Video Article

Integration Free Derivation of Human Induced Pluripotent Stem Cells Using Laminin 521 Matrix

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Abstract

Xeno-free and fully defined conditions are key parameters for robust and reproducible generation of homogenous human induced pluripotent stem (hiPS) cells. Maintenance of hiPS cells on feeder cells or undefined matrices are susceptible to batch variances, pathogenic contamination and risk of immunogenicity. Utilizing the defined recombinant human laminin 521 (LN-521) matrix in combination with xeno-free and defined media formulations reduces variability and allows for the consistent generation of hiPS cells. The Sendai virus (SeV) vector is a non-integrating RNA-based system, thus circumventing concerns associated with the potential disruptive effect on genome integrity integrating vectors can have. Furthermore, these vectors have demonstrated relatively high efficiency in the reprogramming of dermal fibroblasts. In addition, enzymatic single cell passaging of cells facilitates homogeneous maintenance of hiPS cells without substantial prior experience of stem cell culture. Here we describe a protocol that has been extensively tested and developed with a focus on reproducibility and ease of use, providing a robust and practical way to generate defined and xeno-free human hiPS cells from fibroblasts.

Video Link

The video component of this article can be found at https://www.jove.com/video/56146/

Introduction

Since the first derivation of hiPS cell lines by Takahashi *et al.*[1,2], hiPS cells have provided a useful tool for disease modeling, drug discovery and as source material for generating cell therapies in regenerative medicine[3]. hiPS cell culture has long been dependent on co-culture with fibroblast feeder cells[4,5] or on Matrigel[6] and with media formulations containing fetal bovine serum (FBS). Batch-to-batch variances are a common consequence of the undefined nature of these culture conditions, resulting in unpredictable variations, which is a major contributor to the unreliability of these protocols[7]. The development of defined medium such as Essential 8 (E8)[8] and defined cell culture matrices for instance LN-521[9], allows for the establishment of highly reproducible protocols and aid in the robust generation and maintenance of homogenous hiPS cells[7,8,9,10].

Development of integration free reprogramming techniques have been a leap forward. Originally, reprogramming depended on retroviral vectors which randomly integrated into the genome with disruptive effects on genomic integrity[11]. Advances in reprogramming methodologies includes the development of RNA based vectors. RNA vectors have an advantage over the DNA based reprogramming method as unintended integration through genomic recombination is not possible[12]. SeV vectors provide high and transient expression of exogenous factors through single-stranded RNA without a DNA-phase[11]. The reprogramming vectors delivered by the SeV are diluted throughout cell expansion and eventually shed from culture providing a foot-print free way of reprogramming. Thereafter, maintenance of pluripotency is dependent on endogenous expression of the pluripotency genes[2].

As pioneering hiPS cell based therapies are beginning to move into clinical trials, the demands for standardized batches, reproducibility, and safety are essential issues to address[13]. Therefore, products of animal origin should be avoided. For instance, the use of xenogeneic products has been associated with risk of nonhuman pathogen contamination. Also, cells cultured in the presence of animal derived culture components have been shown to incorporate nonhuman siliac acids into cell membranes which threatens to render derived cells immunogenic[14]. Hence, the need to eliminate xenogeneic products is necessary to any future clinical pursuits. This protocol applies xeno-free and defined culture in the maintenance of hiPS cells moving cells closer to clinical compliance.

This protocol describes a consistent, highly reproducible and easy-to-use method that generates standardized hiPS cells from fibroblasts. It also offers a user-friendly culture system for the maintenance of established hiPS cells. This protocol has been used to derive more than 300 hiPS cell lines in the Swedish national human iPS Core facility at Karolinska Institutet of which some lines have previously been described[15,16].

Protocol

The collection of patient material and derivation of hiPS cells is approved by the Ethics Review Board, Stockholm, March 28, 2012, Registration number: 2012/208-31/3. Cell culture steps should be performed in biosafety cabinets unless otherwise mentioned. Always practice sterile handling techniques when working with cells. Allow media, plates and reagents to reach room temperature before starting. Incubate cells at 37 °C, 5% CO_2 in high humidity.

