

# Fast and Efficient Mouse Pluripotency Reprogramming Using a Chemically-Defined Medium

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**Abstract:** The reprogramming of somatic cells to obtain induced pluripotent stem cells (iPSCs) is an important biological and medical breakthrough, providing important applications for fields such as regenerative medicine and disease modeling. However, this promising technology is damped due to its low efficiency and slow kinetics. Therefore, we generated a practical workflow to rapidly and efficiently induce iPSCs from mouse embryonic fibroblasts (MEFs) using iCD1 (iPS chemically-defined medium 1). This protocol can easily be implemented in a standard cell culture laboratory and be applied to cell fate research.

**Keywords:** reprogramming; induced pluripotent stem cells (iPSCs); transcription factors; mouse embryonic fibroblasts (MEFs); fast kinetics



**Citation:** Huang, J.; Yang, X.; Wang, J.; Wu, H.; Pei, D.; Chen, J. Fast and Efficient Mouse Pluripotency Reprogramming Using a Chemically-Defined Medium. *Methods Protoc.* **2022**, *5*, 28. <https://doi.org/10.3390/mps5020028>

Academic Editor: Kejin Hu

Received: 21 January 2022

Accepted: 19 March 2022

Published: 24 March 2022

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## 1. Introduction

A group of cells that are isolated from the inner cell mass at the blastocyst stage, termed pluripotent stem cells, are capable of in vitro self-renewal while maintaining their pluripotency. The blastocyst-derived pluripotent stem cells, named embryonic stem cells (ESCs), retain their differentiation potentials [1,2]. Their ability to differentiate into all adult cell types provides an avenue for developmental biology research and regenerative medicine [3].

Despite wide applications of ESCs, technical difficulties and ethical concerns impede the generation of human ESCs using the previous technologies such as somatic cell nuclear transfer [4] or cell fusion of somatic cells with the existing ESCs [5,6]. Since 2006, the groundbreaking discovery of mouse and human somatic reprogramming by overexpressing four transcription factors Oct4, Sox2, Klf4, and c-Myc (OSKM) [7–11] has substantially addressed the ethical difficulties.

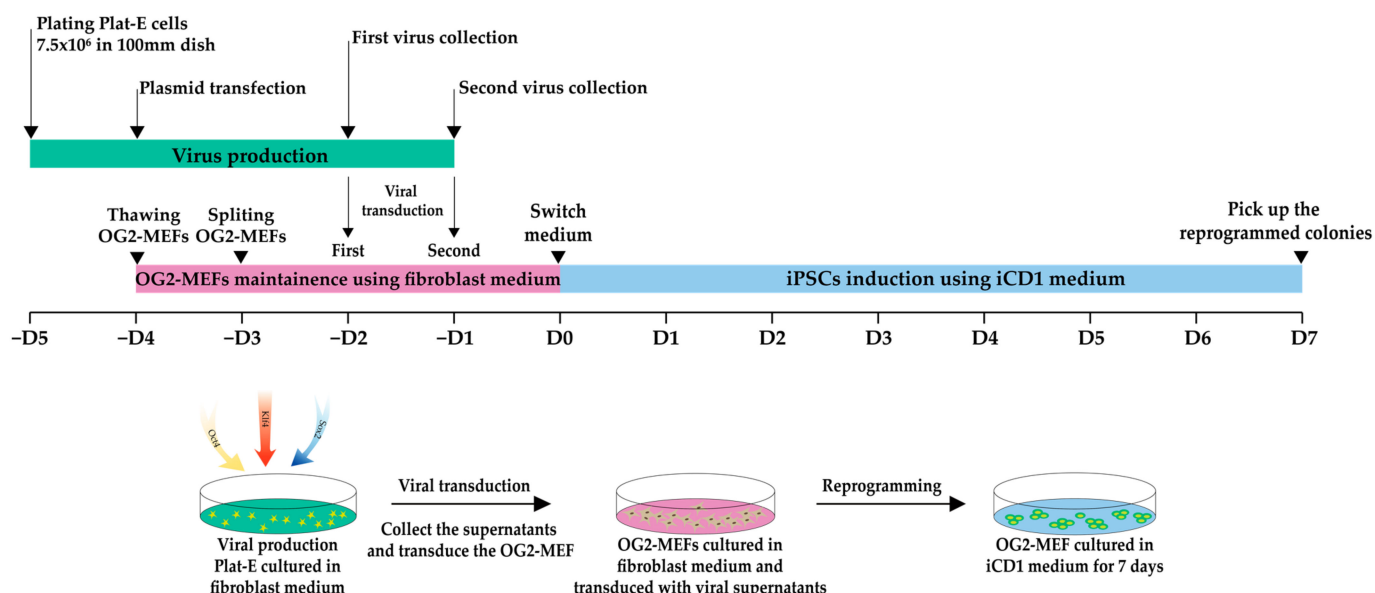
The reprogramming of somatic cells into iPSCs with defined factors is a laborious and inefficient process. Usually, it takes two to three weeks to generate murine iPSCs from MEFs with the requirement of fetal bovine serum (FBS) and feeders to reach only ~0.05% efficiency [12]. Such an inefficient system greatly hinders molecular mechanism investigation of somatic reprogramming, and even the entry of laboratories in this field. Technically, the iPSC generation process includes cell preparation, factor delivery, cell fate induction, as well as culture of the reprogramming and reprogrammed cells. In our experience, a critical practice in cell preparation is the prevention of any mycoplasma contamination, an overlooked factor for the failure of reprogramming. The retroviral [7]

