Induction of human pluripotent stem cells from human somatic cells by chemical reprogramming

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Method Article

Keywords: Reprogramming, small molecules, pluripotency, human somatic cells, human chemically induced pluripotent stem cells, hCiSPCs

Posted Date: April 25th, 2022

DOI: https://doi.org/10.21203/rs.3.pex-1850/v1

Abstract

Pluripotent stem cells can be induced from somatic cells by the cellular intrinsic factors, including oocyte components or transcription factors[1-4]. Previously, we have demonstrated that mouse pluripotent stem cells can be induced from mouse somatic cells by small molecule-based external stimulation, providing a fundamentally different approach for inducing pluripotency[5-8]. Here we demonstrated that human pluripotent stem cells can be induced by small molecules from human somatic cells. This protocol describes the detailed procedures for the induction of human chemically induced pluripotent stem cells (hCiPSCs) from human somatic cells. In this method, the freshly isolated human embryonic fibroblasts (HEFs), human adipose derived mesenchymal stromal cells (hADSCs), and human adult skin fibroblasts are used for the induction of hCiPSCs. Four stages are used for the reprogramming process and the detailed induction medium for each stage is provided. In addition, we provide a troubleshooting list for the induction process.

Introduction

This is a detailed protocol for the induction of human chemically induced pluripotent stem cells (hCiPSCs) from human somatic cells.

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)

2-mercaptoethanol (Gibco, Cat# 21985-023)

Fetal Bovine Serum (FBS) (Vistech, Cat# VIS93526487)

Knockout Serum Replacement (KSR) (Gibco, Cat# 10828028)

N2 supplement (Gibco, Cat# 17502-048)

B27 supplement (Gibco, Cat# 17504-044)

AlbuMAXTM-II (Gibco, Cat# 11021-045)

mTeSR™ Plus Medium (STEMCELL, Cat# 100-0276)

Collagenase IV (Gibco, Cat# 1963347)

0.25% Trypsin-EDTA (Gibco, Cat# 25200-056)

Accutase (Millipore, Cat# SCR005)

ReLeSR™ (STEMCELL, Cat# 05872)

Matrigel (Corning, Cat# 354248)

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)

ABT-869 (WUXI APPTEC)

Thiazovivin (Tzv) (WUXI APPTEC)

EPZ004777 (EPZ) (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)

UNC0224 (U2) (Tocris, Cat# 3861)

VTP50469 (Selleckchem, Cat# S8934)

Valproic acid sodium salt (VPA) (Sigma-Aldrich, Cat#P4543)

Tranylcypromine (Tranyl, T) (Enzo, Cat#BML-EI217-0005)

PD0325901 (WUXI APPTEC)

UNC0379 (Selleckchem, Cat# S7570)

DMH1 (Selleckchem, Cat# S7146)

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)

Equipment

Cell incubator (37°C, 21% O2 and 5% CO2)

Cell incubator (37°C, hypoxia with 5% O2 and 5% CO2)

Water bath (37°C)

Scissor

Centrifuge

Cell counter

Electric pipettor and pipette tips

Pipettor and pipette tips

Inverted microscope

Inverted fluorescence microscope

12-well tissue culture plates

6-well tissue culture plates

100-mm tissue culture dish

15 ml polystyrene conical tubes

50 ml polypropylene conical tubes

Procedure

Isolation and culture of HEFs

Human embryonic fibroblasts (HEFs) were isolated from12- to 18-week-old embryos that obtained from elective termination of pregnancy with informed written consent. Briefly, the donated embryonic dermis tissues (0.5-1 cm^2) were washed twice with PBS (Corning) containing 2% Penicillin-Streptomycin (Gibco), minced by scissors to 1-2 mm^2 and dissociated with 5-10 ml 2 mg/ml collagenase IV solution (Gibco) in a 100-mm dish at 37 ^oC for 1 hour. Next, 10-20 ml 15% FBS-DMEM medium was added and cells were pipetted up and down several times for dissociation. The suspension was collected to 50-ml tube and shook for 1-2 min to release cells. Then the suspension was centrifuged at 400 g for 5 min and cells were resuspended in 15% FBS-DMEM medium after remove the supernatant. Generally, 1-3 x 10^6 cells can be obtained from 0.5-1 cm^2 dermis and are plated in a 100-mm dish (P0) followed by incubation in 37 ^oC with 5% CO_2. The next day, fresh 15% FBS-DMEM medium was changed to remove the non-adherent cells. Primary HEFs usually become confluent in 3-4 days and were ready to passage for reprogramming. 0.25% Trypsin-EDTA (Gibco) was used to dissociate primary HEFs. For hCiPSCs induction, HEFs were seeded at a density of 1.5 x 10^4 cells per well of 12-well plate with 15% FBS-DMEM medium. For culture and expansion, HEFs were seeded at a density of 1.5 x 10^6 cells per 100-mm dish. We recommend to use the primary HEFs for the induction of CiPS cells within 7 passages.

