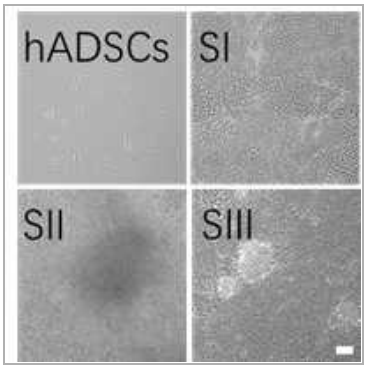

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Protocol for highly efficient and rapid generation of human pluripotent stem cells by chemical reprogramming

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ABSTRACT

We recently established a highly efficient and rapid chemical reprogramming system for generation of human pluripotent stem cells (hCiPSCs) (Liuyang et al., 2023). Here, we describe steps from isolation and culture of somatic cells to the derivation of hCiPSCs cell lines. In this method, human adipose derived stromal cells (hADSCs), and human adult skin fibroblasts (hASFs) are used as starting cells, we detail the preparation of small molecules stock solution and each stage conditional mediums, followed by step-by-step procedures to induction of hCiPSCs from somatic cells.

ATTACHMENTS

Supplementary table - chemicals and medium configuration.xlsx

GUIDELINES

1. We recommend to use the primary isolated hADSCs and hASFs (passages 2-4)

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for the induction of hCiPSCs. For commercially available hADSCs or hASFs, cultivating two passages in Mesenchymal Stem Cell Growth Medium 2 will help to ameliorate the state of cells and improve reprogramming efficiency. The longer passaged cells can be also used for the induction of hCiPSCs, but with quite reduced induction efficiency. Long term passaged commercial cell lines (IMR90 and BJ, etc.) were not recommended for the induction of hCiPSCs.

2. Make sure to culture and expand the primary hADSCs and primary hASFs in Mesenchymal Stem Cell Growth Medium 2
3. Make sure that the hADSCs and hASFs be passaged in time before/when confluent.
4. Make sure to seed the hADSCs or hASFs in 15% FBS-DMEM medium at day-1 when reprogramming and change the medium into Stage 1 medium after 12-24h. Mesenchymal Stem Cell Growth Medium 2 cannot be used to seed the cells at day-1 when reprogramming.
5. An appropriate cell density seeded at day-1 is important for the reprogramming efficiency and induction of the epithelial-like cells in stage I. Primary cells with different proliferation rate could have different appropriate cell density. We highly recommend to optimize the cell density seeded at day-1 when the percentage or area of epithelial-like cells is too low at the end of stage I.
6. The epithelia-like cells generated in stage I growth to approach ~100% confluence is recommended before changing the stage II medium. Cells at the end of stage I may not be firmly attached, so be gentle when changing the medium. Over growth or insufficient reprogramming in each stage would result in low hCiPSCs efficiency.
7. Make sure to change and refresh the reprogramming medium during reprogramming process in every 4 days.In the last four days of stage II, due to the strong proliferation of cells, the medium can be changed every two days, or you can add the same volume of stage II medium to the original well on the penultimate day.
8. Make sure to store all reprogramming regents properly (medium store at 4℃, small molecules dissolved in DMSO store at 4 ℃ for a short time and store at -20 ℃ for a long time, cytokines and enzymes store at 4 ℃ for a short time after be dissolved and store at -20 ℃ for a long time)
9. We recommended to dilute the stock solution of small molecules at proper concentration before being added into the reprogramming medium. Please see the supplementary table (In description section' attachment).
10. Make sure all mediums are prepared freshly and accurately for each component. For example, make sure to add bFGF in stage II reprogramming medium.Also, please add each small molecule after fully dissolved. When some small molecules are added to the medium, solids may precipitate due to the local concentration being too high, please mix well and make sure them fully dissolve before use.
11. Specially, the reprogramming system is quite sensitive to the concentration of CHIR, to make sure the successful induction of hCiPSCs, the volume of the

medium added with CHIR should be at least 50mL.

