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

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.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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 iPSC and iTS-L cells
Protocol for the generation of human induced hepatic stem cells using Sendai virus vectors

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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% CO2, 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

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 vendor; see key resources table in this protocol. Thaw one frozen vial of murine embryonic fibroblasts (MEFs) (3.0 × 10⁶ 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 × 10⁵ cells/dish).
13. Place the MEFs in an incubator at 37°C, 5% CO₂, 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

14. Human hepatocytes are obtained from a vendor; see the key resources table in this protocol. Thaw one frozen vial of human hepatocytes (3.0 × 10⁶ 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% CO₂, 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 |
|---------------------|--------|------------|
| 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)
## 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.

### Critical reagents

#### 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.**

### Critical commercial assays

| 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 (x100) | FUJIFILM Wako Pure Chemical Corporation | Cat# 16823191 |
| Hanks’ Balanced Salt Solution (HBSS) | Life Technologies | Cat# 14025092 |

### Critical reagents

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### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 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

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Cryopreserved Hepatocytes Species:Human, Lot#S1412T, Lot#S1238 and Lot#S1350 | Kaly-Cell | Cat# HHPC-2 M |
| hiPSC Lines 201B7 | CiRA Foundation | N/A |
| MEF cells | ReproCELL Inc. | Cat# RCHEFC003 |

### Oligonucleotides

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| human OCT3/4 forward, GACAGGGGGAGGGGAGGAGCTAGG, human OCT3/4 reverse, CTTCCTCCTAACCACTGGCCCAAC, | Takahashi et al.² | N/A |
| human SOX2 forward, GGGAATGGGAGGGGTGAAAGGG, human SOX2 reverse, TTGCCTGAAGTGTGAGATGTG, | Takahashi et al.² | N/A |
| human KLF4 forward, TGGTATGAGCTTGTGCTGGGCTCC, human KLF4 reverse, AGCATGTGGCCCGAGAAAGGACC, | Takahashi et al.² | N/A |
| human c-MYC forward, GCCTCTGGAAGGAGATCGGAGAC, human c-MYC reverse, TTGAGGGGATCGTCGGGAGTCCGTG, | Takahashi et al.² | N/A |
| human NANOG forward, CACCCCCTCATCTCCACCTCTCC, human NANOG reverse, CGGAAGATCCAACTTACTCTTACC, | Takahashi et al.² | N/A |
| human GDF3 forward, CCTATGCTCGTAAAGGAGGCTGG, human GDF3 reverse, TGCCCAACCCAGCTCCGGGAAGT, | Takahashi et al.² | N/A |
| human REX1 forward, CAGATCCTAACAGCTCCGCAGAT, human REX1 reverse, GCCTACGCAATATAAGGTCCAGA, | Takahashi et al.² | N/A |
| human DNMT3b forward, TGCTGCTCACAGGGCGGATACCTC, human DNMT3b reverse, TCTCTCTAGCTCTAAGGGGCACTCCAAACAC, | Takahashi et al.² | N/A |
| human GAPDH forward, ACCACAGTCATGGGCTACAC, human GAPDH reverse, TCCACCAAACCTGTGTCTTA, | NCBI Reference Sequence NM_004048 |
| human ACTN forward, CAAACCGCGCAAGAGATGAC, human ACTN reverse, AGGAAGGCTGGAAAGAGTGTG, | Kajihara et al.³ | N/A |
| human HNF1β forward, CTGACTCTAGTAAGAGCTGG, human HNF1β reverse, GACTGCCAATTTTTTCTCTCCTATC, | NCBI Reference Sequence NM_000458.3 |
| human HNF4α forward, CAGAAGGAGCTCTTAACCATAGTG, human HNF4α reverse, GTGGCAAGAAGTCTAAGTTTCTCTCTTT, | NCBI Reference Sequence NM_000457.4 |
| human β-ACTIN forward, TGGCAACCAAGACCAATGAAA, human β-ACTIN reverse, CTAAATGCTATGCCCTAGAAGGCA, | NCBI Reference Sequence NM_001101 |

### Other

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| 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/cr/scid/scid/lcl, male, 8 week-old | CLEA Japan | N/A |

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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 \( 1.0 \times 10^5 \) cells/mL in hepatocyte basal medium.
7. Plate cells (\( 1.0 \times 10^5 \) 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 \( \mu L \) of SeV KOS (\( 5.0 \times 10^5 \) CIU), 10 \( \mu L \) of SeV Klfi4 (\( 5.0 \times 10^5 \) CIU), and 10 \( \mu L \) of SeV Myc (\( 5.0 \times 10^5 \) CIU) \((5.0 \times 10^5 \) CIU)/(\( 1.0 \times 10^5 \) 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 iso-flurane inhalation.
20. A total of \( 1.0 \times 10^6 \) 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.