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 and 0.055 mM 2-mercaptoethanol (Gibco).

Isolation and culture of hADSCs

Adult human adipose derived mesenchymal stromal cells (hADSCs) were isolated from donated adult adipose tissue that obtained with informed written consent. Briefly, the tissues (2-4 cm^3) were washed twice with PBS containing 2% penicillin-streptomycin, minced by scissors to 1-2 mm^3 and dissociated with 5-10 ml 2 mg/ml collagenase IV solution in a 100-mm dish at 37 ^oC for 1 hour. Next, 10-20 ml 15% FBS-DMEM medium was added and cells were pipetted up and down several times for dissociation. 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. Then 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. 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. The next day, fresh Mesenchymal Stem Cell Growth Medium 2 was changed to remove the non-adherent cells. 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 HEFs. For hCiPSCs induction, hADSCs were seeded at a density of 1 x 10^4 cells per well of a 12-well plate with 15% FBS-DMEM medium. For culture and expansion, hADSCs were seeded at a density of 1.5 x 10^6 cells per 100-mm dish with Mesenchymal Stem Cell Growth Medium 2. We recommend to use the primary hADSCs for the induction of CiPS cells within 4 passages.

Isolation and culture of hASFs

Human adult skin fibroblasts (hASFs) were isolated from donated adult dermis tissues that obtained with informed written consent. Briefly, 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. Then 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. Next, the pieces were incubated in 37 ^oC with 5% CO_2 for 4-12 hours (do not allow the pieces go to dry out). Then 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). Fresh Mesenchymal Stem Cell Growth Medium 2 was replaced every 2-3 days. Within 4-7 days, outgrowths of fibroblasts would generate. The primary hASFs usually become confluent in 10-14 days and were ready to passage for reprogramming. 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.

Generation of hCiPSCs from HEFs

Medium preparation for hCiPSCs induction

Stage I induction medium:

KnockOut™ DMEM (Gibco) supplemented with 10% Knockout Serum Replacement (KSR) (Gibco), 10% FBS, 1% GlutaMAX™, 1% NEAA, 1% Penicillin-Streptomycin, 0.055 mM 2-mercaptoethanol, 50 μg/ml L-Ascorbic acid 2-phosphate (Vc2P) (Sigma-Aldrich), 5 mM LiCl (Sigma-Aldrich), 1 mM Nicotinamide (NAM) (Sigma-Aldrich), 2 mg/mL AlbuMAX™-II (Gibco) and the small molecules CHIR999021 (10 μM), 616452 (10 μM), TTNPB (2 μM), SAG (0.5 μM), ABT-869 (1 μM), Rock inhibitor (Y-27632 (2 μM) or Tzv (2 μM)).

Stage II induction medium:

KnockOut™ DMEM supplemented with 10% KSR, 10% FBS, 1% GlutaMAX™, 1% NEAA, 1% Penicillin-Streptomycin, 0.055 mM 2-mercaptoethanol, 50 μg/ml Vc2p, 5 mM LiCl, 1 mM NAM, 40 ng/ml bFGF (Origene) and the small molecules CHIR99021 (10-12 μM), 616452 (10 μM), TTNPB (2 μM), SAG (0.5 μM), ABT-869 (1 μM), Y27632 (10 μM), JNKIN8 (1 μM), Tranylcypromine (10 μM), 5-Azacytidine (10 μM).

Note: To enhance the reprogramming efficiency, the small molecules UNC0224 (1 μM), Ruxolitinib (1 μM) and SGC-CBP30 (2 μM) were recommended in stage II induction medium.

Stage III induction medium:

Knockout™ DMEM supplemented with 1% N2 supplement (Gibco), 2% B27 supplement (Gibco), 1% GlutaMAX™, 1% NEAA, 1% Penicillin-Streptomycin, 0.055 mM 2-mercaptoethanol, 50 μg/ml Vc2p, 5 mg/mL AlbuMAX™-II, 20 ng/mL Recombinant Human Heregulinβ-1 (HRG) (PEPROTECH) and the small molecules CHIR99021 (1 μM), 616452 (10 μM), Y-27632 (10 μM), PD0325901 (1 μM), Tranylcypromine (10 μM), VPA (500 μM), DZNep (0.2 μM), EPZ004777 (5 μM), UNC0379 (1 μM).

Stage IV induction medium:

Knockout™ DMEM supplemented with 1% N2 supplement, 2% B27 supplement, 1% GlutaMAX™, 1% NEAA, 1% Penicillin-Streptomycin, 0.055 mM 2-mercaptoethanol, 50 μg/ml Vc2p, 20 ng/mL HRG and the small molecules CHIR99021 (1 μM), Y-27632 (10 μM), PD0325901 (1 μM), IWP-2 (2 μM), SB590885 (0.5 μM). The VPA (500 μM) was included in the first 4 days.

