Induction and maintenance of mouse totipotent stem cells by a defined chemical cocktail

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Method Article

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Abstract

In mice, only the zygotes and blastomeres from 2-cell embryos are authentic totipotent stem cells (TotiSCs), capable of producing all cell types in both embryonic and extraembryonic tissues and forming an entire organism[1]. However, it remains challenging whether and how TotiSCs, representing the very beginning of a life, can be established *in vitro* in the absence of germline cells. Here, we described a step-by-step protocol for induction and long-term maintenance of TotiSCs from mouse pluripotent stem cells (PSCs) by a small-molecule combination of TTNPB, 1-Azakenpaullone, and WS6. These chemically induced totipotent stem cells, designated as ciTotiSCs, resembled mouse totipotent 2C-embryo stage cells at transcriptome, epigenome and metabolome level. In addition, ciTotiSCs exhibited bidirectional developmental potentials and were able to produce both embryonic and extraembryonic cells *in vitro* and in teratoma. Furthermore, following injection into 8-cell embryo, ciTotiSCs contributed to both embryonic and extraembryonic lineages with high efficiency. Our chemical approach for TotiSCs induction and maintenance provides a defined *in vitro* system to manipulate and understand totipotent state towards creating life from non-germline.

Introduction

In mammals, intrinsic totipotency is the capacity by a cell to give rise to all the differentiated cells in both embryonic and extraembryonic tissues and form an organism[1]. In mice, only zygote and its immediate descendent, 2-cell-stage blastomeres, are totipotent[2]. As an embryo further develops, TotiSCs undergo irreversible differentiation into the first two different lineages, the trophectoderm (TE) that will generate the extraembryonic placenta, and the inner cell mass (ICM) that will give rise to the pluripotent epiblasts (EPI) and primitive endoderm (PrE), which are precursors of embryonic fetus and extraembryonic yolk sac, respectively. TotiSCs *in vitro* could so far only be generated transiently through IVF or SCNT using germline cells. Therefore, inducing and maintaining authentic TotiSCs *in vitro* from non-germline represents a holy grail toward creating and understanding life.

Recent studies have reported observations of cells with expression of certain 2-cell-stage genes from mouse PSCs[3], particularly in the presence of specific small molecules, cytokines or transgenes[4,5]. In 2012, Macfarlan *et al.* found that about 0.1-1% cells in mESC and miPSC cultures expressed *MERV-L* and a few other 2C specific genes (named as 2C-like cells/2CLCs), and possessed partial developmental potential of 2C blastomeres[3]. Subsequent studies identified several crucial factors determining 2C-like state, such as DUX[6,7], and revealed that modulating the expression of these factors was able to induce more 2CLCs. In addition, mouse expanded potential stem cells (mEPSCs) were another reported PSCs with developmental potentials into both embryonic and extraembryonic lineages[8,9]. However, contribution of mEPSCs into extraembryonic tissues *in vivo* was challenged by a recent study with stringent criteria[10]. Notably, the transcriptional profiles of 2CLCs and mEPSCs actually more resemble mESCs and post-implantation embryos rather than zygotes or 2C-stage totipotent embryos[10,11], therefore explaining their limited real developmental potentials not as totipotent cells.

The protocol described herein provides a detailed method for the induction and maintenance of authentic TotiSCs from mPSCs by a combination of three small molecules. Those chemically induced TotiSCs/ciTotiSCs closely resemble mouse totipotent 2C-stage embryos in term of transcriptome, epigenome, and metabolome as well as developmental potentials into both embryonic and extraembryonic lineages.

Reagents

Knock-out DMEM (GIBCO, 10829018)

DMEM (Gibco, C11965500BT)

FBS (GIBCO, 1009914),

L-glutaMAX (GIBCO, 35050061),

Penicillin-streptomycin (GIBCO, 10378016),

Non-essential amino acids (NEAA) (GIBCO, 11140050),

Sodium pyruvate (GIBCO, 11360070),

2-mercaptoethanol (ThermoFisher, 21985023),

CHIR-99021 (Selleck, S2924)

PD0325901 (Selleck, S1036)

Gelatin (Sigma, G1890)

KSR (Gibco, 10828010)

CDL (CD lipid concentrate, 500X, Gibco, 11905-031)

N2 (ThermoFisher, A1370701)

Sodium L-ascorbyl-2-phosphate (Selleck, S5115)

Azakenpaullone (Selleck, S7193)

WS6 (Selleck, S7442)

TTNPB (Selleck, S4627)

Trypsin-EDTA (Gibco, 25200072)

D-PBS (Gibco, 14190144)

Equipment

Cell incubator (37°C, 95% air and 5% CO2)

Laminar Flow Hood

Water bath (37°C)

Inverted microscope (Olympus)

Pipette and pipette tips

Centrifuge

15 ml polystyrene conical tubes

12-well tissue culture plates

Procedure

Preparation of culture medium

Feeder cell culture medium: DMEM supplemented with 10% FBS and 1X penicillin-streptomycin.

