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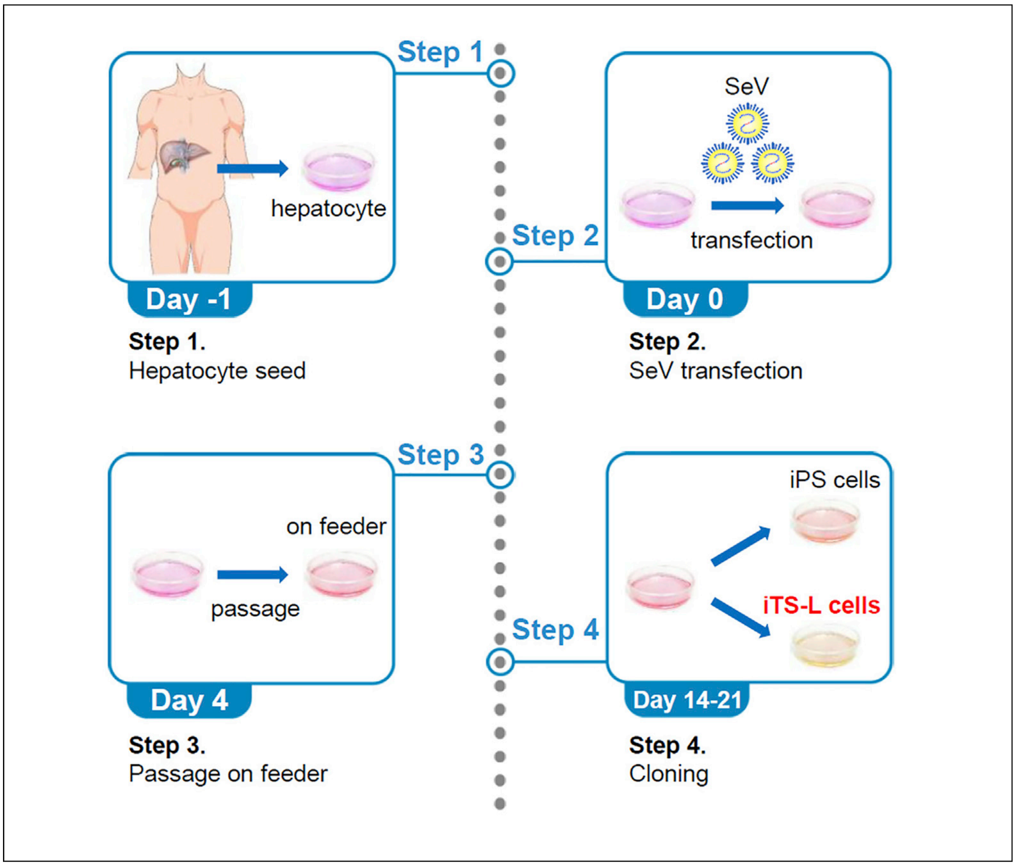
Protocol for the generation of human induced hepatic stem cells using Sendai virus vectors



Hirofumi Noguchi, Yoshiki Nakashima, Masami Watanabe, Masayuki Matsushita, Masayoshi Tsukahara, Issei Saitoh, Chika Miyagi-Shiohira

noguchih@med.u-ryukyu.ac.jp

Highlights

A protocol for reprogramming human hepatocytes to iTS-L cells

Using Sendai virus vectors expressing OCT3/4, SOX2, KLF4, and c-MYC

Steps for culture of human hepatocytes, viral transfection, and culture of iTS-L cells

A protocol for the selection of iPS and iTS-L cells

Our recent study demonstrated the generation of induced tissue-specific stem/progenitor (iTS/iTP) cells by the transient overexpression of reprogramming factors combined with tissue-specific selection. Here, we present a protocol to reprogram human hepatocytes to generate human induced tissue-specific liver stem (iTS-L) cells. Human hepatocytes are transfected with Sendai virus vectors (SeV) expressing OCT3/4, SOX2, KLF4, and C-MYC. iTS-L cells continuously express mRNA of hepatocyte-specific markers (HNF1b and HNF4a) and do not form teratomas.

