Low-molecular-weight inhibitors of cell differentiation enable efficient growth of mouse iPS cells under feeder-free conditions

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Abstract Embryonic stem cells and induced pluripotent stem (iPS) cells are usually maintained on feeder cells derived from mouse embryonic fibroblasts (MEFs). In recent years, the cell culture of iPS cells under serum- and feeder-free conditions is gaining attention in overcoming the biosafety issues for clinical applications. In this study, we report on the use of multiple small-molecular inhibitors (i.e., CHIR99021, PD0325901, and Thiazovivin) to efficiently cultivate mouse iPS cells without feeder cells in a chemically-defined and serum-free condition. In this condition, we showed that mouse iPS cells are expressing the Nanog, Oct3/4, and SSEA-1 pluripo-

tent markers, indicating that the culture condition is optimized to maintain the pluripotent status of iPS cells. Without these small-molecular inhibitors, mouse iPS cells required the adaptation period to start the stable cell proliferation. The application of these inhibitors enabled us the shortcut culture method for the cellular adaptation. This study will be useful to efficiently establish mouse iPS cell lines without MEF-derived feeder cells.

Keywords Induced pluripotent stem cells · Cell culture condition · Serum-free · Feeder-free · Low-molecular-weight compounds

Abbreviations

ES	Embryonic stem
iPS	Induced pluripotent stem
MEFs	Mouse embryonic fibroblasts
SSEA	Stage specific embryonic antigen
HPV	Human papilloma virus
STEMCCA	Stem cell cassette
DMEM	Dulbecco's modified Eagle's medium
KSR	Knockout serum replacement
LIF	Leukemia inhibitory factor
2i	2 Inhibitors (CHIR99021 and PD0325901)
Tzv	Thiazovivin
GSK-3 β	Glycogen synthase kinase 3 β
MAPK	Mitogen-activated protein kinase
ROCK	Rho-associated Kinase
PD	Population doubling

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EBs	Embryoid bodies
AP	Alkaline phosphatase
DAPI	4',6-Diamidino-2-phenylindole
RT	Reverse transcription
PCR	Polymerase chain reaction
cDNA	Complementary DNA

Introduction

The reprogramming of somatic cells to generate induced pluripotent stem (iPS) cells was achieved by expressing four defined transcription factors: Oct3/4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006). Such iPS cells have major potential in the study and therapy of human diseases because they are capable of self-renewal and can generate all the three primary germ layers: ectoderm, mesoderm, and endoderm. iPS cells are usually maintained on feeder cells that are derived from mouse embryonic fibroblasts (MEFs) (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007). However, several feeder-free conditions were developed recently especially for human embryonic stem (ES) cells and iPS cells to ensure biosafe human clinical applications (Xu et al. 2001; Amit et al. 2004; Rosler et al. 2004; Lu et al. 2006; Navarro-Alvarez et al. 2008; Miyazaki et al. 2008; Sun et al. 2009; Kitajima and Niwa 2010; Rodin et al. 2010; Hayashi et al. 2010; Fukusumi et al. 2013). Animal-derived components are relatively high risk for potential infections in clinical regenerative medicine. In addition to regenerative medicine, iPS cells are also expected to play an important role in drug screening. However, the mixed culture condition of iPS cells and the need of feeder cells make the evaluation of the effects of drugs difficult. Furthermore, the establishment of serum- and feeder-free culture conditions is significant to overcome the ethical issues related to animal experiments.

Furue et al. (2005) previously developed a serum-free, chemically defined medium called “ESF7” (now available as ESF-C), which maintains mouse ES cells on collagen-coated dishes. In this manuscript, we show that mouse iPS cells can be cultured in feeder-free conditions as well as on feeder cells using the ESF-C medium containing different kinds of low-molecular-weight cell differentiation inhibitors.