1. Isolation of Human Fibroblasts from Dermal Biopsy

1. **Preparations of culture medium, digestive enzymes, coating of dishes and dissecting tools.**
    1. Prepare Fibroblast medium: 44.5 mL Iscove's Modified Dulbecco's Medium (IMDM), 5 mL Fetal bovine serum (FBS) and 500 µL penicillin/streptomycin (PEST) (see **Table 1**), store at 4 °C.
    2. Prepare digestive enzymes at a working concentration of 1 mg/mL: weigh aliquots of 4 mg of dispase and collagenase type I powder in separate 15-mL tubes and dissolve in 4 mL of DMEM/F12 + 1% PEST. Sterilize the enzymatic solution by passing it through a 0.22 µm strainer. Prepare fresh enzyme solutions every time.
    3. Coat one well in a 6-well tissue culture plate (or 35-mm tissue culture dish) per biopsy dissected with 1 mL of 0.1% gelatin in phosphate buffered saline (DPBS). Incubate at room temperature for 30 min.
    4. Autoclave one set of surgical scissors and forceps per biopsy for dissection.

2. **Biopsy handling**
    NOTE: Process biopsies as fast as possible after being taken. Store in PBS + 1% PEST for up to 48 h at 4 °C. Biopsy should be 2 - 4 mm in diameter in size and in accordance with ethical permits.
    1. Transfer the biopsy into a 35-mm dish. Move the biopsy to a new 35-mm dish and submerge the biopsy in 2 mL of 70% ethanol for 30 s.
    2. Move the biopsy to a third 35-mm dish and wash with 1 mL of sterile Hank's Balanced Salt Solution (HBSS) supplemented with 1% PEST.
    3. Transfer the biopsy to a fourth 35-mm dish, add 1 mL of freshly prepared 0.1% dispase solution.
    4. Cut the skin biopsy into 1 - 2 mm^3 pieces in the dish using surgical scissors and forceps and then transfer biopsy pieces including the dispase solution into a 15-mL tube. Add an additional 2 mL of dispase.
    5. Rinse the 35-mm dish with a 1 mL dispase solution and transfer to the 15-mL tube.
    6. Incubate the biopsy at 4 °C overnight.
    7. Add 4 mL of 0.1% collagenase I to the tube and incubate at 37 °C for 4 h.
    8. Centrifuge the digested cells 300 x g for 3 min, aspirate the supernatant and resuspend the digest in 2 mL of fibroblast medium.
    9. Remove the gelatin solution from the 6-well tissue culture plate. Transfer the cell suspension including any remaining pieces to 6-well tissue culture plate (or 35-mm tissue culture dish) precoated with 0.1% gelatin in DPBS.
    10. Change medium every third day.

3. **Expansion of human fibroblasts**
    NOTE: Fibroblasts are ready to be passaged to a T25 (25 cm^2) tissue culture flask when 80% confluent (**Figure 2B**). Fibroblasts typically reach 80% confluency within 7 days. However, time required can vary greatly but should be no longer than 4 weeks.
    1. Aspirate medium and wash once with 2.5 mL of DPBS.
    2. Remove DPBS and add 1 mL of Trypsin-EDTA (0.05%) and incubate at 37 °C for 5 min or until cells display rounded edges and start to detach.
    3. Add 2 mL of fibroblast medium, if necessary rinse the cells ensuring proper disassociation of cells. Transfer the cell solution to a 15-mL tube and centrifuge at 300 x g for 3 min.
    4. Discard supernatant and resuspend the cell pellet in 5 mL of fibroblast medium and plate fibroblasts in a non-coated cell tissue culture T25 flask. When expanding fibroblasts after this step, dissociate as described above and seed 12 x 10^3 cells/cm^2.
    5. Once confluent cells are ready to be frozen, expand or plate for reprogramming. Freeze down two rounds of fibroblast before any reprogramming is started (refer to next section for freezing procedure). Reprogramming efficiency is generally higher for lower passage fibroblasts, cells at passage 2 - 4 is optimal. However, successful reprogramming of fibroblasts up to passage 10 has been conducted using this protocol.

