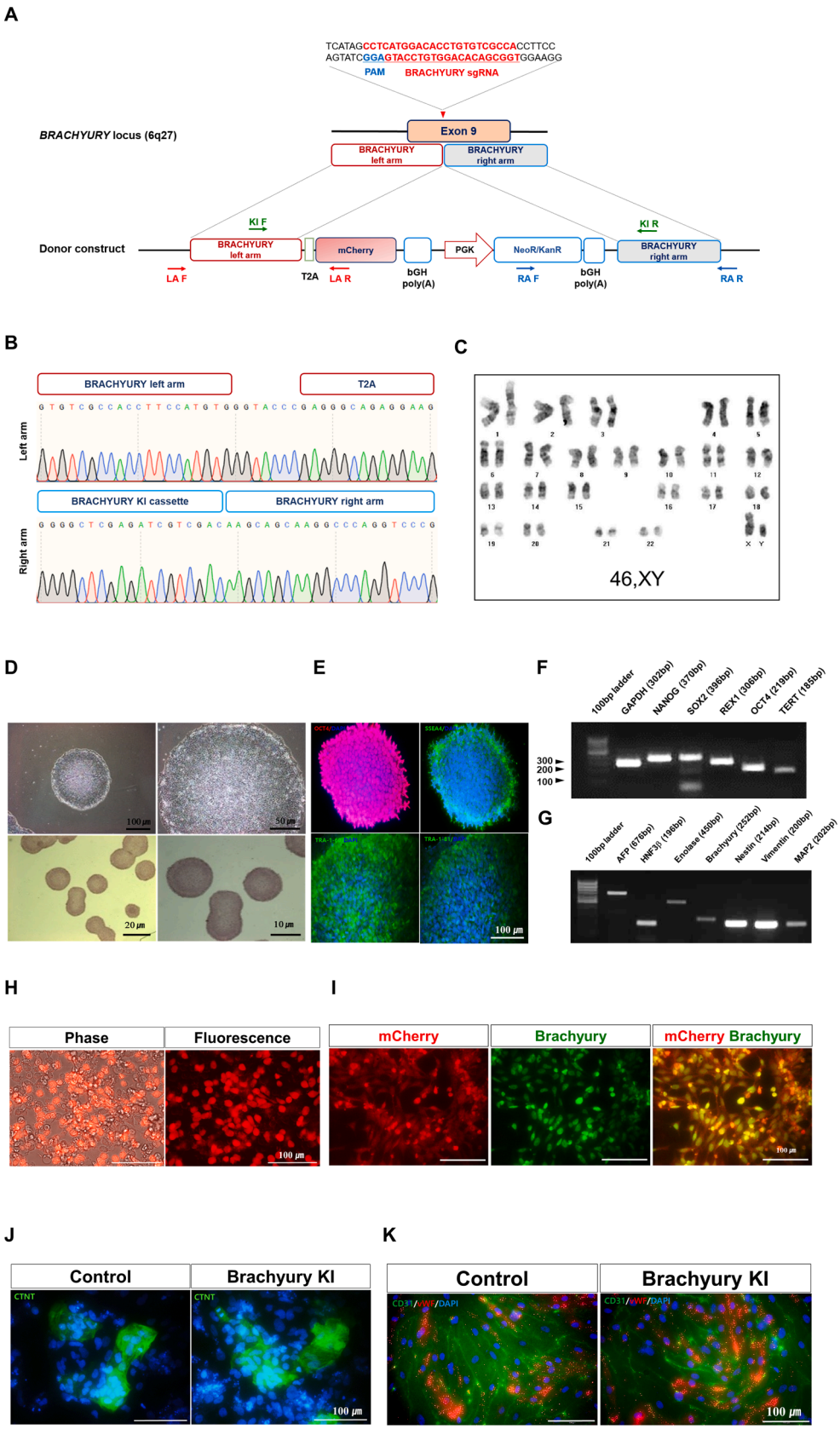
A
TCATAGCCTCATGGACACCTGTGTCGCCACCTTCC
AGTATCGGAGTACCTGTGGACACAGCGGTGGAAGG
PAM
BRACHYURY sgRNA
BRACHYURY locus (6q27)
Exon 9
BRACHYURY left arm
BRACHYURY right arm
KI F
KI R
Donor construct
BRACHYURY left arm
mCherry
PGK
NeoR/KanR
BRACHYURY right arm
LA F
T2A
LA R
bGH poly(A)
RA F
bGH poly(A)
RA R
B
BRACHYURY left arm
T2A
Left arm
BRACHYURY KI cassette
BRACHYURY right arm
Right arm
C
46,XY
D
E
F
100bp ladder
GAPDH (302bp)
NANOG (370bp)
SOX2 (396bp)
REX1 (306bp)
OCT4 (219bp)
TERT (185bp)
300
200
100
G
100bp ladder
AFP (676bp)
HNF3β (196bp)
Enolase (450bp)
Brachyury (252bp)
Nestin (214bp)
Vimentin (200bp)
MAP2 (202bp)
H
Phase
Fluorescence
I
mCherry
Brachyury
mCherry Brachyury
J
Control
Brachyury KI
CTNT
K
Control
Brachyury KI
CD31/vWF/DAPI