Induction process of hCiPSCs from HEFs

Cells were maintained at 37 ℃ with 21% O_2 and 5% CO_2. The induction medium was changed every 3-4 days.

1. HEFs were seeded at a density of 1-1.5 x 10^4 cells per well of a 12-well plate in 15% FBS-DMEM medium. Change the medium into stage I induction medium on the next day.

2. For stage I induction, single layer epithelial-like cells would emerge at day 4-6 and approach 80% confluence at day 8-12, then change the medium into stage II induction medium.

3. For stage II induction, multi-layered colonies appeared after 8-12 days treatment and these cell colonies would continually grow larger. After total 16-20 days' stage II treatment, change the medium into stage III condition.

4. For stage III induction, 12 day's treatment of stage III medium was recommended and then change the medium into stage IV condition.

5. For stage IV induction, VPA (500 μM) was included in the first 4 days. Primary hCiPSC colonies would emerge after 6-8 days' treatment.

Note: At the end of stage IV, immunofluorescent staining of co-expression of OCT4 and NANOG was recommended to confirm the generation of primary hCiPSC colonies. Primary hCiPSC colony number was calculated as the number of the compact OCT4 positive colonies. Reprogramming efficiency was calculated as the number of primary hCiPSC colonies divided by the number of input HEFs.

Generation of hCiPSCs from hADSCs and hASFs

Medium preparation for hCiPSCs induction

Stage I induction medium:

KnockOut™ DMEM supplemented with 10% KSR, 10% FBS, 1% GlutaMAX™, 1% NEAA, 0.055 mM 2-mercaptoethanol, 1% Penicillin-Streptomycin, 50 μg/ml Vc2p, 5mM LiCl, 1 mM NAM, 2 mg/mL AlbuMAX™-II and the small molecules CHIR999021 (10 μM), 616452 (10 μM), TTNPB (2 μM), SAG (0.5 μM), ABT-869 (1 μM), Rock inhibitor (Y-27632 (2 μM) or Tzv (2 μM)), Dot1L inhibitor (EPZ004777 (2 μM) or EPZ5676 (2 μM)), DZNep (0.02 μM), Ruxolitinib (1 μM). For hCiPSCs induction from hASF, 0.5 μM VTP50469 was recommended to enhance the induction efficiency.

Stage II induction medium:

KnockOut™ DMEM supplemented with 10% KSR, 10% FBS, 1% GlutaMAX™, 1% NEAA, 0.055 mM 2-mercaptoethanol, 1% Penicillin-Streptomycin, 50 μg/ml Vc2p, 5 mM LiCl, 1 mM NAM, 100 ng/ml bFGF (Origene) and the small molecules CHIR99021 (12 μM), 616452 (10 μM), TTNPB (2 μM), SAG (0.5 μM), ABT-869 (1 μM), Y-27632 (10 μM), JNKIN8 (1 μM), Tranylcypromine (2 μM), 5-Azacytidine (10 μM), UNC0224 (1 μM), Ruxolitinib (1 μM), BIRB796 (2 μM), Dorsormorphin (0.5 μM), SGC-CBP30 (2 μM). For hCiPSCs induction from hASFs or hADSCs, 0.5 μM VTP50469 was recommended to enhance the induction efficiency in stage II.

Stage III induction medium:

Knockout™ DMEM supplemented with 1% N2 supplement, 2% B27 supplement, 1% GlutaMAX™, 1% NEAA, 0.055 mM 2-mercaptoethanol, 1% Penicillin-Streptomycin, 50 μg/ml Vc2p, 5 mg/mL AlbuMAX™-II, 20 ng/mL HRG and the small molecules CHIR99021 (1 μM), 616452 (10 μM), Y-27632 (10 μM), PD0325901 (1 μM), Tranylcypromine (10 μM), VPA (500 μM), DZNep (0.2 μM), EPZ004777 (5 μM), UNC0379 (1 μM). For hCiPSCs induction from hASFs, 0.5 μM DMH1 was recommended to enhance the induction efficiency.

Stage IV induction medium:

Knockout™ DMEM supplemented with 1% N2 supplement, 2% B27 supplement, 1% GlutaMAX™, 1% NEAA, 0.055 mM 2-mercaptoethanol, 1% Penicillin-Streptomycin, 50 μg/ml Vc2p, 20 ng/mL HRG and the small molecules CHIR99021 (1 μM), Y-27632 (10 μM), PD0325901 (1 μM), IWP-2 (2 μM), SB590885 (0.5 μM). VPA (500 μM), Tranylcypromine (10 μM), DZNep (0.05 μM), and EPZ004777 (5 μM) were included in the first 4 days.

