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Feeder-independent iPS cell derivation from human and mouse adipose stem cells

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Summary

Adipose tissue is an abundantly available source of proliferative and multipotent mesenchymal stem cells with exceptional potential for regenerative therapeutics. We previously demonstrated that both human and mouse adipose-derived stem (ADS) cells can be reprogrammed into induced pluripotent stem (iPS) cells with efficiencies higher than have been reported for other cell types. The ADS-derived iPS cells can be generated in a feeder-independent manner, representing a unique model to study reprogramming and a significant step toward establishing a safe, clinical grade of cells for transplantation. Here we provide a detailed protocol for isolation, preparation and transformation of ADS cells from fat tissue into iPS cells in a feeder- and xenobiotic-free process. This protocol also describes how ADS cells can be used as feeder cells for maintenance of other pluripotent stem cells. ADS derivation is rapid and can be completed in less than 1 week, with mouse and human iPS reprogramming averaging 1.5 and 2.5 weeks, respectively.

INTRODUCTION

Stem cells are ideal and promising sources not only for studying cellular and developmental processes but also for developing regenerative therapeutics. However, many problems remain before these cells can serve as a practical source for clinical applications. The challenges include availability, efficiency, safety and ethical issues. Stem cells present in the human body are usually very limited in number and protocols to fully optimize efficiency of purification and use are still under development. These include development of particular cell lines capable of differentiating into clinically suitable cell types¹, transplantation methods to deliver the cells effectively into desired locations, and introduction of personalized traits tailored for individual needs². Safety is another concern because typical cell culture methods make use of animal sourced products such as serum and mouse feeder cell layers³. In addition, cells to be transplanted typically originate from heterologous sources, increasing the risk of immune rejection⁴. For example, commonly used human embryonic stem (ES) cells express an immunogenic non-human cell surface modification (sialic acid Neu5Gc), presumably due to the use of animal-derived products in the culture media⁵. Furthermore, ethical issues continue to be a matter of debate, especially for the use of human ES cells⁶. Many of these problems, we believe, can potentially be solved by using

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AUTHOR CONTRIBUTIONS

S.S. and Y.K. designed and performed the experimental procedures. S.S., Y.K., and W.T.B. wrote the protocols. R.M.E. is the project leader, obtained funding, reviewed and edited the protocols.

human adipose-derived stem (ADS; also known as processed lipo-aspirate, adipose stromal, or adipose tissue-derived mesenchymal stem) cells⁷.

In addition to mature adipocytes, adipose tissue contains relatively abundant progenitor and mesenchymal stem cell (MSC) populations in the stromal vascular fraction (SVF). It is estimated that as many as 1% of SVF cells are MSCs. In contrast, only 0.001 – 0.002% of cells in the bone marrow, which is considered a standard hub of adult stem cells, represent MSCs⁸. ADS and bone marrow-derived MSC cells are proliferative and ‘multipotent,’ having the capacity to differentiate into limited cell types such as adipocytes, osteocytes, chondrocytes, and myocytes^{9,10}. MSCs from adipose and bone marrow possess many common cell surface markers though a precise definition of ADS cell markers is not well established^{11,12}. It is advantageous to use fat tissue as a potential source for regenerative medicine because the tissue is abundant in this present era of global-wide obesity and relatively easy to obtain. Liposuction, a procedure to remove excess fat tissue, is among the most common plastic surgeries operated in the United States. Additionally, in contrast to bone marrow-derived MSCs that require initial plating with high density (>50,000 cells/cm²), ADS cells can be seeded and maintained as low as 3,000 cells/cm².

The discovery of the ability to create ES cell-like, induced pluripotent stem (iPS) cells by transducing four transcription factors into somatic cells has revolutionized the stem cell field^{13–16}. This technology enables not only the study of cell dedifferentiation and differentiation but also development of patient-specific cells for disease models and regenerative therapies. Unlike ES cells, which are isolated from the blastocysts of an embryo, iPS cells can be derived from adult somatic cells and thus avoid many ethical concerns. We recently investigated if the ‘multipotency’ of ADS cells can be upgraded to ‘pluripotency,’ by introducing the four standard reprogramming factors, Oct4, Sox2, Klf4 and c-Myc⁷. The resultant adipose-derived iPS cells exhibit all the characteristics and morphologies of ES cells from the corresponding species. Mouse and human adipose-derived iPS cells are over 5-fold and 100-fold more efficient in reprogramming than mouse and human fibroblasts, respectively, which are the most commonly used cell lines for iPS generation. Importantly, both mouse and human adipose cells are capable of giving rise to iPS cells without the need for feeder cells. This is due at least in part by the intrinsically high expression of self-renewal supporting factors such as basic FGF, vitronectin, fibronectin and LIF in ADS cells⁷. We found that ADS cells also have the ability to serve as feeders for other pluripotent stem cell lines. This is a key step toward establishing safe, clinical-grade human iPS cell lines because culture of iPS cells typically requires feeder layers of mouse embryonic fibroblasts (MEF) and use of media containing animal components such as serum and bovine serum albumin (BSA)³. We believe that generation of human adipose-derived iPS cells in xenobiotic-free conditions will obviate the development of non-human immunogenic surface markers described above. In addition, use of chemically defined media will ensure quality control, mitigate sources of biological variability and reduced the risk of animal-derived pathogen contamination.