Fig. 1.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-citometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-OCT3/4	1:100	Santa Cruz Biotechnology, Cat#sc-9081, RRID: AB_2167703
	Mouse anti-SSEA4	1:100	Millipore, MAB4304, RRID: AB_177629
	Mouse anti-TRA-1–60	1:100	Millipore, MAB4360, RRID: AB_2119183
	Mouse anti-TRA-1–81	1:100	Millipore, MAB4381, RRID: AB_177638
	Alkaline phosphatase		Sigma-Aldrich, Cat#86R-1KT
Differentiation markers	Rabbit anti-BRACHYURY	1:100 1:100	Santa Cruz Biotechnology, Cat#sc-20109, RRID: AB_2255702
	Mouse anti-cardiac troponin T (cTnT)	1:200 1:500	R&D Systems, Cat# MAB1874, RRID: AB_2206731
	Mouse anti-CD31		BD Biosciences, Cat# 550274, RRID:AB_393571
	Rabbit anti-Von Willebrand factor (vWF)		Millipore, Cat# AB7356, RRID:AB_92216
Secondary antibodies	lexa Fluor 488 donkey anti-rabbit IgG (H + L)	1:200	Thermo Fisher Scientific, Cat#A21206, RRID: AB_2535792
	Alexa Fluor 594 donkey anti-rabbit IgG (H + L)	1:200	Thermo Fisher Scientific, Cat#A21207, RRID: AB_141637
	Alexa Fluor 488 donkey anti-mouse IgG (H + L)	1:200	Thermo Fisher Scientific, Cat#A21202, RRID: AB_141607

Primers		
	Target	Forward/Reverse primer (5′-3′)
Genotyping	KI (3.3 kb), WT (0.6 kb)	GCTCTTTCATGAGCTCTG/ CTTCTTAACCTGAGACTGCC
	LA (1.4 kb)	CATGCTAGCACACCTATG/ GACAGCTTCAAGTAGTCG
	RA (1.7 kb)	GCCGAGAAAGTATCCATC/ GCTTGAATAGGGTGGACAG
House-keeping gene (RT-PCR)	GAPDH (302 bp)	AGCCACATCGCTCAGACACC/ GTACTCAGCGGCCAGCATCG
Pluripotency markers (RT-PCR)	NANOG (370 bp)	CTGAGATGCCTCACACGGAGACTG/ GTCACACCATTGCTATTCTTC
	SOX2 (396 bp)	GGCAGCTACAGCATGATGCAG/ GCTCTGGTAGTGCTGGGACATG
	REX1 (306 bp)	GCGTACGCAAATTAAAGTCCAGA/ CAGCATCCTAAACAGCTCGCAGAAT
	OCT4 (219 bp)	CTACAACGCCTACGAGTCCTACA/ TTCTGGCGCCGGTTACAGAACCA
	TERT (185 bp)	AGCTATGCCCGGACCTCCAT/ GCCTGCAGCAGGAGGATCTT
Differentiation markers (RT-PCR)	Alpa-fetoprotein (AFP) (676 bp)	AGAACCTGTCACAAGCTGTG/ GACAGCAAGCTGAGGATGTC
	HNF3b (196 bp)	CCATTGCTGTTGTTGCAGGGAAGT/ CACCGTGTCAAGATTGGGAATGCT
	Enolase (450 bp)	TGACTTCAAGTCGCCTGATGATCCC/ TGCGTCCAGCAAAGATTGCCTTGTC
	Brachyury (252 bp)	TAAGGTGGATCTTCAGGTAGC/ CATCTCATTGGTGAGCTCCCT
	Nestin (214 bp)	CAGCTGGCGCACCTCAAGATG/ AGGGAAGTTGGGCTCAGGACTGG
	Vimentin (200 bp)	GGGACCTCTACGAGGAGGAG/ CGCATTGTCAACATCCTGTC
	MAP2 (202 bp)	GCATATGCGCTGATTCTTCA/ CTTTCCGTTCATCTGCCATT

using a fluorescence microscope (Olympus FSX 100 system).

3.5. Reverse transcription PCR analysis

Total RNA was isolated from generated cells using the Rneasy Mini kit (Qiagen), and cDNA was synthesized by reverse transcription PCR. The primers used to detect gene expression are listed in Table 2.

3.6. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min and then permeabilized overnight at 4 °C with 0.01% Tween 20, 3% BSA (1:1) blocking solution. The permeabilized cells were incubated overnight at 4 °C with primary antibodies diluted in a 1% Triton X-100, 1% BSA solution. After three washes with dPBS, cells were incubated with secondary antibodies in a 1% Triton X-100, 1% BSA solution for 1 h at room temperature. Cells were washed five times and mounted with DAPI mounting medium (Vector Laboratories). Cell images were acquired with a fluorescence microscope. The antibodies used are listed in Table 2.

3.7. Karyotyping and STR analysis

The karyotype was analyzed following standard protocols for GTG banding. STR analysis for SNUe003-A-2 and parental cells was performed by the DowGene (Korea).

3.8. Mycoplasma testing

Mycoplasma contamination was tested using an e-Myco™ Mycoplasma PCR Detection kit (iNtRON Biotechnology, INC.), as per the manufacturer's instructions.

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.

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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.2021.102321.

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