and lentiviral [13] systems that are widely used in iPSC reprogramming are reliable and commercially available. We recommend the retroviral system as retrovirus transduction is much less cytotoxic than lentivirus. Therefore, the optimization of the reprogramming cell culture is a key process. By screening the chemicals and other components for cell culture, we developed a more efficient and fast way to generate iPSCs. We developed a feeder-independent culture medium termed iCD1 [14] that enhances the reprogramming efficiency up to 10% without the use of oncogene c-Myc.

Here we presented a stepwise method to generate iPSC and compared the efficiency of iCD1 with the traditional method using mES + Vitamin C induction medium. iPSCs can be generated within seven days. The iPSCs that are generated using this method are indistinguishable from embryonic stem cells in colony morphology, cellular functions, cell markers, and transcriptome and are thus suitable for downstream biological research [14]. Our method provides an easy-to-use system for mouse iPSC generation and serves as a starting point for further improvement of iPSC technology.

## 2. Experimental Design

Figure 1 shows the workflow of iPSC generation from OG2-MEFs using iCD1.



**Figure 1.** Workflow of iPSC generation from OG2-MEFs using iCD1. Timeline of viral production, OG2-MEFs preparation and transduction, and iPSC induction. Arrows indicate the key steps at different time points. OG2-MEFs ( $1 \times 10^4$  cells) are split and plated in a 12-well plate.

### 2.1. Materials

Table 1 provides all the consumables and equipment used in Section 3.

### 2.2. Reagents

Table 2 provides all the chemicals, antibodies, recombinant proteins, and plasmids used in Section 3.

### 2.3. Medium Recipes

Table 3 provides all the media, solution and their formula used in Section 3; Table 4 provides complete recipe of iCD1 use in Section 3.

### 2.4. Primers

Table 5 provides all the primers and their sequence used in real-time PCR.