12. When isolate primary hADSCs, make sure to dilute the suspension with enough volume of 15% FBS-DMEM medium and shake it for 1-2 min to release cells into medium before the suspension is centrifuged. This step would influence the isolation efficiency of primary hADSCs.
13. Hypoxia with 5% O2 was only used in stage I induction for reprogramming of hADSCs and hASFs (dispensable, only used for enhancing and accelerating reprogramming). After stage I induction, cells were cultured in 21% O2 condition (hypoxia has negative effect in stage II, stage III).
14. Primary hCiPS colonies are naïve state. They cannot be picked and culture directly to mTeSR™ Plus Medium.

MATERIALS

Reagents

DMEM (Gibco, Cat# C11965500BT)
Mesenchymal Stem Cell Growth Medium 2 (Promo Cell, Cat# C-28009)
KnockOutTM DMEM (Gibco, Cat# 10829018)
GlutaMAXTM (Gibco, Cat# 35050-061)
MEM Non-Essential Amino Acids Solution (NEAA) (Gibco, Cat# 11140050)
Penicillin-Streptomycin (Gibco, Cat# 15140-122)
Fetal Bovine Serum (FBS) (Vistech, Cat# VIS93526487)
Knockout Serum Replacement (KSR) (Gibco, Cat# 10828028)
B27 supplement (Gibco, Cat# 17504-044)
AlbuMAXTM-II (Gibco, Cat# 11021-045)
mTeSR^{TM} Plus Medium (STEMCELL, Cat# 100-0276)
Collagenase IV (Gibco, Cat# 1963347)
0.25% Trypsin-EDTA (Gibco, Cat# 25200-056)
Accutase (Millipore, Cat# SCR005)
ReLeSR^{TM} (STEMCELL, Cat# 05872)
Matrigel (Corning, Cat# 354248)
Laminin-521 (STEMCELL, Cat# 77004)
PBS (Corning, Cat# 05418005)
DMSO (Dimethyl sulfoxide) (Sigma-Aldrich, Cat# D2650)
CHIR99021 (CHIR, C) (WUXI APPTEC)
616452 (6) (WUXI APPTEC)
TTNPB (N)(WUXI APPTEC)
SAG (WUXI APPTEC)
EPZ5676 (MCE, Cat# HY-15593)
Ruxolitinib (Ruxo) (Selleckchem, Cat# S1378)
3-deazaneplanocin A (DZNep) (WUXI APPTEC)
Y-27632 (WUXI APPTEC)
5-Azacytidine (5azaC, 5) (Sigma-Aldrich, Cat# A2385)
JNKIN8 (J) (Selleckchem, Cat# S4901)

BIRB796 (BIRB) (Selleckchem, Cat# S1574)
SGC-CBP30 (CBP30) (Selleckchem, Cat# S7256)
Dorsomorphin (DM)(MCE, Cat# HY-13418A)
VTP50469 (Selleckchem,Cat# S8934)
Valproic acid sodium salt (VPA) (Sigma-Aldrich, Cat#P4543)
Tranylcypromine (Tranyl, T) (Enzo, Cat#BML-EI217-0005)
PD0325901 (WUXI APPTEC)
IWP-2 (Selleckchem, Cat# S7085)
SB590885 (Selleckchem, Cat# S2220)
L-Ascorbic acid 2-phosphate (Vc2P) (Sigma-Aldrich, Cat# A8960)
LiCl (Sigma-Aldrich, Cat# L4408)
Nicotinamide (NAM) (Sigma-Aldrich, Cat# 72340)
Recombinant Human FGF2 (Origene, Cat# TP750002)
Recombinant Human Heregulinβ-1 (HRG) (PEPROTECH, Cat# 100-03)
BMP4 (Stemimmune, Cat# HST-B4-0100)
AKT Kinase Inhibitor (AKTi) (MCE, Cat# HY-10249A)
SETD2-IN-1 (SET) (MCE, Cat# HY-136328)
5-Iodotubercidin (5ITU) (MCE, Cat# HY-15424)
CX4945 (MCE, Cat# HY-50855)
Triton X-100 (Sigma-Aldrich, Cat# T8787)
Donkey serum (Jackson Immuno Research, Cat# 017-000-121)
More detailed information about chemicals can refer to supplementary table (In description section' attachment).