Here we describe detailed protocols of ADS cell isolation and subsequent iPS cell line derivation. Use of ADS cells for supporting heterologous pluripotent stem cells as feeders is also described. In addition to retrovirus production of reprogramming factors, derivation of mouse ADS-derived iPS cells in feeder-free conditions is described, which produces about a 0.25% reprogramming efficiency. We also compare derivation of human ADS-derived iPS cells in feeder-dependent, feeder-free or xeno-free conditions. The reprogramming efficiencies are approximately 1.0% with feeder cells and ~0.008% without feeders or xenobiotics. The relative abundance and ease of isolation and derivation of ADS cells offers an ideal system for the study of the molecular mechanisms of cellular reprogramming and translation of stem cells into cell-based therapies.

MATERIALS

REAGENTS

- Ethanol 70% **CAUTION** It is highly flammable, and a skin and eye irritant.
- Hank's balanced salt solution (HBSS; Invitrogen, cat. no. 14175-095)
- Surgical scissors and forceps **CAUTION** Sterilize by autoclave.
- Sterile serological pipettes (5, 10, and 25 ml; Thermo Fisher, cat. no. 13-678-11)
- Scintillation vials (Research Products International, cat. no. 121040) **CAUTION** Sterilize by autoclave.
- Bovine serum albumin (BSA; Sigma, cat. no. A7906)
- D-glucose (Sigma, cat. no. G7528)
- Adenosine (Sigma, cat. no. A9251)
- Type I collagenase (Worthington Biochemical, cat. no. LS004196)
- Disposable sterile filter (0.22 μ m, 500 ml, Thermo Fisher, cat. no. 09-740-32)
- Sterile syringe filter (0.2 μ m and 0.45 μ m, VWR, cat. no. 28144-040 and 28144-007)
- Syringes (Becton Dickinson, various sizes)
- Kimwipes (Thermo Fisher, cat. no. 06-666)
- Nylon mesh (250 μ m; Small Parts, Inc, cat. no. CMN-0250) **CAUTION** Cut in appropriate sizes and sterilize by autoclave.
- Falcon 100 μ m cell strainers (BD, cat. no. 352360)
- Sterile polypropylene centrifuge tubes (15 ml and 50 ml; Corning, cat. no. 430790 and 430828)
- Bacterial Petri dishes (6cm and 10cm; Thermo Fisher, cat. no. 08-757-100)
- Cell culture dishes (6cm, 10cm, 6-well & 12-well; Thermo Fisher, cat. no. 08-772B, 08-772-23, 07-200-80 & 07-200-81)
- DMEM (Invitrogen, cat. no. 11965-092)
- Heat-inactivated Fetal Bovine Serum (FBS; Invitrogen, cat. no. 10438-026)
- Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen, cat. no. 14040-117)
- 0.25% (wt/vol) Trypsin/EDTA (Invitrogen, cat. no. 25200-056)
- DMEM no phenol red (Invitrogen, cat. no. 31053-028)
- Sodium azide (Sigma, cat. no. S8032) **CAUTION** It is highly toxic. Use protective equipment to minimize exposure.
- BD IMag Streptavidin Particles Plus – DM (BD Biosciences cat. no. 557812)
- Lineage markers (CD31, CD45 and Ter119), Sca-1, CD24, CD29, CD73, CD90, and CD105 antibodies conjugated with biotin or various fluorophores are available from various companies including BD Biosciences, eBioscience and BioLegend
- Bambanker (Wako Chemicals USA, Inc. cat.no. 302-14681)

- Human ADS cells can be obtained from PromoCell (hMSC-AT; cat. no. C-12977), Invitrogen (StemPro® hADSC kit; cat. no. R7788-110), or ZenBio (Adult stem cells; cat. no. ASC-F)
- Mitomycin C (Thermo Fisher, cat. no. AC22694)
- Retroviral constructs pMX-Oct4, pMX-Sox2, pMX-Klf4, pMX-cMyc, pUMVC3-gag/pol, pCMV-VSV-G, and pMX-GFP (see REAGENT SETUP)
- Phosphate-buffered saline (PBS; e.g., Invitrogen, cat. no. 10010-056)
- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
- 0.05% (wt/vol) Trypsin/EDTA (Invitrogen, cat. no. 25300-054)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668)
- TrypLE (Invitrogen, cat. no. 12604)
- EmbryoMax® ES Cell Qualified 0.1% Gelatin Solution (Millipore, cat. no. ES-006-B) (see REAGENT SETUP)
- Knockout (KO) DMEM (Invitrogen, cat. no. 10829-018)
- DMEM NUTRIENT MIX F12 (DMEM/F-12) (Invitrogen, cat. no. 11330057)
- KO Serum Replacement (KO-SR; Invitrogen, cat. no. 10828-028)
- EmbryoMax® ES Cell Qualified FBS (Millipore, cat. no. ES-009-B)
- GlutaMAX (Invitrogen, cat. no. 35050-038)
- Non-essential amino acid (NEAA) solution (Invitrogen, cat. no. 11140-050)
- EmbryoMax® ES Cell Qualified 2-Mercaptoethanol (100X) (Millipore, cat. no. ES-007-E) **CAUTION** It is toxic. Avoid inhalation, ingestion or contact with skin.
- EmbryoMax® ES Cell Qualified Nucleosides (100X) (Millipore, cat. no. ES-008-D)
- ESGRO® (LIF) (Millipore, cat. no. ESG1107)
- 293T cells (ATCC, cat. no. CRL-11268)
- Polybrene (10 mg ml⁻¹; Chemicon, cat. no. TR-1003-6)
- EmbryoMax® ES Cell Qualified Fetal Bovine Serum (Millipore, cat. no. ES-009-B)
- EmbryoMax® Primary MEF feeder cells, mitomycin C treated (Millipore, cat. no. PMEF-CF)
- StainAlive DyLight 488 mouse anti-human TRA-1-81 antibody (Stemgent, cat. no. 09-0069)
- Matrigel hES qualified matrix (BD Biosciences, cat. no. 354277)
- mTeSR1 (StemCell Technologies, cat. no. 05850)
- CELLstart (Invitrogen, cat. no. A10142-01)
- Stemedia NutriStem xeno-free medium (Stemgent, cat. no. 01-0005)
- Synthemax 6-well plates (Corning, cat. no. 3876XX1)