4. **Freezing and thawing of fibroblasts**
    1. Prepare fresh fibroblast freezing medium: 90% FBS + 10% dimethylsulfoxide (DMSO). Keep at 4 °C (**Table 1**). Once confluent, the T25 tissue culture flask can be frozen into three cryovials. Prepare 500 µL of fibroblast freezing medium per vial frozen.
    2. To freeze cells, dissociate cells as described in Step 1.3, count cells using a hemocytometer and transfer portions of 3 x 10^5 cells to 15-mL tubes. Spin tubes at 300 x g for 3 min. Aspirate supernatant.
    3. Resuspend cell pellets in 500 µL of 4 ˚C fibroblast freezing medium and transfer into cryovials. Place the vials in a freezing container and incubate cells at -80 °C for 24 h before transferring them to liquid nitrogen for long term storage.
    4. To thaw the cells, submerge the bottom part of the cryovial in 37 °C water. Once liquefied, transfer the cell-suspension to a 15-mL tube and add 5 mL of fibroblast medium. Spin cells 300 x g for 3 min.
    5. Remove the supernatant and resuspend the cell pellet in 5 mL of fibroblast medium. Transfer cells to a non-coated T25 tissue culture flask and incubate in 37 °C.

2. SeV Vector Reprogramming of Fibroblasts

1. **Preparation of vector aliquots**
   CAUTION: Handle the SeV under biosafety level 2 containment. Refer to manufacturer's protocol and local guidelines[17]. Virus titer varies between batches.
   1. Before preparing aliquots calculate the amount of vector needed for 5 x 10^4 cells according to manufacturer's protocol (*e.g.*, CytoTune 2.0). The virus titer generally varies from 8 x 10^7 - 1.5 x 10^8. Thaw and combine vectors 5:5:3 (KOS MOI = 5, hc-myc MOI = 5 and hKlf4 = MOI 3). Portion the amount calculated for the reprogramming of 5 x 10^4 cells into autoclaved 1.5 mL tubes. Dilute virus aliquots with fibroblast medium to a final volume of 250 µL for reprogramming.
      NOTE: Each vial is valid for reprogramming of one well of a 24-well tissue culture plate with 5 x 10^4 fibroblasts. Store virus aliquots at -80 °C.

2. **SeV Vector transduction**
   1. Aspirate cell culture medium and wash with 1 mL of DPBS. Aspirate DPBS and add 1 mL of Trypsin-EDTA (0.05%). Incubate at 37 °C for 5 min or until cells are detached.
   2. Add 2 mL of fibroblast medium and resuspend the cells and transfer to a 15-mL tube. Centrifuge 300 x g for 3 min.
   3. Aspirate the supernatant and resuspend the pellet in 2 mL of fibroblast medium.
   4. Count fibroblasts by using a hemocytometer and seed 5 x 10^4 cells in one well of a 24-well tissue culture plate. Incubate cells at 37 °C overnight.
   5. Aspirate cell culture medium, add 250 µL of the SeV solution and incubate at 37 °C overnight.
   6. Change medium daily to fresh fibroblast medium. Allow transduced cells to grow for 7 days before being passaged to LN-521 coated plates. Prepare LN-521 plates the day before passage. Refer to 2.3.1 for coating procedure.

3. **Replating of transduced fibroblasts**
   1. Preparation of LN-521 coated dishes: Dilute LN-521 in DPBS to a final concentration of 0.63 µg/cm^2. Pipette 2 mL of the LN-521 solution per 60-mm tissue culture dish. Seal the 60-mm tissue culture dish using parafilm. Incubate overnight at 4 °C.
   2. Prepare Essential 8 medium (E8) aliquots for easier handling: 48.5 mL of E8 medium, 1 mL of E8 supplement and 500 µL of PEST (**Table 1**).
   3. 7 days after transduction, passage the cells to a LN-521 coated 60 mm tissue culture dish. Add 250 µL of Trypsin-EDTA (0.05%) and incubate at 37 °C for 5 min or until cells have detached. Add 2 mL of fibroblast medium and centrifuge cells for 3 min at 300 x g.
   4. Aspirate the supernatant. Resuspend pellet in 2 mL of fibroblast medium and count cells in a hemocytometer. Aspirate LN-521 solution from the precoated plate and seed 4 x 10^4 cells in 5ml of fibroblast medium in the LN-521 precoated 60 mm dish. Add Rho-kinase inhibitor Y-27632 (ROCKi) to a final concentration of 5 µM. Incubate in 37 °C overnight.
   5. Importantly, the next day, change medium to E8 medium. Change E8 medium daily. hiPS cell colonies will emerge within 2 - 3 weeks.