Noguchi et al., STAR Protocols 3, 101884
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101884

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Protocol for the generation of human induced hepatic stem cells using Sendai virus vectors

Hirofumi Noguchi,[1,6,7,*] Yoshiki Nakashima,[1,2] Masami Watanabe,[3] Masayuki Matsushita,[4] Masayoshi Tsukahara,[2] Issei Saitoh,[5] and Chika Miyagi-Shiohira[1]

[1]Department of Regenerative Medicine, Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan

[2]Kyoto University Center for iPS Cell Research and Application Foundation (CiRA Foundation), Facility for iPS Cell Therapy (FiT), Kyoto 606-8397, Japan

[3]Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8558, Japan

[4]Department of Molecular and Cellular Physiology, Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan

[5]Division of Pediatric Dentistry, Graduate School of Medical and Dental Science, Niigata University, Niigata 951-8514, Japan

[6]Technical contact: noguchih@med.u-ryukyu.ac.jp

[7]Lead contact

*Correspondence: noguchih@med.u-ryukyu.ac.jp
https://doi.org/10.1016/j.xpro.2022.101884

SUMMARY

Our recent study demonstrated the generation of induced tissue-specific stem/progenitor (iTS/iTP) cells by the transient overexpression of reprogramming factors combined with tissue-specific selection. Here, we present a protocol to reprogram human hepatocytes to generate human induced tissue-specific liver stem (iTS-L) cells. Human hepatocytes are transfected with Sendai virus vectors (SeV) expressing OCT3/4, SOX2, KLF4, and c-MYC. iTS-L cells continuously express mRNA of hepatocyte-specific markers (HNF1β and HNF4α) and do not form teratomas.

For complete details on the use and execution of this protocol, please refer to Nakashima et al. (2022).[1]

BEFORE YOU BEGIN

Prepare the media below. Prewarm the medium intended for cell culture at 37°C at least 30 min prior to beginning each section of this protocol. Refer to the key resources table for a complete list of materials.

1. Human hepatocyte culture medium: Kaly-Cell Thawing Medium (KLC-TM), Kaly-Cell Seeding Medium (KLC-SM), hepatocyte basal medium.
2. Human embryonic stem (ES)/iPS/iTS-L cell culture medium: Primate ES Cell Medium with 5 ng/mL bFGF.
3. Mouse embryonic fibroblast (MEF) culture medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S).
4. All cell types are cultured in an incubator at 37°C, 5% CO_2, and 85% humidity.

Institutional permissions

All experimental protocols were in accordance with the guidelines for the care and use of laboratory animals set by Research Laboratory Center, Faculty of Medicine, and the Institute for Animal Experiments, Faculty of Medicine, University of the Ryukyus (Okinawa, Japan).

Mouse embryonic fibroblasts (MEFs) thawing and culturing

Timing: 1 day

5. Add 1 mL of 0.1% gelatin solution to the 60 mm dishes and incubate the dish for 30 min at 37°C.
6. MEFs (inactivated by mitomycin) are obtained from a vender; see key resources table in this protocol. Thaw one frozen vial of murine embryonic fibroblasts (MEFs) (3.0×10^6 cells) in a 37°C water bath.
7. Transfer the content of the vial into a 15 mL tube containing 10 mL of MEF culture medium.
8. Centrifuge the samples at 700 × *g* for 5 min to pellet the cells.
9. Remove the supernatant.
10. Resuspend the cell pellet in 5 mL of MEF culture medium using a 5 mL pipette to a single cell suspension, by pipetting up and down 5–7 times.
11. Aspirate the gelatin solution from the 60 mm dishes.
12. Transfer the cell suspension into 60 mm dishes (6.0×10^5 cells/dish).
13. Place the MEFs in an incubator at 37°C, 5% CO_2, and 85% humidity. Feeders can be used up to 5 days after preparation. The cells are then renewed with fresh MEF culture medium every two days.