EQUIPMENT

- Water bath shaker **CRITICAL** We recommend using a reciprocal shaker that moves in a horizontal direction instead of an orbital one in order to ensure an optimal digestion.
- Biosafety cabinet with aspirator for tissue culture
- Incubator: 37 °C, 90% humidity, 5% CO₂
- Water bath: 37 °C
- Cell counter or hemocytometer
- Pipettes
- Centrifuge
- Inverse phase-contrast microscope
- Stereomicroscope
- Cryovials
- Freezing container

REAGENT SETUP

Mice—C57BL/6J (Jackson Lab stock no. 000664) or Oct4-EGFP (Jackson Lab strain name B6;129S4-Pou5f1^{tm2Jae}/J; stock no. 008214; isolated cells show green fluorescence upon successful reprogramming) **CAUTION** Any work involving use of animals must be reviewed and approved by the Institutional Animal Care and Use Committee.

Collagenase solution—Immediately prior to the digestion, prepare a fresh solution of 2.5 µg/ml collagenase (wt/vol), 1% BSA, 50 µg/ml D-glucose and 200 nM adenosine in HBSS. Filter sterilize.

Erythrocyte lysis buffer—Prepare 154 mM NH₄Cl and 20 mM Tris. Adjust pH to 7.4 and filter sterilize. Stable at room temperature for up to 4 weeks.

Retroviral vectors—pMX vectors containing the cDNAs of mOct4 (Plasmid 13366), mSox2 (Plasmid 13367), mKlf4 (Plasmid 13370), mc-Myc (Plasmid 13375) (Addgene), hOct4 (Plasmid 17217), hSox2 (Plasmid 17218), hKlf4 (Plasmid 17219), hc-Myc (Plasmid 17220), retroviral gag-pol packaging plasmid (Plasmid 8449), VSV-G expression plasmid (Plasmid 8454) (Addgene), pMX-GFP (Cell Biolabs). **CAUTION** Since retrovirus pseudotyped with VSV-G can infect humans, any procedure involving this virus must be performed under Biosafety Level 2 containment.

Complete DMEM media for 293T cells and MEF—High-glucose DMEM, 10% FBS (vol/vol), 1× GlutaMAX, 1× NEAA, penicillin/streptomycin (100 U ml⁻¹ and 100 µg ml⁻¹, respectively). To prepare 500 ml of the media, mix 50 ml FBS, 5 ml GlutaMAX, 5 ml NEAA solution and 5 ml penicillin/streptomycin, and then fill up to 500 ml with DMEM. It can be stored at 4 °C for 4 weeks.

Expansion DMEM media for mouse ADS cells—To increase growth of mADS cells, supplement complete DMEM media with 5 ng/ml basic FGF. Use of heat-inactivated FBS is recommended. Expansion media may be stored at 4 °C for up to 2 weeks.

Media for human ADS cells (“hADSC” media)—While Expansion DMEM media is good for culturing human ADS cells, we found that Mesenchymal Stem Cell Growth Medium (PromoCell, cat. no. C-28010) achieves optimum growth for regular culture conditions. For xeno-free conditions, use StemPro MSC SFM XenoFree (Invitrogen, cat. no. A10675-01).

Cell sorting buffer—DMEM without phenol red, 2% heat inactivated FBS, and 0.1% sodium azide. It can be stored at 4°C for up to 4 weeks.

Gelatin-coated culture dishes—Add a 0.1% gelatin solution to coat the dish well, ensuring that the entire bottom surface is coated. Incubate for at least 30 min at 37 °C. Immediately before cell plating, aspirate the solution.

mESC media (serum base)—To prepare 500 ml of ESC media, mix 400 ml KO-DMEM, with 75 ml ES Cell Qualified FBS, 5 ml GlutaMAX, 5 ml Nucleotides, 5 ml penicillin/streptomycin, 5 ml NEAA solution, 5 ml diluted β-mercaptoethanol (100X) and 50 μl LIF (from 1×10^6 U/ml stock; 1,000 U/ml final). Store ESC media at 4 °C for up to 1 week.

mESC media (KO-SR base)—To prepare 500 ml of ESC media, mix 400 ml KO-DMEM, with 75 ml KOSR, 5 ml GlutaMAX, 5 ml Nucleotides, 5 ml penicillin/streptomycin, 5 ml NEAA solution, 5 ml β-mercaptoethanol (100X) and 50 μl LIF (1,000 U/ml final). Store at 4 °C for up to 1 week.

hESC media—To prepare 500 ml of hESC media, mix 385 ml of DMEM/F-12 base media with 100 ml knockout serum replacement, add 5 ml NEAA solution, 5 ml Glutamax, 5 ml β-mercaptoethanol (100X) and FGF2 to final concentration of 10 ng/ml (add 100 ul of a 50 ug/ml stock of FGF2). Sterilize by filtering through 0.22 μm vacuum filter unit. Make fresh and store at 4°C for up to 2 weeks.