3. Picking of Colonies and Expansion of hiPS Cells

NOTE: The following steps are done outside of the biosafety cabinet under a stereo microscope. The use of hairnet and procedure masks are recommended. Work carefully not to contaminate the cell culture.

1. Prepare LN-521 coated tissue culture 24-well plates the day before picking. Dilute LN-521 in DPBS 0.63 µg/cm^2. Pipette 250 µL of the LN-521 solution into one well of a 24-well tissue culture plate. Seal plates using parafilm. Incubate overnight at 4 °C.
   NOTE: Colonies are ready to be picked when they reach a size > 1 mm in diameter. Colonies should display sharp edges and grow in homogeneous monolayers (**Figure 2D**).
2. Aspirate the LN-521 solution and add 500 µL of E8 into one well of a 24-well tissue culture plate.
3. Cut colonies into smaller pieces with a scalpel in a grid like pattern under a stereomicroscope. Mechanically scrape the cell sheets from the dish and transfer the sheets using a 200 µL pipette to the prepared well (**Figure 2D-2E**). Pick colonies into separate wells.
4. Allow cells to attach without changing medium for 48 h. Thereafter, change E8 medium daily.
5. When the colonies have reached a size of > 5 mm, enzymatically passage cells as single cells to a new well of a LN-521 coated plate.
6. Aspirate the cell culture medium from cells and wash with 250 µL of DPBS.
7. Aspirate DPBS. Add 250 µL of dissociation reagent and incubate for 3 min at 37 °C.
8. Observe that the cells have started to round up. Detach cells from the plate by rinsing the cells with dissociation reagent a few times.
9. Add 500 µL of E8 medium to a 15-mL tube. Transfer the cells in the dissociation reagent to the tube and centrifuge for 3 min at 300 x g.
10. Aspirate the LN-521 solution from the LN-521 precoated well and add 500 µL room temperature E8 medium.
11. Aspirate the supernatant and resuspend the cell pellet in 500 µL of E8 medium.
12. Count the cells using a hemocytometer and seed 2.5 x 10^4 - 5 x 10^4 cells in the prepared LN-521 precoated well (1.25 x 10^4 - 2.5 x 10^4 cells/cm^2). Cell culture format can easily be upscaled to suit the intended purpose.
13. Add ROCKi to a final concentration of 10 µM. Incubate cells at 37 °C.
14. Change E8 medium daily. Cells are usually ready to passage after 4 - 6 days. Enzymatically passage cells as single cells as described above when about 90% confluent.
    NOTE: The reprogramming vectors are progressively diluted during hiPS cell expansion and not detectable after passage 12. Cells that are impartially reprogrammed commonly cease to proliferate around passage 6 - 10 or lose their characteristic pluripotent stem cell morphology (**Figure 3B**).
15. **Freezing and thawing of hiPS cells**
    1. To freeze cells, enzymatically dissociate cells as single cells as described above, count cells in a hemocytometer and transfer 2.5 x 10^5 cells to a 15-mL tube containing 1 mL of E8 medium. Centrifuge at 300 x g for 3 min. Aspirate the supernatant.
    2. Resuspend cell pellet in 250 µL of 4 °C PSC cryomedium and transfer to a cryovial. Place the vial in a freezing container and incubate cells at -80 °C for 24 h before transferring them to liquid nitrogen for long term storage.