Human hepatocyte thawing and culturing

Timing: 1 week

14. Human hepatocytes are obtained from a vender; see the key resources table in this protocol. Thaw one frozen vial of human hepatocytes (3.0×10^6 cells) in a 37°C water bath.
15. Transfer the content of the vial into a 15 mL tube containing 10 mL of KLC-TM medium.
16. Centrifuge the samples at 700 × *g* for 5 min to pellet the cells.
17. Remove the supernatant.
18. Resuspend the cell pellet in 5 mL of KLC-SM medium using a 5 mL pipette to a single cell suspension pipetting up and down 5–7 times.
19. Transfer the cell suspension into two collagen-coated 100 mm dishes and add 7.5 mL of KLC-SM medium to each dish (final 10 mL/dish).
20. Place the human hepatocytes in an incubator at 37°C, 5% CO_2, and 85% humidity. Renew the cells with fresh KLC-SM medium after 6 and 24 h. After 48 h, change the medium to hepatocyte basal medium. Renew with fresh hepatocyte basal medium every two days.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Gelatin	FUJIFILM Wako Pure Chemical Corporation	Cat# 190-15805
Y-27632	FUJIFILM Wako Pure Chemical Corporation	Cat# 257-00511
KLC-TM medium	KaLy-Cell	Cat# KLC-TM
KLC-SM medium	KaLy-Cell	Cat# KLC-SM
Hepatocyte basal medium	Lonza	Cat# CC-3199
Primate ES Cell Medium	ReproCELL	Cat# RCHEMD001
Freezing medium for human ES/iPS cells (DAP213)	ReproCELL	Cat# RCHEFM001
Recombinant human bFGF (FGF2)	ReproCELL	Cat# RCHEOT002
D-PBS(-)	Nacalai Tesque	Cat# 11482-15

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
0.05% trypsin/EDTA	Thermo Fisher Scientific	Cat# 25300054
DMEM	Wako	Cat# 043-30085
Fetal bovine serum	Thermo Fisher Scientific	Cat# 10270-106
CytoTune-iPS 2.0	Medical & Biological Laboratories Co., Ltd.	Cat# IDT-DV0304
Penicillin−streptomycin solution (×100)	FUJIFILM Wako Pure Chemical Corporation	Cat# 16823191
Hanks' Balanced Salt Solution (HBSS)	Life Technologies	Cat# 14025092
Critical commercial assays		
SuperPREP II Cell Lysis & RT Kit for quantitative PCR	TOYOBO CO., LTD.	Cat# SCQ-401
Luna Universal qPCR Master Mix	New England Biolabs Inc.	Cat# M3003E
TaqMan Array 96-Well FAST Plate(Human Stem Cell Pluripotency)	Applied Biosystems	Cat# 4418722
TaqMan™ Fast Advanced Master Mix	Thermo Fisher Scientific	Cat# 4444963
Experimental models: Cell lines		
Cryopreserved Hepatocytes Species:Human, Lot#S1412T, Lot#S1238 and Lot#S1350	KaLy-Cell	Cat# HHCPC-2 M
hiPSC Lines 201B7	CiRA Foundation	N/A
MEF cells	ReproCELL Inc.	Cat# RCHEFC003
Oligonucleotides		
human OCT3/4 forward, GACAGGGGGAGGGGAGGAGCTAGG, human OCT3/4 reverse, CTTCCCTCCAACCAGTTGCCCCAAAC,	Takahashi et al.[2]	N/A
human SOX2 forward, GGGAAATGGGAGGGGTGCAAAAGAGG, human SOX2 reverse, TTGCGTGAGTGTGGATGGGATTGGTG,	Takahashi et al.[2]	N/A
human KLF4 forward, TGATTGTAGTGCTTTCTGGCTGGGCTCC, human KLF4 reverse, ACGATCGTGGCCCCGGAAAAGGACC,	Takahashi et al.[2]	N/A
human c-MYC forward, GCGTCCTGGGAAGGGAGATCCGGAGC, human c-MYC reverse, TTGAGGGGCATCGTCGCGGGAGGCTG,	Takahashi et al.[2]	N/A
human NANOG forward, CAGCCCCGATTCTTCCACCAGTCCC, human NANOG reverse, CGGAAGATTCCCAGTCGGGTTCACC,	Takahashi et al.[2]	N/A
human GDF3 forward, CTTATGCTACGTAAAGGAGCTGGG, human GDF3 reverse, GTGCCAACCCAGGTCCCGGAAGTT,	Takahashi et al.[2]	N/A
human REX1 forward, CAGATCCTAAACAGCTCGCAGAAT, human REX1 reverse, GCGTACGCAAATTAAAGTCCAGA,	Takahashi et al.[2]	N/A
human DNMT3b forward, TGCTGCTCACAGGGCCCGATACTTC, human DNMT3b reverse, TCCTTTCGAGCTCAGTGCACCACAAAAC,	Takahashi et al.[2]	N/A
human GAPDH forward, ACCACAGTCCATGCCATCAC, human GAPDH reverse, TCCACCACCCTGTTGCTGTA,	NCBI Reference Sequence	NM 004048
human β-ACTIN forward, CAACCGCGAGAAGATGAC, human β-ACTIN reverse, AGGAAGGCTGGAAGAGTG,	Kajihara et al.[3]	N/A
human HNF1β forward, CTGACTACCAGCTAACTCCAGTCTC, human HNF1β reverse, GACTGCAACTTTTTCTTCTGCTATC,	NCBI Reference Sequence	NM_000458.3
human HNF4α forward, GAACAGGAGCTCTTAACTACAGTGG, human HNF4α reverse, CTGTCAAGAGTCATGAATTCTCCTT,	NCBI Reference Sequence	NM_000457.4
human β-ACTIN forward, TGGCACCCAGCACAATGAA, human β-ACTIN reverse, CTAAGTCATAGTCCGCCTAGAAGCA,	NCBI Reference Sequence	NM_001101
Other		
LightCycler 96 Real-Time PCR system	Roche	Cat# 05 815 916 001
Invitrogen™ EVOS™ FL Auto Imaging System	Thermo Fisher Scientific	Cat# AMAFD1000
C.B-17/Icr-scid/scidJcl, male, 8 week-old	CLEA Japan	N/A