Matrigel-coated culture dishes—Refer to the manufacturer’s recommendation on handling and thawing matrigel. It is important to thaw on ice and keep all components chilled that are to be used for aliquoting or diluting matrigel. We recommend aliquoting into 1 mg each (volume will depend on each production lot – see the product’s certificate of analysis for protein concentration). Store aliquots at –80°C. Dilute 1 mg of matrigel into 24 ml of ice-cold DMEM/F-12 media and mix by pipeting up and down. Immediately pipette 2 ml per well (6-well format) or 5 ml per 6-cm dish of the matrigel solution. Swirl to evenly coat surface. Allow product to gel and coat surface at 37°C for 1 – 48 h. Prepare for cell culture by first aspirating excess matrigel solution, then add sufficient volume of fresh hES cell media of choice to cover the surface. Return the plate/dish to the incubator to warm and CO₂ equilibrate. Passage cells onto the prepared surface after 10 minutes and up to several hours.

PROCEDURE

Overview

- Step 1 Isolation of mouse ADS cells
- Step 2 Isolation of human ADS cells
- Step 3 ADS cell expansion
- Step 4 Retrovirus production

Step 5 Mouse iPS cell derivation

Step 6 Human iPS cell derivation

1. Isolation of mouse ADS (mADS) cells **TIMING** 5 – 7 hours

- i. Sacrifice each mouse (C57BL/6J or Oct4-EGFP mice, 8–10 weeks old) by carbon dioxide asphyxiation. Wipe with 70% ethanol and open abdominal area. Using sterilized forceps and scissors, dissect perigonadal (epididymal in male and parametrial in female) and/or subcutaneous inguinal fat pads and place into 6 cm petri dishes containing HBSS. **CRITICAL STEP** We advise proceeding immediately to digestion of fat tissue on the day of harvest. Chilling or freezing the tissue for storage purposes makes subsequent collagenase digestion more difficult.
- ii. Add an equal volume of collagenase solution (to weight of fat pads) into autoclaved scintillation vials and warm to 37°C.
- iii. Rinse fat pads in HBSS, dry with Kimwipes and place into collagenase solution.
- iv. Finely mince the tissue with a sterilized scissor.
- v. Shake vials (~100 rpm) in a 37°C water bath shaker for 30 to 60 min. Check digests every 10 min and stop reaction when complete. **CRITICAL STEP** The digestion efficiency varies with speed and type of shakers and batch of collagenase used. You should see a white fat layer separated and, when the vials settle, it floats on top of the solution.

TROUBLESHOOTING

- vi. Transfer and filter the digested solution through a 250 µm nylon filter. Then filter through a 100 µm cell strainer and transfer the filtrate into 15 ml or 50 ml centrifuge tubes, depending on the volume. Centrifuge at 400g for 5 min at room temperature.
- vii. Carefully aspirate the floating layer containing mature adipocytes and aqueous supernatants, leaving the pellet. This pellet is the stromal vascular fraction (SVF).
- viii. Resuspend the pellet with 10 ml HBSS. Centrifuge again at 400g for 5 min.
- ix. Repeat the washing step three times.
- x. If the pellet appears red, rupture the red blood cells by adding 10 ml erythrocyte lysis buffer. Pipet up and down to resuspend the pellet. The solution should become red. Leave for 5 min at room temperature and centrifuge for 5 min. Aspirate the supernatant.
- xi. Resuspend the pellet in 10 ml Expansion DMEM media and plate onto Petri dishes.
- xii. Incubate at 37°C in a 5% CO₂ incubator for 1 hour. The majority of hematopoietic lineage cells such as monocytes/macrophages will attach to the Petri dish at this stage.
- xiii. Transfer non-adherent cells (containing ADS cell populations) to 10 cm regular culture dishes and incubate at 37°C in a 5% CO₂ incubator. We typically culture cells isolated from 2 – 3 g fat in one 10 cm dish.

- xiv. Change the media after 24 h. After that, feed cells every 3 d until cells reach around 80% confluency. mADS cells should exhibit a large and flat fibroblastic morphology (Fig. 1a).

TROUBLESHOOTING

- xv. Aspirate the media and wash with D-PBS three times. Add a minimum amount of 0.25% trypsin/EDTA just barely covering the bottom surface (e.g. 1 ml for 10 cm dishes). Incubate at 37°C for no longer than 5 min. Gently tap the dish to dislodge the cells and observe by microscope. Ignore firmly adhering cells, if any, as these are often of the hematopoietic lineage. Add an equal amount of Expansion DMEM media to inactivate the trypsin.

- xvi. Transfer to the 15 ml tube and centrifuge at 400g for 5 min. Aspirate the supernatant and leave cell pellets.

2. **Isolation of human ADS (hADS) cells** A detailed protocol for isolating human cells was previously published in *Nature Protocols*¹⁷ and other journals^{9,18–20}. Briefly, the protocol for isolating hADS is similar to Step 1 for mADS, except that the working volume is typically much larger. In addition, if lipoaspirate samples are used, no mincing with scissors is necessary as the liposuction procedure itself results in the mincing of the tissue. Alternatively, hADS cell lines isolated from fat tissue can be obtained from commercial sources (See MATERIALS). The typical morphology of hADS culture is shown in Fig. 1b. Commercially available media can also be used for hADS cells in place of Expansion DMEM media.

CAUTION If human tissue samples are used for ADS cell isolation, assure that the procedures is first reviewed and approved by the appropriate Human Subjects Institutional Review Board.

3. **ADS cell expansion** Isolated cells can be further passaged (A), sorted by cell surface markers (B), frozen for storage (C), or prepared as feeder cell layers (D).

A. Further passaging **TIMING** 1 – 3 weeks

- i. Resuspend cells in 1 ml Expansion DMEM media. Count the cell number using a hemocytometer or an automated cell counter. Plate at a density of 5,000 cells per cm² culture area.
- ii. Passage for 2 – 3 times at 80% confluence. This will ensure that the majority of populations are ADS cells. Cells may be used for iPS reprogramming up to passage 5 as long as they are in the proliferative phase.