3. To thaw cells, prepare a precoated LN-521 well of a 24-well tissue culture plate by aspirating the LN-521 solution and adding 250 µL of E8 medium. Submerge the bottom part of the cryovial in 37 °C water until only a small icicle remains.
4. Add 250 µL of E8 Medium to the cryovial and transfer the cell-suspension to a 15-mL tube and add 1 mL of E8 medium. Spin cells 300 x g for 3 min.
5. Remove the supernatant and resuspend the cell pellet in 250 µL of room temperature E8 medium. Transfer the cell suspension to the prepared well and add of 1% cell viability supplement or 10 µM ROCKi. Incubate the tissue culture plate at 37 °C.

Representative Results

From biopsy to hiPS cells

The entire process from biopsy to established hiPS cells, clear of reprogramming vector and ready for characterization, takes approximately 16 weeks (**Figure 1**). A more detailed timeline is specified in **Figure 2A**. Approximately 4 weeks is needed to establish and expand fibroblast cultures. The first hiPS cell colonies started to emerge about three-weeks post Sendai vector transduction. Colonies were picked mechanically for the first passage and then passaged enzymatically as single cells.

Establishment of human fibroblast cultures

When the human fibroblast cultures had grown to confluency and displayed accepted morphology, they were first expanded and frozen for two passages before being plated for transduction (**Figure 2B**).

Reprogramming of human fibroblasts using SeV vectors

Increased levels of cell death were found in the days following SeV transduction and slight changes to fibroblast morphology were observed. Emergence of the first hiPS colonies was detected from day 12 post-transduction (**Figure 2A, 2C**). Colonies ready to be picked were expected about three to 4-weeks post-transduction (**Figure 2A, 2D**). Seeding cells in the amounts specified in this protocol resulted in an abundance of colonies emerging (<20). Selective picking of colonies displaying the favorable morphology is recommended. Colony features preferred included flattened compact cells mass, growth as homogeneous monolayers, distinguishable individual cells with sharp edge towards surrounding fibroblasts (**Figure 2D**). The hiPS cell colonies were cut in a grid-like pattern using a scalpel and mechanically passaged (**Figure 2E**). Picked hiPS clones were left for 48 hours to adhere to the plate without medium change. hiPS cell clones were highly homogeneous however few fibroblasts originating from the reprogramming plate was tolerated during the first two passages (**Figure 2F**). Picking of colonies with fussy borders and large uncompact heterogeneous cell mass should be avoided (**Figure 2G**). Obtained hiPS cell lines grew as dense monolayers, with large nucleus-to-cytoplasm ratio and had defined luminescent borders. In contrast, adhered non-homogenous colonies that did not fulfill these criteria were discarded (**Figure 2H**) to avoid cultures of mixed cell populations.

Validation of hiPS cell clones

Pluripotency characterization of derived cells was performed after the SeV vector was lost and the maintenance of the pluripotent state was driven by the expression of endogenous factors. Before proceeding with hiPS cell validation, ensure the SeV vector expression has been lost. Following this protocol, SeV vector RNA is no longer detectable by passage 12 according to our experience, thus a suitable starting point of characterization (**Figure 3A**). Immunocytochemistry staining for pluripotency markers OCT4, SSEA4 and NANOG should yield a homogenously positive result (**Figure 3B**). Cell cultures containing partially reprogrammed cells that did not express pluripotency factors were discarded (**Figure 3C**). mRNA expression of endogenous pluripotency genes is shown by real time-PCR (RT-PCR) in **Figure 3D**.

Pluripotent stem cells should be readily able to differentiate into the three germ layers. hiPS cells differentiation potential was assessed by formation of Embryoid bodies (EB)[18]. Free floating EBs generated from hiPS cells are shown in **Figure 3E** and mRNA expression of germ layer specific markers after 21 days of EB differentiation is represented in **Figure 3F**.