STEP-BY-STEP METHOD DETAILS

Reprogramming of human hepatocytes

Timing: 3–4 weeks

Human hepatocytes are reprogrammed into iPS/iTS-L cells using Sendai virus (SeV) vectors expressing OCT3/4, SOX2, KLF4, and c-MYC from a vender; see key resources table in this protocol.

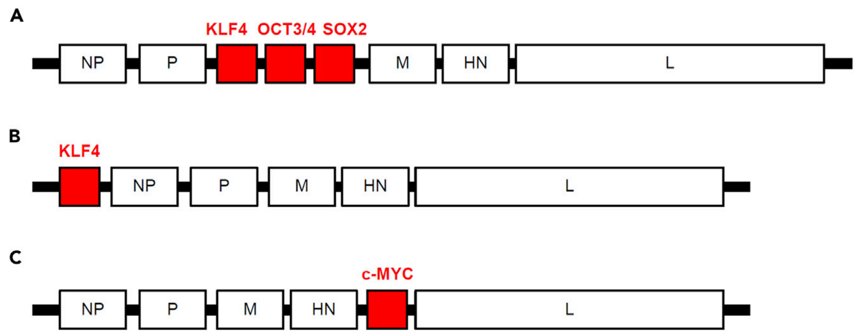

Figure 1. Schematic Representation of Sendai Virus (SeV) vectors

(A) SeV vector that allows the expression of human KLF4, OCT3/4, and SOX2 proteins.

(B) SeV vector that allows the expression of human KLF4 protein.

(C) SeV vector that allows expression of human c-MYC protein. NP; Nucleocapsid Protein. P; Phosphoprotein. M; Matrix protein. HN; Hemagglutinin-Neuraminidase. L; Large protein.

1. On Day -1, wash human hepatocytes twice with 10 mL of phosphate buffered saline (PBS).
2. The cells are then dissociated with 0.05% trypsin/EDTA for 5 min.
3. Add 5 mL of hepatocyte basal medium and break up cell aggregates by pipetting up and down with a 5 mL pipette.
4. The samples are then centrifuged at 700 × *g* for 5 min to pellet the cells.
5. Remove the supernatant.
6. Count cells and dilute to 1.0×10^5 cells/mL in hepatocyte basal medium.
7. Plate cells (1.0×10^5 cells/well) in 6-well plates.
8. Human hepatocytes are placed in an incubator at 37°C, 5% CO_2, and 85% humidity for 24 h.
9. On Day 0, prepare a 15 mL tube containing 2 mL of hepatocyte basal medium with 10 μL of SeV KOS (5.0×10^5 CIU), 10 μL of SeV Klf4 (5.0×10^5 CIU), and 10 μL of SeV Myc (5.0×10^5 CIU) ((5.0×10^5 CIU)/(1.0×10^5 cells)=5 multiplicity of infection (MOI)) (Figure 1).
10. Aspirate the culture medium.
11. Add 2 mL hepatocyte basal medium with SeV.
12. Place the 6-well plate in an incubator at 37°C, 5% CO_2, and 85% humidity for 24 h.
13. On Day 1, aspirate the culture medium and renew with fresh hepatocyte basal medium.
14. Renew with fresh hepatocyte basal medium daily for 3 days.
15. On Day 4, dissociate the hepatocytes and plate the hepatocytes in 60 mm dish containing MEFs using human ES/iPS/iTS-L cell culture medium.
16. The 60 mm dish is then placed in an incubator at 37°C, 5% CO_2, and 85% humidity.
17. Change human ES/iPS/iTS-L cell culture medium daily.
18. On Days 14–21, the reprogrammed cells should now transform to a round morphology. Every single clone should be independently expanded for characterization and freezing (Figure 2).