B. Cell sorting **TIMING** 2 – 5 hours

It is also possible to bypass culturing onto the plates before this step. In this case, proceed directly from Step 1.x. above. Cell sorting may be performed by using either magnetic beads or a fluorescence activated cell sorter (FACS) machine.

- i. Resuspend cells in 1 ml Expansion DMEM media. Count the cell number. Centrifuge at 400g for 5 min and resuspend cells in the cell sorting buffer at a concentration of 2×10^7 cells per ml.
- ii. Follow the manufacturer's or FACS facility's protocols specific for magnetic bead separation or FACS sorting. For

example, we use the IMag Streptavidin Particles Plus – DM system (See MATERIALS) to get rid of lineage markers by magnetic separation. The antibodies for the lineage markers used are biotinylated anti-mouse CD31 (endothelial), CD45 (hematopoietic) and Ter119 (erythrocytes)²¹. Alternatively, a positive selection for stem cell-specific markers may be performed. These include Sca-1, CD24, CD29, CD73, CD90, and CD105. Removing the lineage positive cells or selecting by stem cell markers at an initial passage results in a better iPS reprogramming efficiency. However, we find that passaging unsorted cells 2 – 3 times as described in (A) gives a comparable efficiency.

C. Freezing cells for storage **TIMING** 1 hour

- i. Resuspend cells in 1 ml Bambanker, which is a serum-free cell freezing medium. Count the cell number. Adjust to 1×10^6 cells per ml of Bambanker. Store 1 ml each cryovial.
- ii. Freeze cells at -80°C . We find that human and mouse ADS cells can be stored in the -80°C freezer without significant loss of cell viability for longer than one year. For long-term storage, cells can be frozen in liquid nitrogen.

PAUSE POINT To replat frozen cells, quickly thaw the cells in a 37°C water bath. Then transfer and add 4 ml of Expansion DMEM media in a drop-wise manner, and centrifuge at 400g for 5 min. Cells are plated at a density of 5,000 cells per cm^2 .

D. Preparing ADS cells for feeder layers **TIMING** 1 hour

- i. Mouse or human ADS cells are collected as pellets with media added in tubes or frozen in storage cryovials as described above.
- ii. For mitotic inactivation, gamma-irradiate the ADS cells either in tubes or in cryovials on dry ice by exposing them to 6000 rad of irradiation following the instructions provided with the specific model of irradiator unit used.
PAUSE POINT We have achieved successful mitotic inactivation by irradiating both fresh cell pellets and frozen cryovials on dry ice. Alternatively, one can use mitomycin C treatment following the manufacturer's recommendations.
CAUTION This procedure involves a lethal dose of radiation. Strict adherence to regulatory guidelines and appropriate safety training are required.
- iii. Immediately freeze the irradiated cells for storage according to Step 3.C. above, or proceed to the next step.
- iv. At least one day prior to starting culture of ES or iPS cells, plate mitotically inactivated ADS cells at densities between 1.0×10^4 and 6.0×10^4 cells per cm^2 . The optimal densities depend on the pluripotent cell lines used and need to be empirically determined.

- v. Culture for up to 7 – 10 days using standard feeder-dependent ES/iPS culture conditions.

4. **Retrovirus production** **TIMING** 1 week For virus to be used to reprogram cells on feeders or feeder-free, follow the standard method (option A). For xeno-free virus production, follow option (B).

CAUTION All work involving active virus supernatants or concentrated virus particles must be performed in an appropriate biological safety cabinet using adequate personal protection equipment (Biosafety Level 2 containment).

A. Standard virus production protocol

- i. Thaw a vial of 293T cells. Seed out $\sim 1.2 \times 10^5$ cells /ml in each tissue culture dish. For example, 10 ml ($\sim 1.2 \times 10^6$) for a 10 cm dish.
- ii. When cells reach more than 90% confluence (24 – 48hr), aspirate the media, gently wash with PBS, and add 1 ml of 0.05% Trypsin/EDTA. Incubate for 2 min at room temperature. Gently tap the tissue culture plate from side to side to ensure that all cells are detached.
- iii. Add 9 ml of Complete DMEM media and transfer to a 15 ml tube.
- iv. Centrifuge at 400g for 2 min.
- v. Resuspend in 10 ml Complete DMEM media and determine cell numbers.
- vi. Seed 1.2×10^6 cells in 10 cm dishes and place in a 37 °C, 5% CO₂ incubator. **CRITICAL STEP** Adjust the number of cells to seed according to the rate of cell proliferation and desired time of transfection.
- vii. When cells reach over 90% confluence, prepare the Lipofectamine 2000 and DNA complex according to the manufacturer's instruction. For the 10 cm dish, mix 15 µg pMX, 10 µg gag-pol and 5 µg VSV-G expressing plasmids, and 45 µl Lipofectamine for cotransfection.
- viii. Gently add the Lipofectamine/DNA complex dropwise onto the media.
- ix. Place in a 37 °C, 5% CO₂ incubator overnight.
- x. The next day, carefully change the media (10 ml per 10 cm dish) and incubate at 32 °C in a 5% CO₂ incubator overnight. Although virus is more stable at 32°C, 37°C is also acceptable.
CRITICAL STEP Ensure that nearly 100% of cells are transfected by using a GFP reporter construct such as pMX-GFP.
- xi. After ~24 h, carefully collect the viral supernatant. Add fresh Complete DMEM media to the dish.
CRITICAL STEP Be careful that cells do not detach from the culture dishes.