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**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.
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 iPSCs.

22. The iPSC/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 | Final concentration | Amount |
|----------------|---------------------|--------|
| Reagent        |                     |        |
| Luna Universal qPCR Master Mix   | 1 X                | 10 μL  |
| Forward primer (10 μM)           | 0.25 μM            | 0.5 μL |
| Template DNA               | < 100 ng           | variable |
| ddH₂O                       | N/A                | variable |
| Total                      | N/A                | 20 μL  |

| PCR cycling conditions | Temperature | Time | Cycles |
|------------------------|-------------|------|--------|
| Steps                  |             |      |        |
| 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

iPSC/iTS-L cells can be generated and passaged within 3–4 weeks. iPSC/iTS-L clones can be expanded for characterization. We recommend the following characterization assays for distinguishing iPSC/iTS-L cells: quantitative RT–PCR for the detection of markers of hepatic stem cells (iPSCs: NANOG(+), OCT3/4(+), HNF1β(−), and HNF4α(x)/iTS-L: NANOG(±) (less than 1/4 that of iPSCs), OCT3/4(±) (less than 1/4 that of iPSCs), HNF1β(+) and HNF4α(+) and teratoma formation using immunodeficient mice. Commercially available iPSC cells and original hepatocytes should be used as positive/negative controls for expression.
LIMITATIONS
Although the generation efficiency of human iPS cells is low and reprogramming rates vary from 10% to 0.0001%, the efficiency of iTS cells is relatively higher.

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. 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 $1 \times 10^4$ cells/cm². 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**
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**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.

**REFERENCES**

1. Nakashima, Y., Miyagi-Shiohira, C., Saitoh, I., Watanabe, M., Matsushita, M., Tsukahara, M., and Noguchi, H. (2022). Induced hepatic stem cells are suitable for human hepatocyte production. iScience 25, 105052.

2. Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., et al. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.

3. Kajihara, R., Numakawa, T., Odaka, H., Yaginuma, Y., Fusaki, N., Okumya, T., et al. (2020). Novel Drug Candidates Improve Ganglioside Accumulation and Neural Dysfunction in GM1 Gangliosidosis Models with Autophagy Activation. Stem Cell Reports 14, 909–923.

4. Gonzalez-Muñoz, E., Arboleda-Estudillo, Y., Otu, H.H., and Cibelli, J.B. (2014). Cell reprogramming. Histone chaperone ASF1A is required for maintenance of pluripotency and cellular reprogramming. Science 345, 822–825.

5. Gonzalez-Munoz, E., and Cibelli, J.B. (2018). Somatic cell reprogramming informed by the oocyte. Stem Cells Dev. 27, 871–887.

6. Noguchi, H., Miyagi-Shiohira, C., Nakashima, Y., Kinjo, T., Kobayashi, N., Saitoh, I., Watanabe, M., Shapiro, A.M.J., and Kin, T. (2019). Induction of expandable tissue-specific progenitor cells from human pancreatic tissue through transient expression of defined factors. Mol. Ther. Methods Clin. Dev. 13, 243–252.

7. Halley-Stott, R.P., and Gurdon, J.B. (2013). Epigenetic memory in the context of nuclear reprogramming and cancer. Brief. Funct. Genomics 12, 164–173.

8. Kim, K., Doi, A., Wen, B., Ng, K., Zhao, R., Cahan, P., Kim, J., Aryee, M.J., Ji, H., Ehrich, L.I.R., et al. (2010). Epigenetic memory in induced pluripotent stem cells. Nature 467, 285–290.

9. Streckfuss-Bömeke, K., Wolf, F., Azizian, A., Staake, M., Tiburec, M., Wagner, S., Hubscher, D., Dreisell, R., Chen, S., Jende, J., et al. (2012). Comparative study of human-induced pluripotent stem cells derived from bone marrow cells, hair keratinocytes, and skin fibroblasts. Eur. Heart J. 34, 2618–2629.