Isolation and culture of hADSCs

1 Adult human adipose derived stromal cells (hADSCs) were isolated from donated adult adipose tissue that obtained with informed written consent.

1. The tissues (2-4 cm^3) were washed twice with PBS containing 2% penicillin-streptomycin, minced as much as possible with scissors to 1-2 mm^3.
2. Dissociated with 5-10 ml 2 mg/ml collagenase IV solution in a 100-mm dish at 37 ^oC for 1-1.5 hour.
3. 10-20 ml 15% FBS-DMEM medium was added and cells were pipetted up and down several times for dissociation.
4. The suspension was collected to two 50-ml tubes and diluted to 30-40 ml each with 15% FBS-DMEM medium, followed by shaking for 1-2 min to release cells.
5. The suspension was centrifuged at 400 g for 5 min and cells were resuspended in Mesenchymal Stem Cell Growth Medium 2 (Promo Cell) after removing the supernatant.
6. Generally, 1-3 x 10^6 cells can be obtained from 2-4 cm^3 adipose tissue and are plated in a 100-mm dish (P0) followed by incubation in 37 ^oC with 5% CO_2.
7. The next day, fresh Mesenchymal Stem Cell Growth Medium 2 was changed to remove the non-adherent cells.

8. Primary hADSCs usually become confluent in 3-5 days and were ready to passage for reprogramming. The 0.25% Trypsin-EDTA was used to dissociate primary hADSCs.
9. For hCiPSCs induction, hADSCs were seeded at a density of 1 x 10^4 cells per well of a 12-well plate or 5 x 10^3 cells per well of a 24-well plate with 15% FBS-DMEM medium.
10. For culture and expansion, hADSCs were seeded at a density of 1.5 x 10^6cells per 100-mm dish with Mesenchymal Stem Cell Growth Medium 2. We recommend to use the primary hADSCs for the induction of CiPS cells between passages 2 to 4.

The 15% FBS-DMEM medium: high glucose DMEM (Gibco) supplemented with 15% Fetal Bovine Serum (FBS) (Vistech), 1% GlutaMAX™ (Gibco), 1% MEM Non-Essential Amino Acids Solution (NEAA) (Gibco), 1% Penicillin-Streptomycin.

Isolation and culture of hASFs

2 Human adult skin fibroblasts (hASFs) were isolated from donated adult dermis tissues that obtained with informed written consent.

1. The tissues (0.5-1 cm^2) were washed twice with PBS containing 2% penicillin-streptomycin, minced by scissors to 0.5-1 mm^2 pieces.
2. The pieces were placed in the 100-mm cell culture dish and 1 drop of 15% FBS-DMEM medium was put onto each piece of tissue.
3. The pieces were incubated in 37 ^oC with 5% $CO_2$for 4-12 hours (do not allow the pieces go to dry out).
4. 3-5 ml of Mesenchymal Stem Cell Growth Medium 2 was mildly added to the dish (do not allow the pieces detached from the dish).
5. Fresh Mesenchymal Stem Cell Growth Medium 2 was replaced every 2-3 days.
6. Within 4-7 days, outgrowths of fibroblasts would generate.
7. The primary hASFs usually become confluent in 10-14 days and were ready to passage for reprogramming.
8. We passaged the hASFs both for reprogramming and expansion in the same way to hADSCs mentioned above.

Commercial human adult dermal fibroblasts (Lonza-CC2511) and ADSCs (Lonza-PT5006) were also cultured in Mesenchymal Stem Cell Growth Medium 2 and passaged both for reprogramming and expansion in the same way to hADSCs mentioned above.
We recommended to use the primary isolated hADSCs (passages 2-4) for the induction of hCiPSCs. If the primary hASFs were used, the cells passages between 2 to 4 were recommended. For these commercially available hADSCs or hASFs, cultivating two passages in Mesenchymal Stem Cell Growth Medium 2 will help to improve the state of these cells and reprogramming efficiency. The longer passaged cells can be also used for the induction of hCiPSCs, but with quite reduced induction efficiency. Long term passaged commercial cell lines (IMR90 and BJ, etc.) are not recommended for the induction of hCiPSCs.