Induction process of hCiPSCs from hADSCs and hASFs

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. ADSCs and hASFs were seeded at a density of 1 x 10^4 cells per well of a 12-well plate in 15% FBS DMEM medium. Change the medium into stage I induction medium on the next day.

2. For stage I induction, single layer epithelial-like cells induced from hADSCs would emerge at day 4-6 and approach 80% confluence at day 8-12. For ASFs, epithelial-like cells would approach 80% confluence at day 12-20. Then change the medium into stage II induction medium.

3. For stage II induction, multi-layered cell colonies appeared after 8-12 days treatment and these cell colonies would continually grow larger. After total 20 days' treatment of stage II medium, change the medium into stage III induction medium.

4. For stage III induction, 12-day's treatment of stage III induction medium was recommended. Then change the medium into stage IV condition.

5. For stage IV induction, VPA (500 μM), Tranylcypromine (10 μM), DZNep (0.05 μM), and EPZ004777 (5 μM) were included in the first 4 days of stage IV induction medium. Primary hCiPSC colonies would emerge after 6-8 days' treatment.

Derivation and culture of human CiPS cell lines

After 8-12 days stage IV condition treatment, cells were dissociated by Accutase (Millipore) and replated at a ratio from 1:3 to 1:12 on feeder layers of mitomycin C (Sigma-Aldrich)-treated MEFs (2-3 x 10^4 per cm^2) in the modified stage IV condition: Knockout DMEM supplemented with 1% N2 supplement, 2% B27 supplement, 1% GlutaMAX™, 1% NEAA, 1% Penicillin-Streptomycin, 0.055 mM 2-mercaptoethanol, 50 μg/ml Vc2p, 2 mg/mL AlbuMAX™-II and the small molecules CHIR99021 (1 μM), PD0325901 (0.5 μM), IWP-2 (2 μM), Y-27632 (10 μM), HRG (20 ng/mL), and bFGF (100 ng/mL, Origene). Cells were incubated under 21% O_2, 5% CO_2 at 37 ^oC and the medium was changed every day. After 3-7 days, compact CiPS cell colonies appeared. After 10-12 days, these colonies were manually picked up and mechanically dissociated into small clamps and transferred onto Matrigel (Corning) coated plates in mTeSR™ Plus

Medium (STEMCELL) 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.

Human CiPSCs were maintained in mTeSR™ Plus Medium on Matrigel coated plates under 21% O_2, 5% CO_2 at 37 ^oC. 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, human CiPS cells 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.

Troubleshooting

1. We recommended to use the primary isolated hADSCs (within 4 passages) for the induction of hCiPSCs. If the primary HEFs were used, the cells within 7 passages were recommended. If the primary hASFs were used, the cells within 4 passages were recommended. The longer passaged cells (up to 10 passages) can be also used for the induction of hCiPSCs, but with 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 HEFs in 15% FBS-DMEM medium.

3. Make sure to culture and expand the primary hADSCs and primary hASFs in Mesenchymal Stem Cell Growth Medium 2.

4. Make sure that the primary HEFs, primary hADSCs and primary hASFs be passaged in time before/when confluent.

5. Make sure to seed the primary HEFs, primary hADSCs or primary hASFs in 15% FBS-DMEM medium at day-1 when reprogramming and change the medium into Stage 1 medium in 24 h after cells were attached. Mesenchymal Stem Cell Growth Medium 2 cannot be used to seed the cells at day-1 when reprogramming.

6. 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 of epithelial-like cells is too low at the end of stage I.

7. The primary HEFs, primary hADSCs and primary hASFs with different genetic backgrounds could have different kinetics and growth rate during reprogramming. We highly recommend to adjust the treatment time especially in stage I. The epithelia-like cells generated in stage I growth to approach 80%

confluence is recommended before changing the stage II medium. Over growth or insufficient reprogramming in each stage would result in low hCiPSCs efficiency.

8. Make sure to change and refresh the reprogramming medium during reprogramming process in every 4 days.

9. 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)

10. We recommended to dilute the stock solution of small molecules at proper concentration before be added into the reprogramming medium: small molecules dissolved in DMSO diluted to 5,000-10,000x of final concentration used in reprogramming process, small molecules or cytokines dissolved in H_2O diluted to 1,000x of final concentration used in reprogramming process.

11. Make sure all mediums are prepared properly and accurately for each component. For example, make sure to add bFGF in stage II reprogramming medium.

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, not used in stage I induction of HEFs). After stage I induction, cells were cultured in 21% O2 condition (hypoxia has negative effect in stage II, stage III and stage IV).

Time Taken

Anticipated Results

If the experiments were performed correctly as described above, primary hCiPSC colonies can be induced and detected by immunofluorescent staining with co-expression of OCT4/NANOG.

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