Materials and methods

Mouse iPS cell induction

A human papilloma virus-derived E6 gene was introduced to MEFs (E6-MEFs) to extend the cell proliferation until cellular senescence (Yamamoto et al. 2003). Then, STEMCCA-loxP lentiviral vector (STEMCCA means a “stem cell cassette”), which is composed of Yamanaka 4 factors and self-cleaving 2A peptides, was introduced into E6-MEFs to generate iPS cells (Sommer et al. 2009).

Cell culture

MEFs and feeder cells were cultured in MEF medium consisting of Dulbecco’s modified Eagle’s medium (DMEM; #08459-35, Nacalai Tesque, Kyoto, Japan) supplemented with 10 % fetal bovine serum (FBS; #12483-020, Invitrogen, Carlsbad, CA, USA) and 100× antibiotic–antimycotic mixed solution (#02892-54, Nacalai Tesque). The experimental conditions for the established mouse iPS cells are as follows: (1) mouse iPS cells were cultured on MEF feeder cells in mouse iPS cell medium [DMEM with 15 % Knockout Serum Replacement (referred to as KSR, #10828-028, Invitrogen), 0.22 mM 2-mercaptoethanol (#21438-82, Nacalai Tesque), 100 × MEM Non-essential Amino Acids Solution (#139-15651, Wako Pure Chemical Industries, Osaka, Japan), 100× antibiotic–antimycotic mixed solution and 1,000× leukemia inhibitory factor (LIF, Human, recombinant, Culture Supernatant, #125-05603, Wako Pure Chemical Industries)]; (2) mouse iPS cells were cultured on collagen (Cellmatrix Type I-A, Nitta Gelatin, Osaka, Japan)-coated dishes in ESF-C medium (#2004-05, Cell Science and Technology Institute, Sendai, Japan) containing 1,000 × LIF; (3) the same cell culture of point (2) containing three low-molecular-weight inhibitors [1.5 μM CHIR99021 (#163-24001, Wako Pure Chemical Industries), 0.5 μM PD0325901 (hereinafter the combination of these two inhibitors are referred to as 2i for short, #13034, Cayman Chemical, Ann Arbor, MI, USA), and 0.5 μM Thiazovivin (referred to as Tzv, #04-0017, Stemgent, Cambridge, MA, USA)]. The preparation of MEFs and feeder cells were described in our previous report (Donai et al. 2013).

Population doubling (PD) assay

Mouse iPS cells were seeded at a density of 5.0×10^4 cells per well in a 6-well plate. We evaluated three independent mouse iPS clones. The passages of the mouse iPS cells were carried out until one well of the 6-well plate became confluent. The cell number was counted using an automatic cell counter (Countess, Invitrogen), and the cell growth was evaluated with triplicated experiments. The passage process was repeated up to the 6th passage. Cell PD was obtained from the number of cells using the formula described in a previous report as follows: $PD = \log_2(a/b)$, where 'a' is the number of the cells counted in passage and 'b' represents the number of seeded cells (Qin et al. 2012). The results of PD assay are shown as means with standard deviations. Statistical significance was evaluated using Student's *t* test ($P < 0.05$).

Embryoid bodies (EBs) formation

To form EBs, $2\text{--}3 \times 10^6$ iPS cells were seeded into a Petri dish (for bacterial culture). After 3–4 days, floating EBs were picked up and transferred to 0.1 % gelatin-coated 24-well plates and cultured for 3–4 additional days. EB medium contained DMEM with 15 % FBS, 100× antibiotic–antimycotic mixed solution, 0.22 mM 2-mercaptoethanol and 100× MEM non-essential amino acids solution.

Alkaline phosphatase (AP) staining and Immunocytochemistry

Detailed method for detection of AP activity and pluripotent markers with immunocytochemistry was previously described in our publication (Donai et al. 2013). EBs on gelatin-coated dishes were stained with β III tubulin monoclonal antibody (1:600 dilution, #MAB1637, Millipore, Billerica, MA, USA), 4',6-diamidino-2-phenylindole (DAPI) solution (1:1,000 dilution, #340-07971, Wako Pure Chemical Industries) and Alexa Fluor 568 (1:500 dilution, #A21124, Invitrogen).