- xii. After another 24 h, collect the viral supernatant again.
- xiii. Filter the viral supernatant through a 0.45- μ m syringe filter.
- xiv. Add 0.5 μ l polybrene of 10 mg/ml stock for each ml of viral supernatant, giving a final polybrene concentration of 5 μ g/ml.

CRITICAL STEP The use of fresh virus supernatant increases the efficiency of iPS generation. While pseudotyped retrovirus can be frozen in aliquots at -80°C , avoid repeated freeze-thaw cycles.

TROUBLESHOOTING

- B. Xeno-free virus production protocol** This protocol is identical to option (A) except that xeno-free media such as StemPro MSC SFM XenoFree is used instead of the Complete DMEM media to incubate the transfected 293T cells (after Step 4.A.x.). Carefully wash the cells with D-PBS before the media change. Virus is thus produced in xeno-free conditions.

5. Mouse iPS cell derivation **TIMING** 1 – 2 weeks

- i. Trypsinize or thaw mADS at passages 2 – 3 and plate 40,000 cells per well in 12-well dishes. Save one well for using GFP virus to check transduction efficiency and another well for counting the initial cell number to calculate reprogramming efficiency.
- ii. The following day, the cells should be around 70% confluent. Add fresh retroviral supernatant from Step 4 that has been filtered with polybrene added (5 μ g/ml). Use 0.25ml of each transcription factor per well in a 12-well dish. Thus, when using the four factor combination, the total volume will be 1 ml.
- iii. Keep cells at 32°C or 37°C in a 5% CO_2 incubator overnight.
- iv. Remove the supernatant and wash with PBS twice. Add Expansion DMEM media.
- v. After 1 d, trypsinize the cells and resuspend in 1 ml mESC (serum base) media.
- vi. Plate out 600 μ l of infected mADS cells in a 6-well plate treated with gelatin.
- vii. After 2 d, change media to mESC (KO-SR base) media and change media every other day. After 5–7 d, small colonies will be visible (Fig. 2a).
- viii. After 9 d, large, fast-growing reprogrammed colonies are visible (Fig. 2b). Change media daily once many colonies are growing. These cells can be manually picked, subcloned, cultured and characterized depending on the purpose of the experiments. Fig. 2c shows typical mouse iPS colonies. Further details can be found in the published protocols^{14,22}.

TROUBLESHOOTING

6. Human iPS cell derivation

There are 3 different methods used for derivation of human iPS cell lines from hADS cells, derivation on feeder cells (option A), feeder-free derivation (option B)

or xeno-free derivation (option C). **CRITICAL STEP** Prior training and experience with the basic culturing techniques for hES or human iPS cells is essential.

CAUTION Use of human ES and iPS cells must be approved by the institutional stem cell research oversight committee or equivalent, and conform to regulatory and ethical guidelines.

A. Derivation of human iPS cells on feeder cells. TIMING 2 – 3 weeks

- i. Passage 1×10^5 hADS cells per well of a 6-well plate. Incubate overnight at 37°C in 5% CO₂. **CRITICAL STEP** hADS cells must be actively proliferating to achieve robust reprogramming. Try to avoid cell cultures that have been passaged more than 4–5 times.
- ii. The following day, transduce the hADS culture with established retroviral reprogramming factors Oct4, Sox2, Klf4 and c-Myc, or GFP control. The cells should be 60–70% confluent. **CRITICAL STEP** The success of reprogramming depends on access to good virus. Follow the protocol in Step 4 to make in-house, ideally with the help of experienced personnel, or purchase from a proven vendor.
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- iii. Mix equal volumes of freshly prepared (or freshly thawed) supernatants of the four retroviral preparations from Step 4. Typically, a total volume of 2 – 4 ml of the combined viral supernatant solution (0.5 – 1 ml each factor) will be needed to reprogram $1 - 2 \times 10^5$ hADS cells.
- iv. Aspirate the hADSC media and pipette 2 – 4 ml of the combined and pre-warmed viral supernatant solution onto the well. **CRITICAL STEP** Avoid warming the entire stock of viral supernatant. Only warm the necessary volume of viral supernatant to be used for this step to 32°C for 5 – 10 minutes.
- v. Centrifuge the plate of hADS cells with viral supernatants at 800g at room temperature for 60 minutes. This ‘spin-fection’ procedure increases transduction efficiencies. **CAUTION** Use biocontainment covers on the microplate carriers when centrifuging samples with active virus particles.
- vi. Immediately following the centrifugation, return the plate to the 37°C incubator. 2 ml of fresh hADSC media can be added (optional).
- vii. If working with unproven or lower titer viral supernatants, repeat Steps iii. to vi. up to three times. If the viral supernatants have already been proven to reprogram cells using lower volumes this step can be skipped.
- viii. Plate out MEF feeder cells on the same day that the final viral transduction is performed. Thaw a tube of MEFs according to the manufacturer’s instruction. Seed a 10 cm gelatin-coated dish with 1.2×10^6 MEF cells and incubate overnight at 37°C, 5% CO₂. Prepare one 10 cm dish for each well of hADS cells to be reprogrammed.

- ix. The day following the last virus transduction or by day 5 at the latest, passage the transduced hADS cells using TrypLE (trypsin replacement) onto the prepared MEF feeder plate. Follow the trypsin passaging protocol described in Steps 1. xv. and xvi. One well of hADS cells can be passaged to one 10 cm dish of MEF feeder cells.
- x. The next day switch the media from hADSC to hES cell media.
- xi. Feed cells with 10 ml of fresh hES cell media every day.
- xii. Use phase-contrast microscopy to look for emerging iPS cell colonies daily or every other day. Expect to see iPS cell colonies that have characteristic hES cell morphology around 2 weeks following the virus transduction.
- xiii. Allow the colonies to expand to medium to large sizes over a period of 1 – 3 weeks with daily feeding.
- xiv. Select medium to large sized colonies for manual passaging that display typical hES cell morphology. (Optional) A supplement to morphology based colony selection is the use of a live cell staining antibody for an endogenous pluripotency marker such as TRA-1-81 StainAlive following the manufacturer's recommendation. This can be a helpful aid for those with limited experience in identifying appropriate colonies for selection.