Medium preparation for hCiPSCs induction

3 For more intuitive medium configuration, please refer to the supplementary table(In description section' attachment).
Please prepare each medium freshly and shake well after configuration and before use. Prepared induction media can be stored at 4°C for up to two weeks.
Please do not prepare all the media at one time, the media containing small molecules has a certain use time limit.

3.1 Stage I induction medium:

KnockOut^{TM} DMEM supplemented with 1% KSR, 2% B27 1% GlutaMAX^{TM}, 1% NEAA, 1% Penicillin-Streptomycin, 50 µg/ml Vc2p, 5mM LiCl, 1 mM NAM, 20 ng/mL BMP4 and the small molecules CHIR999021 (5 µM), 616452 (10 µM), TTNPB (2 µM), SAG (0.5 µM), EPZ5676 (2 µM), DZNep (0.05 µM), Ruxolitinib (1 µM), VTP50469 (0.5 µM), AKT Kinase Inhibitor (1 µM), JNKIN8 (0.2 µM), and SETD2-IN-1(0.2 µM)

3.2 Stage II induction medium:

KnockOut^{TM} DMEM supplemented with 2% B27, 1% GlutaMAX^{TM}, 1% NEAA, 1% Penicillin-Streptomycin,50 µg/ml Vc2p, 200 ng/ml bFGF (Origene) and the small molecules CHIR99021 (5 µM), 616452 (10 µM), TTNPB (2 µM), SAG (0.5 µM), JNKIN8 (0.5 µM), EPZ5676 (2 µM), DZNep (0.2 µM), Ruxolitinib (1 µM), BIRB796 (2 µM), SGC-CBP30 (2 µM), Dorsormorphin (0.5 µM), VTP50469 (0.5 µM), 5-Iodotubercidin (0.5 µM), 5-Azacytidine (2 µM), AKT Kinase Inhibitor (0.2 µM), and CX-4945 (1 µM).

3.3 Stage III induction medium:

Knockout^{TM} DMEM supplemented with 2% B27 supplement, 1% GlutaMAX, 1% NEAA, 1% Penicillin-Streptomycin, 5% Knockout Serum Replacement (KSR), 50 µg/ml Vc2p, 20 ng/mL Recombinant Human Heregulinβ-1 (HRG) and the small molecules CHIR99021 (1 µM), Y-27632 (10 µM), PD0325901 (1 µM), SB590885 (0.5 µM). During the induction process of stage III, VPA (1000 µM), Tranylcypromine (10 µM), DZNep (0.2 µM), and EPZ5676 (2 µM) were included in the medium for the first 4 days. Subsequently, VPA (500 µM) was included in the next 4 days while Tranylcypromine, DZNep, and EPZ5676 were removed. The stage III induction medium without VPA, Tranylcypromine, DZNep, or EPZ5676 could be applied for additional 2-4 days to allow the primary hCiPSC colonies to grow larger.

Induction process of hCiPSCs from hADSCs and hASFs

4 Hypoxia with 5% $O_2$was used in stage I induction. After stage I induction, cells were cultured in 21% O_2 condition. The induction medium was changed every 3-4 days.

1. hADSCs and hASFs were seeded in high-glucose DMEM supplemented with 15% FBS at a density of 1 x 10^4 cells per well of a 12-well plate or 5-6 x 10^3cells per well of a 24-well plate, and would be changed into freshly prepared stage I induction medium after12-24h. Please

ensure that the cell density is appropriate and cells are evenly attached to the bottom of each well. An appropriate cell density seeded at day-1 is important for the reprogramming efficiency and induction of the epithelial-like cells in stage I. We highly recommend to optimize the cell density seeded at day-1 when the percentage or area of epithelial-like cells is too low at the end of stage I.