Reverse transcription (RT) and quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from cells confluent in a 6-well plate and a Petri dish using the TaKaRa

FastPure RNA Kit (#9190, TaKaRa Bio, Shiga, Japan) according to the manufacturer's protocol. Complementary DNA (cDNA) synthesis and PCR were performed in a similar way as in our previous report (Donai et al. 2013).

Results and discussion

We initially investigated whether mouse iPS cells could maintain their specific morphologies in each culture condition. Figure 1 shows the cell morphology and alkaline phosphatase (AP) staining under the conditions 1 and 2. In condition 1, using feeder cells as control, the cells showed mouse ES cell-like morphology, characterized by larger nuclei, scant cytoplasm, dome-shaped colonies, and strong positive AP staining (Fig. 1a, b). In condition 2 (collagen coating + ESF-C), mouse iPS cells showed colonies with scattering morphologies (Fig. 1c), and relatively weak AP activity (Fig. 1d). Then, we compared the cell proliferation between condition 1 and 2 using the corresponding PD values. The cell numbers in condition 1 exponentially increased (Fig. 1e, white circle) and showed aggressive growth potential represented by >1.5 population doubling per day (Fig. 1f, open bar). In condition 2, the cell growth of mouse iPS cells was remarkably delayed (Fig. 1e, black circle). The delayed iPS cell growth restored after passage three, suggesting that the cellular adaptation of mouse iPS cells to the feeder-free condition was necessary to ensure a stable cell growth. The delayed cell growth in the early passages of mouse iPS cells was apparent when the PD value was normalized by the culture day (PD per day, Fig. 1f). Furthermore, although we tried to maintain the three independent mouse iPS clones under feeder-free conditions, one of the mouse iPS clones did not show cell growth and was unable to survive in ESF-C medium (data not shown).

In condition 3, we added three inhibitors (CHIR99021, PD0325901 and Thiazovivin) to the ESF-C medium. CHIR99021 is reported as the inhibitor of glycogen synthase kinase 3 β (GSK-3 β) signaling, which has a large impact to maintain pluripotency of ES and iPS cells effectively (Sato et al. 2004; Besser 2004; Polychronopoulos et al. 2004; Ying et al. 2008). PD0325901 targets mitogen-activated protein kinase (MAPK/ERK kinase) (Sebolt-Leopold and Herrera 2004; Bain et al. 2007). In combination with

Fig. 1 Morphology of mouse iPS cell colonies in condition 1 and condition 2. *Condition 1*, on feeders in mouse iPS cell medium with LIF; *Condition 2*, on collagen-coated dishes (feeder-independent) in ESF-C medium (serum-free) with LIF. **a**, **b** Morphology of mouse iPS cell colonies (**a**) and AP staining (**b**), respectively, in condition 1, showing compact colonies and strong reactivity. **c**, **d** Morphology of mouse iPS cell colonies (**c**) and AP staining (**d**), respectively, in condition 2. Shape scattering of colonies and weak AP signals are evident. **e** The population doublings under condition 1 and condition 2. $n = 3 \times 3$ **f** Population doubling per day. *Open and closed bars* correspond to condition 1 and 2, respectively. The cell growth was partly recovered at late passages in condition 2