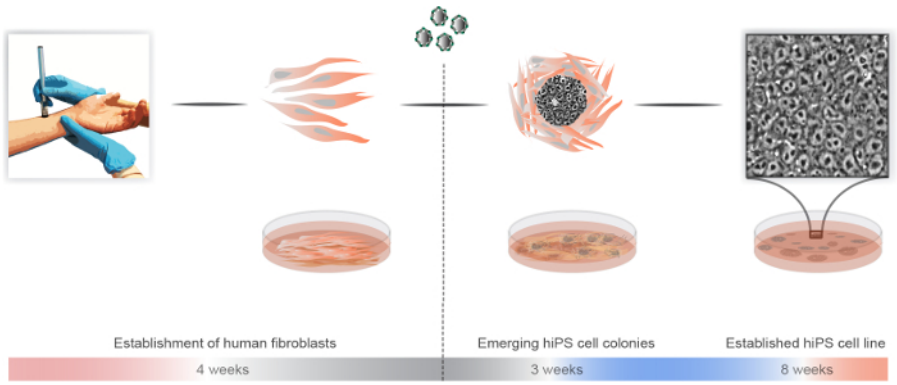

Figure 1: Flow Chart from Skin Biopsy to hiPS Cells.
Overview of the key steps in the protocol. The illustration includes biopsy collection, isolation of fibroblasts, SeV transduction, picking of colonies and the clonal expansion of hiPS cells. Please click here to view a larger version of this figure.