Teratoma formation assay

Timing: 10–15 weeks

The colonies similar to human ES cells or gut tube endodermal (GTE) cells (Figure 3) are selected for further cultivation and evaluation. Colonies similar to human ES cells should be iPS cells and generate teratomas. The colonies similar to GTE cells should be iTS-L cells and generate no teratomas.

19. Immunodeficient male mice (age: 7 weeks; C. B-17/Icr-scid/scidJcl) are anesthetized with isoflurane inhalation.
20. A total of 1.0×10^6 or more cells in 0.1 mL of cold Hanks' balanced salt solution (HBSS) are subcutaneously injected into the shoulders and buttocks using a 22 G injection needle.

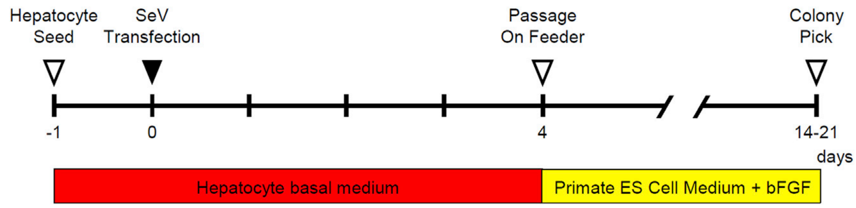

Figure 2. Time schedules for the induction of iPS/iTS-L cells transfected with SeV vectors
Open arrowheads indicate the times of cell seeding, passaging, and colony selection. Solid arrowheads indicate the times of transfection.

21. The mice are examined daily, and tumors are extracted at 10 or 15 weeks after surgery.

Quantitative RT−PCR

Timing: 1–2 days

The iTS-L cells continuously express HNF1β and HNF4α mRNA but not iPS cells.

22. The iPS/iTS-L cells are cultured in Primate ES Cell Medium to approximately 80% confluence.
23. RNA is prepared using a SuperPREP II Cell Lysis & RT Kit for quantitative PCR according to the manufacturer's instructions.
24. Real-time PCR analyses are performed using a LightCycler 96 Real-Time PCR system. The PCR protocol is as follows. Luna Universal qPCR Master Mix is used according to the manufacturer's instructions.

PCR master mix

Reagent	Final concentration	Amount
Luna Universal qPCR Master Mix	1×	10 μL
Forward primer (10 μM)	0.25 μM	0.5 μL
Forward primer (10 μM)	0.25 μM	0.5 μL
Template DNA	< 100 ng	variable
ddH_2O	N/A	variable
Total	**N/A**	**20 μL**

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	15 s	40 cycles
Annealing	60°C	60 s	
Denaturation	95°C	15 s	1 [Melt Curve Stage]
Annealing	60°C	60 s	
Denaturation	95°C	15 s	

EXPECTED OUTCOMES

iPS/iTS-L cells can be generated and passaged within 3–4 weeks. iPS/iTS-L clones can be expanded for characterization. We recommend the following characterization assays for distinguishing iPS/iTS-L cells: quantitative RT−PCR for the detection of markers of hepatic stem cells (iPS: NANOG(+), OCT3/4(+), HNF1β(-), and HNF4α(-)/iTS-L: NANOG(±) (less than 1/4 that of iPS), OCT3/4(±) (less than 1/4 that of iPS), HNF1β(+) and HNF4α(+)) and teratoma formation using immunodeficient mice.[1] Commercially available iPS cells and original hepatocytes should be used as positive/negative controls for expression.