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- xv. Colonies selected for sub-cloning can be expanded, characterized and frozen for cryopreservation using the same methodology for human ES cells. Fig. 3a shows images of iPS development at different stages. **PAUSE POINT** Follow standard practices for hES cells as previously detailed in Nature Protocols^{23–25}.

B. Derivation of human iPS cells in feeder-free conditions. TIMING 3 – 8 weeks

- i. Follow the protocol for the feeder-based derivation Steps 6.A.i – vii.
- ii. Prepare matrigel coated plates as described in REAGENT SETUP.
- iii. The day following the final viral transduction, passage cells onto matrigel-coated plates (6-well format) or dishes (6 cm dish). The number of transduced cells to seed is preferably between 10,000 – 20,000 cells per cm².
- iv. The day following passaging onto matrigel, start transitioning to a feeder-free hES cell media, like mTeSR1. For the first feeding with mTeSR1, add a 1:1 mix of mTeSR1 and hADSC media. The following day, feed with 75% mTeSR1 and the 3rd day, complete the transition to mTeSR1.

- v. Continue feeding every day with 2 ml per well or 5 ml per 6 cm dish.
- vi. Several colonies (approximately 1 in 10,000 transduced cells) appear in a significantly longer timeframe than Step 6.A (3 – 8 weeks).
- vii. Pick individual colonies and passage directly to freshly coated matrigel wells and continue to culture in mTeSR1 or other feeder-free media for expansion and characterization (Step 6.A.xv.). A typical iPS colony is shown in Fig. 3b.

C. Derivation of human iPS cells in xeno-free conditions. TIMING 3 – 8 weeks

- i. Precoat the culture dishes with humanized xenobiotic-free extracellular matrix such as CELLstart according to the manufacturer's instruction. Use xeno-free media such as StemPro MSC SFM XenoFree for derivation and propagation of hADS cells. **CRITICAL STEP** hADS cells must be actively proliferating to achieve robust reprogramming. Plan to start the reprogramming experiment as soon as sufficient cells are available since xeno-free media typically will not support the same level of expansion.
- ii. Use virus produced in xeno-free conditions as described in Step 4.B. for transducing reprogramming factors.
- iii. Follow the protocol described in Step 6.B., with the substitution of xeno-free media (e.g. NutriStem) for mTeSR1 and xeno-free matrix (e.g. CELLstart) for matrigel to coat the dishes. Alternatively, synthetic polymer-coated dishes can be used as recently reported^{26,27}. The percentage of emerging iPS colonies should be similar to the feeder-free derivation (Step 6.B.). In general, iPS colonies derived in the xeno-free condition exhibit flat morphology (Fig. 3c).

TROUBLESHOOTING

TIMING

- Step 1, isolation of mouse ADS cells: 5 – 7 hours
- Step 3 (A), ADS cell expansion: 1 – 3 weeks (1–3 passages)
- Step 3 (B), Cell sorting of ADS cells: 2 – 5 hours
- Step 3 (C), Freezing ADS cells: 1 hour
- Step 3 (D), Preparing ADS cells for feeder layers: 1 hour
- Step 4, Retrovirus production: 1 week
- Step 5, Mouse iPS cell derivation: 1 – 2 weeks (until mechanical picking of iPS cells)
- Step 6 (A), Derivation of human iPS cells on feeder cells: 2 – 3 weeks (until mechanical picking of iPS cells)
- Step 6 (B), Derivation of human iPS cells in feeder-free conditions: 3 – 8 weeks
- Step 6 (C), Derivation of human iPS cells in xeno-free conditions: 3 – 8 weeks

TROUBLESHOOTING

Troubleshooting advice is shown in Table 1.

ANTICIPATED RESULTS

Isolated ADS cells should proliferate well, with a typical doubling period of 2 to 3 days. ADS cells are positive for cell surface antigens Sca-1, CD29, CD73 and CD90, and negative for CD31 and CD45. The CD34 marker is present in the initial culture, but is quickly lost after one to two passages. ADS cells are phenotypically and functionally very similar to bone marrow-derived MSCs. The authenticity of the mesenchymal origin of ADS cells can be tested by differentiation of these cells into those of the mesodermal cell lineage (i.e. adipocytes, osteoblasts and chondrocytes) by the addition of well-defined standard adipogenesis, osteogenesis and chondrogenesis cocktails, respectively^{19,20,17}.

Mouse iPS cells can be produced from ADS cells with an efficiency range of 0.1 – 0.5%. The presence of MEF feeders during iPS derivation does not affect the percentage of emerging colonies, but appears to better support the maintenance of self-renewal of many colonies long term. Mouse ADS-derived iPS cells can be stained for pluripotent markers including alkaline phosphatase, Nanog and SSEA1. At least several good colonies of iPS cells need to be maintained and stored. Mouse iPS cells can be further characterized for their pluripotency by *in vitro* differentiation through embryoid body (EB) formation (1 – 2 weeks), *in vivo* differentiation through teratoma formation (2 – 4 weeks), and/or generation of chimeric mice through injection into blastocyst (1 – 2 months).