2. For stage I induction, single layer epithelial-like cells would emerge at day 4-6. When the epithelial-like cells reach ~100% confluence (usually after 8-10 days' stage I induction for hADSCs and 9-16 days for hASFs). Change the medium into freshly prepared stage II induction medium. Cells at the end of stage I may not be firmly attached, so be gentle when changing the medium.
3. For stage II induction, multi-layered cell colonies appeared after 4-6 days treatment and these cell colonies would continually grow larger. In the last four days of stage II, due to the strong proliferation of cells, the medium can be changed every two days, or you can add the same volume of stage II medium to the original well on the penultimate day. It is normal that there is cell death in late stage II.
4. Change the medium into freshly prepared stage III induction medium after 8-12 days' treatment of stage II medium when thecolonies grow larger. The induction days of stage II is important for the induction efficiency of hCiPSCs. You can switch stage II to stage III after 8, 10 or 12 days of stage II induction, and compare efficiency.
5. For stage III induction, VPA (1000 µM), Tranylcypromine (10 µM), DZNep (0.2 µM), and EPZ5676 (2 µM) were included in the induction medium for the first 4 days. Subsequently, VPA (500 µM) was included in the next 4 days while Tranylcypromine, DZNep, and EPZ5676 were removed. The stage III induction medium without VPA, Tranylcypromine, DZNep, or EPZ5676 could be applied for additional 2-4 days to allow the primary hCiPSC colonies to grow larger. In stage III, it is normal that there will be a large amount of cell death.
6. Immunofluorescent staining of OCT4 at the end of stage III is recommended and the OCT4-positive colonies were regarded as primary hCiPSC colonies. Please leave at least one well of living cells for establishment of hCiPS cell lines.

Derivation and culture of hCiPS cell line

5

1. Preparation plates coated with Laminin-521. Briefly, thaw the Laminin-521 at 2-8°C before use. Dilute it in PBS to a final concentration of 5-10 µg/mL. Gently mix and immediately add 0.5mL diluted Laminin-521 to 12-well plate. Incubate at 2-8 °C overnight.
2. Preparation medium for derivation hCiPS cell lines.
3. After 8-12 days stage III condition treatment, cells were dissociated by Accutase (Millipore) and replated at a ratio from 1:3 to 1:12 on Laminin-521 (STEMCELL) coated plates in the medium for derivation hCiPS cell lines: Knockout DMEM supplemented with 2% B27 supplement, 1% GlutaMAX, 1% NEAA, 1% Penicillin-Streptomycin, 50 µg/ml Vc2p, 2 mg/mL AlbuMAX^{TM}-II (AlbuMAX^{TM}-II could be replaced by 4% KSR) and the small molecules CHIR99021 (1 µM), PD0325901 (0.5 µM), Y-27632 (10 µM), HRG (20 ng/mL), and bFGF (100 ng/mL). Cells were incubated under 21% O2, 5% CO2 at 37°C and the medium was changed every day.
4. After 7-12 days, single compact hCiPSC colony was mechanically picked and dissociated into

small clamps and transferred onto Matrigel coated plates in mTeSR™ Plus Medium supplemented with Y-27632 (10 µM).

5. After 24 hours, the culture was replaced by fresh mTeSR™ Plus Medium without Y-27632.

hCiPS cell lines were maintained in mTeSR™ Plus Medium on Matrigel coated plates under 21% O_2, 5% CO_2 at 37 ^{o}C. The medium was changed every day. Cells were passaged when they reach ~85% confluence. This typically occurred at day 3–7 after passaging with split ratios of around 1:10 to 1:20. For passaging, hCiPS cell lines were dissociated by ReLeSR^{TM}(STEMCELL), and the detached cell aggregates were transferred onto Matrigel-coated plates in mTeSR™ Plus Medium supplemented with Y-27632 (10 µM). Allow the colonies to attach to the culture plate for 24 hours before replacing the spent medium with fresh mTeSR™ Plus Medium without Y-27632.