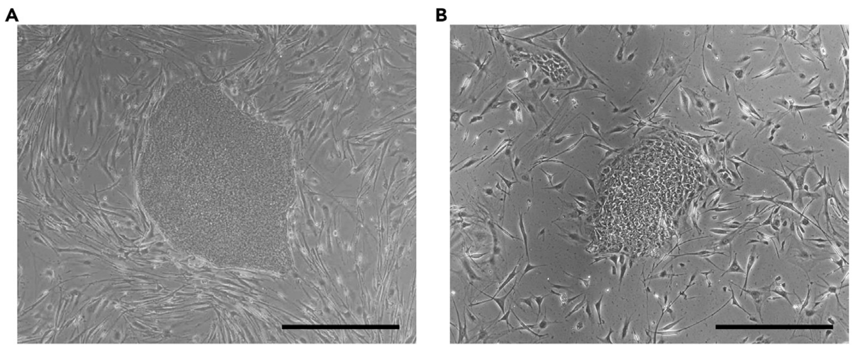

Figure 3. The morphologies of iPS and iTS-L cells
(A) The morphology of iPS cells.
(B) The morphology of iTS-L cells. The morphologies of iPS cells are similar to those of human ES cells. The morphologies of iTS-L cells are similar to those of gut tube endodermal cells. Scale bars = 200 μm.

LIMITATIONS

Although the generation efficiency of human iPS cells is low and reprogramming rates vary from 10% to 0.0001%,[4,5] the efficiency of iTS cells is relatively higher.[6]

The following limitations should be mentioned specifically. First, iPS/iTS-L clones should be expanded 3–5 passages before characterization to distinguish iPS/iTS-L cells. In low-passage iPS/iTS-L cells, transgenes derived from SeV may remain. The remaining reprogramming genes may change the characterization of iPS/iTS-L cells. Second, based on our experience, low passage hepatocytes (passages 2–5) should be used for reprogramming. Enzymatic dissociation or passaging and long-term culture have been described to affect the epigenetic state of the cell and to hinder efficient reprogramming.[7–9] Third, this protocol renders efficient reprogramming when using hepatocytes; other cell types for the generation of other induced tissue-specific stem cells may require further optimization.

TROUBLESHOOTING

Problem 1

Human hepatocytes do not proliferate properly (related to step 18 of "before you begin").

Potential solution

The cells are not diluted to less than 1×10^4 cells/cm^2. Low confluency gives rise to poor cell proliferation and early senescence. When human hepatocytes do not proliferate properly after 3–5 days of cell culture, we recommend replating the cells into new dishes at a higher cell density.

Problem 2

Excessive cell death after SeV transfection (related to step 9).

Potential solution

Check the confluency of human hepatocytes at the moment of transfection. The uneven distribution of human hepatocytes may result in cell death after SeV transfection. It should be over 50% for proper survival after transduction.

Problem 3

The generation efficiency of human iPS/iTS-L cells is extremely low (related to step 9).

Potential solution

Increase SeV at 6–10 MOI.

Problem 4

iTS-L cells do not grow well (related to step 18).

Potential solution

Increase the number of cells initially applied to the well and thereby increase cell density.

Problem 5

iTS-L cells spontaneously differentiate (related to step 18).

Potential solution

New bFGF and Primate ES Cell Medium are prepared.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hirofumi Noguchi noguchih@med.u-ryukyu.ac.jp.

Materials availability

All material used are listed in the key resources table, and any further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

Data and code availability

This protocol does not include the generation of datasets.

ACKNOWLEDGMENTS

This work was supported in part by JSPS KAKENHI (grant numbers JP22K08759, JP21K19537, JP20H03745, and JP19K09051), the Okinawa Science and Technology Innovation System Construction Project (OSTC), and the Japan Agency for Medical Research and Development (AMED) (grant number JP22bm0104001).

AUTHOR CONTRIBUTIONS

Conceived and Designed Experiments, H.N.; Performed Experiments, H.N., Y.N., C.S.; Analyzed the Data, H.N., Y.N., C.S.; Wrote the Manuscript, H.N.; Funding Acquisition, H.N., M.W., M.M., M.T., I.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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