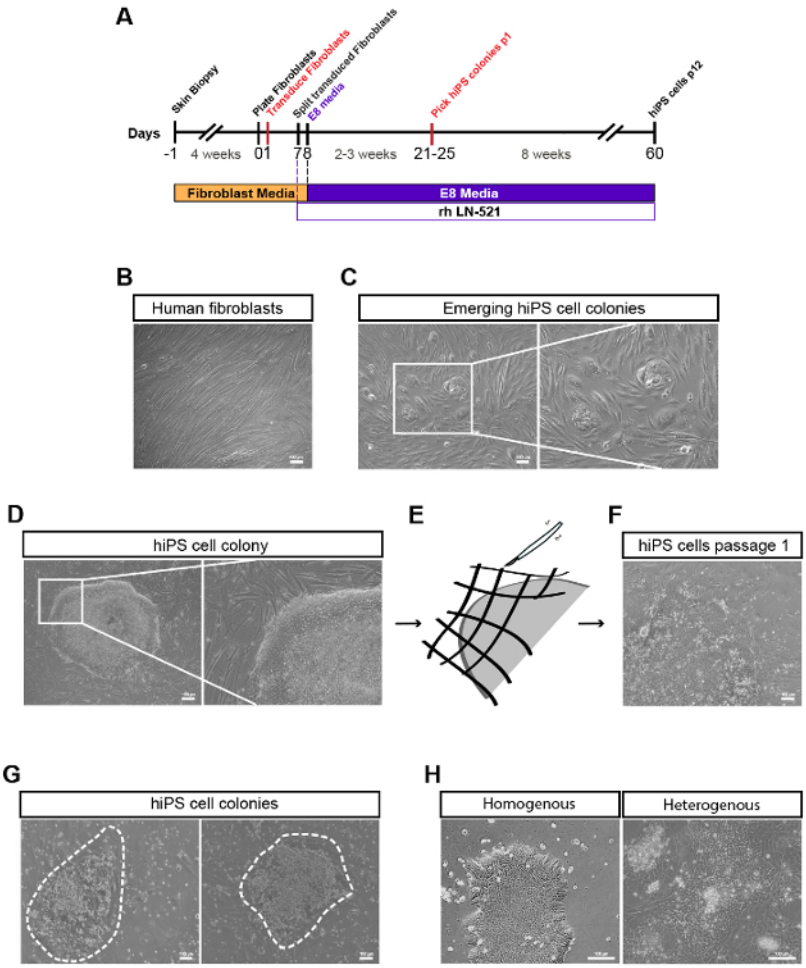

Figure 2: Critical Steps in the Generation of hiPS Cells.
(A) Timeline highlighting important time points in the protocol including fibroblast plating, SeV transduction, medium changes and coatings. Passage 12 of hiPS cells is highlighted as the start of characterization experiments. **(B)** Bright field image of confluent fibroblasts in culture with typical morphology. **(C)** Emergence of hiPS cell colonies from transduced fibroblasts. **(D)** Ready to pick colony displaying preferential features, flattened compact cell mass, distinguishable individual cells with sharp edges towards surrounding fibroblasts (Passage 0). **(E)** hiPS cell colonies are cut in a grid-like pattern and mechanically passaged. **(F)** Adhered colony after a few days of growth. The adhered hiPS cells are surrounded by an unusually high number of fibroblasts originating from the reprogramming plate. The fibroblasts can be scraped of the plate and will most likely disappear with subsequent single cells passages (Passage 1). **(G)** Colonies displaying morphology not reminiscent of fully reprogrammed cells; fussy borders, large uncompact heterogeneous cell mass. **(H)** Bright field image exemplifying an early passage homogeneous fully reprogrammed hiPS cell line compared to a highly heterogeneous cell line. Scale bars indicate 100 µm. Please click here to view a larger version of this figure.

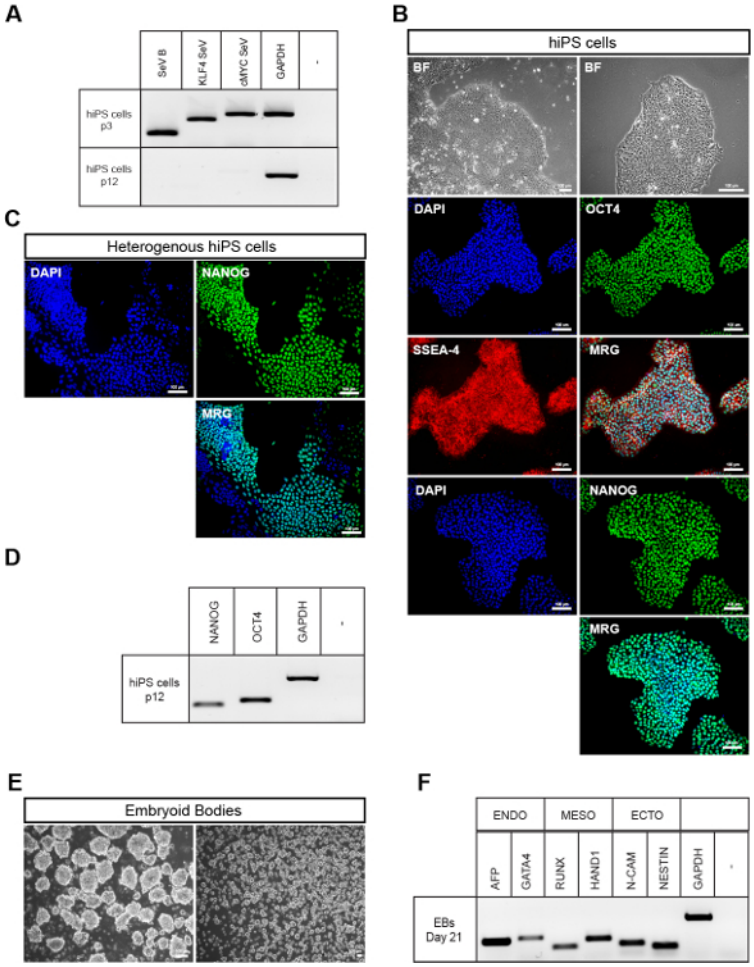

Figure 3: Characterization of hiPS Cells.
(A) RT-PCR of SeV vector specific markers. hiPS cells at passage 3 are used as positive control for the reprogramming vectorsl. At passage 12 cells are negative for expression of virus specific markers Sendai specific backbone (SeV B), Sendai specific KLF4 (KLF4 SeV), Sendai specific cMYC (cMYC SeV), PCR control (GAPDH) and no template (-). **(B)** Representative example of fully reprogrammed homogeneous hiPS cell line at passage 12. Bright field image of hiPS cells reveal cells grow in tight monolayers with sharp luminescing edges and with large nuclei and small cytoplasm. Immunocytochemistry staining of pluripotency markers OCT4, NANOG, SSEA4, and nuclear staining 4',6-diamidino-2-phenylindole (DAPI) demonstrating homogeneous positive expression. **(C)** Cell line exhibiting heterogeneous expression for pluripotency marker NANOG. The culture contains partially reprogrammed cells and should be discarded. **(D)** RT-PCR of mRNA expression of pluripotency markers NANOG, OCT4, PCR control (GAPDH) and no template (-) indicating the expression of pluripotency markers. **(E)** Free floating embryoid bodies generated from hiPS cells. **(F)** RT-PCR after 21 days of embryoid body differentiation show that derived hiPS cells are capable of differentiation to the three germ layers. Endodermal markers AFP and GATA4, mesodermal markers RUNX and HAND1, ectodermal markers NCAM and NESTIN, PCR control (GAPDH) and no template (-). Scale bars indicate 100 µm. Please click here to view a larger version of this figure.