The efficiencies of human iPS cell derivation are around 0.5 – 1.5% in the feeder-dependent condition, and around 0.008% in the feeder-free and xeno-free conditions. However, efficiencies are highly affected by many variables such as retrovirus titer, transduction efficiency, cell viability and proliferation states, choice of media, growth factors, matrix and feeder cells, etc. It is best to pick and maintain as many colonies as possible to ensure the derivation of iPS cells that have the same morphology and gene/protein expression profiles as the ES cells. It is important to karyotype the cells to check for any chromosomal abnormalities after extended passages. Common pluripotency tests of human iPS cells are positive staining for alkaline phosphatase activity or pluripotency markers including SSEA3, SSEA4, TRA-1-60 and TRA-1-81 (1 day), *in vitro* differentiation through EB formation (2 – 4 weeks) and *in vivo* differentiation assays through teratoma formation (4 – 8 weeks) by injecting cells subcutaneously into immunodeficient animals (e.g. SCID mice).

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Figure 1. Morphology of ADS cells in culture. **(a)** Mouse ADS cells grown in Expansion DMEM media. **(b)** Human ADS cells grown in hADSC media.

Figure 2. Derivation of mouse ADS-derived iPS cells. **(a)** An intermediate iPS colony at day 5 post-infection. Colonies are derived from mADS cells that were isolated from Oct4-EGFP mice. Note that the colony in the middle is weakly positive for GFP (right panel). **(b)** A developing iPS colony derived from Oct4-EGFP mADS cells at day 9. The colony is positive for GFP, indicating Oct4 activation. **(c)** An iPS colony (left) is picked and expanded in culture (right).

Figure 3. Derivation of human ADS-derived iPS cells. **(a)** Development of human iPS colonies at different stages. **(b)** Morphology of a hADS-derived iPS colony generated in the feeder-free condition. **(c)** A larger magnification of a hADS-derived iPS colony produced in the xeno-free condition. Note that xeno-free iPS cells generally appear flat.

TABLE 1

Troubleshooting tips

Step	Problem	Possible reasons	Possible solutions
Step 1 (v)	Collagenase digestion of fat tissue is incomplete.	<ol style="list-style-type: none"> 1 Lot to lot variability of collagenase. 2 Presence of inhibitory substances in the digestion buffer. 	<ol style="list-style-type: none"> 1 Extend the digestion time (e.g. 2 h). Enzymatic activities of collagenase are highly variable among commercial sources and different lots. Prior to the experiment, it is best to test their activities and use a verified source of collagenase. 2 Lower the amount of BSA and avoid serum components in the digestion buffer.
Step 1 (xiv)	Cells are contaminated with bacteria or fungi.	Tools, tubes and solutions used are not sterilized.	Include antibiotics and/or antimycotics to the media. Make sure all the tools including surgical scissors, forceps, nylon mesh, tubes, etc. are autoclaved, and solutions are filtered prior to the experiment. Be especially careful during the incubation step in the water bath, which is often the origin of contamination.
Step 1 (xiv)	Cells grow too slowly or undergo senescence.	<ol style="list-style-type: none"> 1 Proliferation rates vary between different individuals and sources of fat depots/states. 2 Cells are passaged more than 5 times. 	<ol style="list-style-type: none"> 1 Use Expansion DMEM media that is supplemented with basic FGF. If cell growth is still slow, consider adding additional growth factors in the media, such as EGF (5 ng/ml) and TGF beta (0.25 ng/ml). 2 Use cells passaged less than 4 times.
Step 4 (xiv)	Low infectivity of virus is obtained.	<ol style="list-style-type: none"> 1 Low viral titer. 2 Unhealthy ADS cells. 	<ol style="list-style-type: none"> 1 Ensure that 293T cells are healthy and transfection efficiency is close to 100% by examining GFP control. Use low-passage 293T cells. 2 Check if infectivity of other cell lines is high. If so, adapt the ADS culture to achieve optimum log-phase growth and ensure that more than 50% of cells can be differentiated to mature adipocytes with a standard adipogenesis cocktail in one week.
Step 5 (viii) and Step 6A (ii)	No or low number of iPS colonies are found.	<ol style="list-style-type: none"> 1 Low viral infection efficiency or error in expression constructs. 2 Diminished mitotic activity of ADS cells due to inappropriate culture procedures or conditions. 	<ol style="list-style-type: none"> 1 Check if efficient infection of all the transcription factors is achieved. Perform control infection of fibroblasts to ensure that viral constructs are working. Check biological titer of virus. Make all 4 viral-factors simultaneously to minimize variation. 2 Try seeding ADS cells on feeders 1–4 d after the last viral transduction using different seeding densities. Ensure that ADS cells are healthy at low passage and proliferating well. Selection of an appropriate FBS batch or culture medium for ADS cells is also important to optimize their growth.
Step 6A (xiv)	Poor recovery of picked iPS cells is obtained.	Immature iPS cell colonies are selected or inappropriate picking methods are employed.	Increase the number of colonies to be picked to get a better chance of obtaining good colonies. Avoid picking iPS cell colonies too early. Often the first colonies to emerge are transformed and not bona fide iPS colonies. Obtaining appropriate training on manual picking techniques is necessary.
Step 6C (iii)	Poor recovery of picked xeno-free iPS cells is observed following passaging.	iPS cells generated in xeno-free conditions are generally very sensitive to enzymes such as trypsin, collagenase and dispase.	Avoid using enzyme. Use manual passaging techniques. Selecting the best colonies (fast-proliferating with little differentiation and characteristics identical to hES cells) is critical. Also, the pairing of xeno-free media and matrix coating is important. Test different combinations for their recovery. We have obtained optimal results using NutriStem media and Synthemax plates, but results can be cell-line specific.