2i/LIF mESC medium: Knock-out DMEM supplemented with 15% FBS, 1X L-glutaMAX, 1X penicillin-streptomycin, 1X non-essential amino acids (NEAA), 1X sodium pyruvate, 0.1 mM 2-mercaptoethanol, 1000 U/mL mouse leukemia inhibitory factor (mLIF), 3 μM CHIR-99021 and 1 μM PD0325901.

ciTotiSC medium: Knock-out DMEM supplemented with 5% KSR, CDL, 1% N2, 1X L-glutaMAX, 1X penicillin-streptomycin, 1X non-essential amino acids (NEAA), 1X sodium pyruvate, 0.1 mM 2-mercaptoethanol and 1000 U/mL mouse leukemia inhibitory factor (mLIF), 50 ng/ml Sodium L-ascorbyl-2-phosphate, 2.5 μM 1-Azakenpaullone, 0.5 μM WS6 and 0.2 μM TTNPB.

Note: 50 nM ~ 200 nM TTNPB was recommended to be tested in different cell lines.

Derivation of ciTotiSCs from mESCs:

Day 0:

1. Pre-coat 12-well plate with 1 ml 0.1% gelatin per well and incubate the plate at 37 °C for 30 min.

2. Remove the gelatin solution and seed inactivated MEF feeders at a density of $2X10^5$ per well in gelatin pre-coated 12-well plate with feeder cell culture medium.

3. Incubate the feeder cells overnight in incubator at 37 °C with 5% CO_2 and 95% air.

Day 1:

4. Aspirate out the feeder cell culture medium, change to 1 ml pre-warmed ciTotiSC medium, and equilibrate the culture medium in the incubator for 10 min.

5. mESCs are routinely cultured on inactivated MEF feeders using 2i/LIF mESC medium, and are passaged every 2-3 days using 0.05% trypsin-EDTA. For ciTotiSC derivation, upon mESCs reaching 80% confluency, aspirate out the medium, wash once with DPBS, and then incubate the cells with 500 µl 0.05% trypsin-EDTA at 37 °C for 3 min.

6. Add 2 ml 2i/LIF mESC medium, pipette cells up and down gently into single cells, transfer cell suspension to a 15 ml polystyrene conical tube and centrifuge cells at 1,000 rpm for 5 min.

7. Aspirate out the supernatant, resuspend the dissociated cell pellet with pre-warmed ciTotiSC medium and seed the cells with split ratios of 1:10~1:15 on inactivated MEF feeders.

8. Incubate the cells in incubator overnight at 37 °C with 5% CO_2 and 95% air, and change the medium every day with freshly pre-warmed ciTotiSC medium.

Day 3/4:

9. After culturing with ciTotiSC medium for 2-3 days, ciTotiSC colonies will arise and reach 70-80% confluency.

10. Aspirate out ciTotiSC medium, wash once with DPBS, and then incubate the cells with 500 µl 0.05% trypsin-EDTA at 37 °C for 3 min.

11. Add 2 ml fresh ciTotiSC medium, pipette cells up and down gently into single cells, transfer cell suspension to a 15 ml polystyrene conical and centrifuge cells at 1,000 rpm for 5 min.

13. Aspirate out the supernatant, resuspend the dissociated cell pellet in pre-warmed ciTotiSC medium and seed the cells with split ratios of 1:3~1:5 on inactivated MEF feeders.

14. Incubate the cells in incubator overnight at 37 °C with 5% CO_2 and 95% air, change the medium every day with freshly pre-warmed ciTotiSC medium.

15. ciTotiSCs are passaged every 2-4 days at high densities (1:3-1:5) when they reach 70-80% confluency.

Note: To ensure ciTotiSCs remain healthy, do not culture them on feeder cells pre-plated for more than one day in advance. To enhance the survival of passaged ciTotiSCs, Y27632 can be added on the first day after passaging.

Troubleshooting

Time Taken

Anticipated Results

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