Discussion

The expected result of this protocol is the successful generation of several clonally derived hiPS cell lines. Importantly, the method for the maintenance of and expansion of established hiPS cells here described is reliable and can be performed with little prior experience of stem cell culture. Enzymatic single cell passaging with ROCKi together with the LN-521 matrix is known to maintain cells as karyotypically normal, pluripotent and readily able to differentiate while avoiding induced heterogeneity that colony based passaging can stimulate[10,19,20]. hiPS cells cultured on LN-521 can be passaged as single cells without the addition of ROCKi[10], the addition of ROCKi does however make the protocol easier for less experienced handlers and reduces the time required for hiPS cell derivation.

Reprogramming efficiency is generally not an issue with this protocol; SeV vectors have relatively high reprogramming efficiency > 1% for fibroblasts[11]. The emergence of an abundance of colonies (<20) is expected. It is recommended to pick three times the number of colonies needed. It has been observed that a portion of picked clones fail to adhere after picking. Extra colonies could also be needed to compensate for partially reprogrammed colonies. Partially reprogrammed colonies are in some cases capable to adhere and grow supported by exogenous expression of pluripotency genes by the SeV vectors. As the SeV vectors are diluted, these cells will cease proliferating and dissociate, usually apparent around passage 6-8. Heterogeneity of the culture is also a sign of partial reprogramming and heterogeneous lines should be discarded (**Figure 2H**).

The optimal choice of reprogramming vector and culture conditions is dependent on the purpose of the cells generated. However, to avoid batch-to-batch variances influencing results, defined conditions are recommended. The choice of a non-integrating vector system is suggested to conserve genomic integrity of reprogrammed cells. SeV vectors were chosen in this protocol due to the robustness, relatively high efficiency and low hands on time required. The SeV vectors are diluted during cell divisions and are not detectable around passage 12, thus ensuring that generated data is not influenced by exogenous expression. Because of the meticulous demands put on cells intended for clinical purposes, SeV reprogramming can be a barrier to clinical translation since proving the complete shedding of all vector material is difficult. mRNA mediated reprogramming can be advantageous if deriving cells with intended use in cell therapies[12]. These methods are however far more labor intensive and more complicated to use[21]. The method here described for fibroblast culture is also unsuitable for clinical applications. As both FBS and gelatin are xenogeneic and undefined, consider switching these to defined xeno-free products[22].

Sterile handling techniques and regular mycoplasma infection testing is recommended while working with any form of cell culture. Human skin contains many microorganisms that could thrive in the conditions cells are cultured in if not properly handled. It is therefore recommended to be extra vigilant in the handling of skin biopsies and during the first days of culture following biopsy processing. The establishment of fibroblast culture from skin biopsies is, indeed, a time-consuming process. After processing the skin biopsy, there will initially be weak signs of fibroblast cells adhering, thus wait at least one week to find a few fibroblast cells displaying expected morphology in the culture. The time needed for the establishment of fibroblasts *in vitro* cultures from biopsies is dependent on a variety of factors including size of biopsy, age of donor and how the biopsy has been handled since it was taken. It is preferred to reprogram early passage fibroblasts for optimal reprogramming efficiency[23].

In summary, we here describe an established robust and easy-to-use method for generation of highly homogeneous hiPS cells.

Disclosures

The authors have no conflicts of interests to report.

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