CHIR99021, PD0325901 affects the self-renewal of ES and iPS cells to prevent cell differentiation (Lin et al. 2009). Thiazovivin acts as a Rho-associated Kinase inhibitor, which can improve the survival of ES and iPS cells from single cell dissociation (Lin et al. 2009; Xu et al. 2010). The iPS cell colonies were more rounded with clearer boundaries than those obtained under condition 2 (Fig. 2a). In agreement with the improved morphology, the reactivity against AP of the cells in the ESF-C in the presence of these three inhibitors as well as on the feeder cells became strong (Fig. 2b). Furthermore, mouse iPS cells showed enhanced cell proliferation compared to those in condition 2 (Fig. 2c). Although the cell proliferation speed in condition 3 was significantly lower than that

in condition 1, the PD value of condition 3 was similar to that of condition 1. As shown in Fig. 2d, the effect of these low-molecular inhibitors (2i + Tzv) became more apparent with increasing PD per day. Based on these results, we concluded that 2i + Tzv enabled us to efficiently maintain mouse iPS cells in the chemically defined medium and in feeder-free conditions without requiring the cellular adaptation period. We evaluated the expression of pluripotent markers of mouse iPS cells under condition 3 by immunocytochemistry (Fig. 2e). The detection of the expression of markers in mouse iPS cells was in good agreement with that of mouse ES cells. Briefly, the mouse iPS cell colonies in condition 3 showed immunoreactivity against Nanog, Oct3/4, and SSEA-1 antibodies. From

Fig. 2 The small-molecule inhibitors improve the morphology of mouse iPS cell colonies, their proliferation, and the expression of pluripotent markers. Condition 3, on collagen-coated dishes (feeder-independent) in ESF-C medium (serum-free) with LIF, 2i and Tzv. **White bar** = 50 μ m. **a, b** Morphology of mouse iPS cell colonies and AP staining, respectively, under condition 3. The cells formed dome-shaped colonies with clear edges and displayed strong AP activity. **c** The number of population doubling under condition 1 and condition 3. The growth speed

in condition 3 showed enhanced proliferation similar to that of condition 1. **d** Population doubling per day. Mouse iPS cells showed good cell proliferation starting from early passages. *Open and closed bars* represent condition 1 and 3, respectively. **e** Expression of pluripotent markers in mouse iPS cells. Nanog- and Oct 3/4-positive staining, which could be overlapped with DAPI blue counterstaining, was detected in the nuclei. Stage-specific embryonic antigen (SSEA)-1 protein localizes at the cell surface. **White bar** = 100 μ m. (Color figure online)

Fig. 3 In vitro differentiation of iPS cells. **a, b** Morphology of EBs floating in a Petri dish after 4 days incubation. **Left panel (a)** EBs derived from iPS cells cultivated under condition 1; **Right panel (b)** EBs derived from iPS cells cultivated under condition 3. **White bar** 100 μ m. **c** Detection of stem cell-specific genes by real-time quantitative polymerase chain reaction (RT-

qPCR) between iPS cells and EBs in condition 3. **White column** iPS cells (undifferentiated state); **Black column** EBs (differentiated state). $n = 3$ **d** immunostaining of β III tubulin (ectoderm) marker in EBs cultivated 4 days on gelatin-coated dishes derived from condition 3. **White bar** 50 μ m

these data, we concluded that our mouse iPS cells maintained a pluripotent state even in the absence of serum and feeder cells.

To validate whether iPS cells have differentiation capacity in feeder-independent culture, we tried to form EBs after incubation in condition 1 and 3. We could not detect any difference in EBs formation between condition 1 and 3 (Fig. 3a, b). After EBs formation, we detected lower expression levels of stem cell-specific genes compared with pluripotent state (Fig. 3c). Furthermore, we also detected β III tubulin (ectoderm) marker by immunostaining (Fig. 3d). These findings indicate that differentiation capacity is still intact even in chemically-defined and serum-free condition.

In this study, we demonstrated the efficient expansion of mouse iPS cells on collagen-coated dishes under serum- and feeder-free conditions in combination with three small-molecule cell differentiation inhibitors. We could not observe any morphological or cell proliferation change until passage 10. We also confirmed the effect of the inhibitors on the cell growth of mouse ES cell line E14tg2a. They expectedly showed growth pattern similar to that of iPS cells under the presence of these inhibitors (data not shown). Collagen coating is a simple method and does not require special techniques. This method provides a useful means for using mouse iPS cells to assess novel drugs and animal-derived components without adaptation periods.

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References

- Amit M, Shariki C, Margulets V, Itskovitz-Eldor J (2004) Feeder layer- and serum-free culture of human embryonic stem cells. *Biol Reprod* 70:837–845. doi:[10.1095/biolreprod.103.021147](https://doi.org/10.1095/biolreprod.103.021147)
- Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P (2007) The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408:297–315. doi:[10.1042/BJ20070797](https://doi.org/10.1042/BJ20070797)
- Besser D (2004) Expression of nodal, lefty-a, and lefty-B in undifferentiated human embryonic stem cells requires activation of Smad2/3. *J Biol Chem* 279:45076–45084. doi:[10.1074/jbc.M404979200](https://doi.org/10.1074/jbc.M404979200)
- Donai K, Kuroda K, Guo Y, So KH, Sone H, Kobayashi M, Nishimori K, Fukuda T (2013) Establishment of a reporter system to monitor silencing status in induced pluripotent stem cell lines. *Anal Biochem* 443:104–112. doi:[10.1016/j.ab.2013.08.014](https://doi.org/10.1016/j.ab.2013.08.014)
- Fukusumi H, Shofuda T, Kanematsu D, Yamamoto A, Suemizu H, Nakamura M, Yamasaki M, Ohgushi M, Sasai Y, Kanemura Y (2013) Feeder-free generation and long-term culture of human induced pluripotent stem cells using pericellular matrix of decidua derived mesenchymal cells. *PLoS ONE* 8:e55226. doi:[10.1371/journal.pone.0055226](https://doi.org/10.1371/journal.pone.0055226)
- Furue M, Okamoto T, Hayashi Y, Okochi H, Fujimoto M, Myoishi Y, Abe T, Ohnuma K, Sato GH, Asashima M, Sato JD (2005) Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In Vitro Cell Dev Biol Anim* 41:19–28. doi:[10.1290/0502010.1](https://doi.org/10.1290/0502010.1)
- Hayashi Y, Chan T, Warashina M, Fukuda M, Ariizumi T, Okabayashi K, Takayama N, Otsu M, Eto K, Furue MK, Michiue T, Ohnuma K, Nakauchi H, Asashima M (2010) Reduction of N-glycolylneuraminic acid in human induced pluripotent stem cells generated or cultured under feeder- and serum-free defined conditions. *PLoS ONE* 5:e14099. doi:[10.1371/journal.pone.0014099](https://doi.org/10.1371/journal.pone.0014099)
- Kitajima H, Niwa H (2010) Clonal expansion of human pluripotent stem cells on gelatin-coated surface. *Biochem Biophys Res Commun* 396:933–938. doi:[10.1016/j.bbrc.2010.05.026](https://doi.org/10.1016/j.bbrc.2010.05.026)
- Lin T, Ambasudhan R, Yuan X, Li W, Hilcove S, Abujarour R, Lin X, Hahm HS, Hao E, Hayek A, Ding S (2009) A chemical platform for improved induction of human iPSCs. *Nat Methods* 6:805–808. doi:[10.1038/nmeth.1393](https://doi.org/10.1038/nmeth.1393)
- Lu J, Hou R, Booth CJ, Yang SH, Snyder M (2006) Defined culture conditions of human embryonic stem cells. *Proc Natl Acad Sci USA* 103:5688–5693. doi:[10.1073/pnas.0601383103](https://doi.org/10.1073/pnas.0601383103)
- Miyazaki T, Futaki S, Hasegawa K, Kawasaki M, Sanzen N, Hayashi M, Kawase E, Sekiguchi K, Nakatsuji N, Suemori H (2008) Recombinant human laminin isoforms can support the undifferentiated growth of human embryonic stem cells. *Biochem Biophys Res Commun* 375:27–32. doi:[10.1016/j.bbrc.2008.07.111](https://doi.org/10.1016/j.bbrc.2008.07.111)
- Navarro-Alvarez N, Soto-Gutierrez A, Yuasa T, Yamatsuji T, Shirakawa Y, Nagasaka T, Sun SD, Javed MS, Tanaka N, Kobayashi N (2008) Long-term culture of Japanese human embryonic stem cells in feeder-free conditions. *Cell Transpl* 17:27–33
- Polychronopoulos P, Magiatis P, Skaltsounis AL, Myrianthopoulos V, Mikros E, Tarricone A, Musacchio A, Roe SM, Pearl L, Leost M, Greengard P, Meijer L (2004) Structural basis for the synthesis of indirubins as potent and selective inhibitors of glycogen synthase kinase-3 and cyclin-dependent kinases. *J Med Chem* 47:935–946. doi:[10.1021/jm031016d](https://doi.org/10.1021/jm031016d)
- Qin XY, Fukuda T, Yang L, Zaha H, Akanuma H, Zeng Q, Yoshinaga J, Sone H (2012) Effects of bisphenol A exposure on the proliferation and senescence of normal human

- mammary epithelial cells. *Cancer Biol Ther* 13:296–306. doi:[10.4161/cbt.18942](https://doi.org/10.4161/cbt.18942)
- Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K (2010) Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol* 28:611–615. doi:[10.1038/nbt.1620](https://doi.org/10.1038/nbt.1620)
- Rosler ES, Fisk GJ, Ares X, Irving J, Miura T, Rao MS, Carpenter MK (2004) Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn* 229:259–274. doi:[10.1002/dvdy.10430](https://doi.org/10.1002/dvdy.10430)
- Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH (2004) Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 10:55–63. doi:[10.1038/nm979](https://doi.org/10.1038/nm979)
- Sebolt-Leopold JS, Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat Rev Cancer* 4:937–947. doi:[10.1038/nrc1503](https://doi.org/10.1038/nrc1503)
- Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G (2009) Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells* 27:543–549. doi:[10.1634/stemcells.2008-1075](https://doi.org/10.1634/stemcells.2008-1075)
- Sun N, Panetta NJ, Gupta DM, Wilson KD, Lee A, Jia F, Hu S, Cherry AM, Robbins RC, Longaker MT, Wu JC (2009) Feeder-free derivation of induced pluripotent stem cells from adult human adipose stem cells. *Proc Natl Acad Sci USA* 106:15720–15725. doi:[10.1073/pnas.0908450106](https://doi.org/10.1073/pnas.0908450106)
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676. doi:[10.1016/j.cell.2006.07.024](https://doi.org/10.1016/j.cell.2006.07.024)
- Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872. doi:[10.1016/J.Cell.11.019](https://doi.org/10.1016/J.Cell.11.019)
- Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* 19:971–974. doi:[10.1038/nbt1001-971](https://doi.org/10.1038/nbt1001-971)
- Xu Y, Zhu X, Hahm HS, Wei W, Hao E, Hayek A, Ding S (2010) Revealing a core signaling regulatory mechanism for pluripotent stem cell survival and self-renewal by small molecules. *Proc Natl Acad Sci USA* 107:8129–8134. doi:[10.1073/pnas.1002024107](https://doi.org/10.1073/pnas.1002024107)
- Yamamoto A, Kumakura S, Uchida M, Barrett JC, Tsutsui T (2003) Immortalization of normal human embryonic fibroblasts by introduction of either the human papillomavirus type 16 E6 or E7 gene alone. *Int J Cancer* 106:301–309. doi:[10.1002/ijc.11219](https://doi.org/10.1002/ijc.11219)
- Ying QL, Wray J, Nichols J, Battle-Morera L, Doble B, Woodgett J, Cohen P, Smith A (2008) The ground state of embryonic stem cell self-renewal. *Nature* 453:519–523. doi:[10.1038/nature06968](https://doi.org/10.1038/nature06968)
- Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917–1920. doi:[10.1126/science.1151526](https://doi.org/10.1126/science.1151526)