**Table 1.** Information on all consumables and equipment.

| Name                                    | Source                   | Identifier | Location              |
|---|--------------------------|------------|-----------------------|
| Pipette tips 10 µL                      | Corning                  | T-300      | Glendale, AZ, USA     |
| Pipette tips 200 µL                     | Corning                  | T-200-Y    | Glendale, AZ, USA     |
| Pipette tips 1 mL                       | Corning                  | T-1000-B   | Glendale, AZ, USA     |
| Microtubes                              | Corning                  | MCT-150-C  | Glendale, AZ, USA     |
| 15 mL tubes                             | Corning                  | 430790     | Glendale, AZ, USA     |
| 50 mL tubes                             | Corning                  | 430828     | Glendale, AZ, USA     |
| Cell culture multiwell plate, 6 well    | Greiner                  | 657160     | Kremsmünster, Austria |
| Cell culture multiwell plate, 12 well   | Greiner                  | 665180     | Kremsmünster, Austria |
| 60 mm cell culture dishes               | Greiner                  | 664160     | Kremsmünster, Austria |
| 100 mm cell culture dishes              | Greiner                  | 628160     | Kremsmünster, Austria |
| 0.45 µm filter                          | Millipore                | SLHVR33RB  | Burlington, MA, USA   |
| 4 °C refrigerator                       | Haier                    | HYCD-290   | Guangzhou, China      |
| −20 °C freezer                          | Haier                    | HYCD-290   | Guangzhou, China      |
| −80 °C freezer                          | Thermo Fisher Scientific | 995        | Waltham, MA, USA      |
| Heracell 240i Incubator                 | Thermo Fisher Scientific | 51026331   | Waltham, MA, USA      |
| Microscope                              | ZEISS                    | Vert.A1    | Oberkochen, Germany   |
| Low-speed centrifuge                    | ZONKIA                   | SC-3612    | Anhui, China          |
| QuantStudio™ 3 Real-Time PCR Instrument | Applied Biosystems       | A28132     | Waltham, MA, USA      |

**Table 2.** Information on all chemicals, antibodies, recombinant proteins, and plasmids.

| Name   | Source               | Identifier | Location            |
|--|----------------------|------------|---------------------|
| Dulbecco's Phosphate-Buffer Saline (DPBS)            | HyClone              | SH30028.02 | Logan, UT, USA      |
| DMEM High Glucose                                    | HyClone              | SH30022.01 | Logan, UT, USA      |
| FBS (for mES + Vitamin C medium)                     | Lonsera              | S711-001s  | Shanghai, China     |
| FBS (for fibroblast medium)                          | NATOCOR              | SFBE       | Córdoba, Argentina  |
| GlutaMAX   | GIBCO                | 35050079   | Waltham, MA, USA    |
| Non-Essential Amino Acids Solution (NEAA)            | GIBCO                | 11140076   | Waltham, MA, USA    |
| Sodium Pyruvate                                      | GIBCO                | 11360070   | Waltham, MA, USA    |
| β-Mercaptoethanol                                    | GIBCO                | 21985-023  | Waltham, MA, USA    |
| Trypsin-EDTA (0.25%)                                 | GIBCO                | 25200114   | Waltham, MA, USA    |
| DAPI   | Sigma                | D9542      | Burlington, MA, USA |
| GSA  | ZSGB-BIO             | ZLI-9022   | Beijing, China      |
| Triton x-100   | Sigma                | T9284      | Burlington, MA, USA |
| Nanog Polyclonal Antibody                            | BETHYL               | A300-397   | Montgomery, TX, USA |
| Alexa Fluor 568 Goat anti-Rabbit                     | Invitrogen           | A11011     | Waltham, MA, USA    |
| ChamQ™ SYBR qPCR Master Mix kit                      | Vazyme               | Q311       | Nanjing, China      |
| HiScript II Q RT SuperMix for qPCR kit               | Vazyme               | R222-01    | Nanjing, China      |
| TRI Reagent  | MRC                  | TR118-200  | Cincinnati, OH, USA |
| CHIR99021  | Sigma                | SML1046    | Burlington, MA, USA |
| Thiamine hydrochloride                               | Sigma                | T1270      | Burlington, MA, USA |
| 2-Phospho-L-ascorbic acid trisodium salt (Vitamin C) | Sigma                | 49752      | Burlington, MA, USA |
| Lithium chloride                                     | Sigma                | L4408      | Burlington, MA, USA |
| Polybrene  | Sigma                | TR1003     | Burlington, MA, USA |
| Sodium phosphate dibasic                             | Sigma                | S7907      | Burlington, MA, USA |
| Potassium chloride                                   | Sigma                | P9333      | Burlington, MA, USA |
| HEPES  | Sigma                | H7523      | Burlington, MA, USA |
| D-(+)-Glucose  | Sigma                | G6152      | Burlington, MA, USA |
| Sodium chloride                                      | Sigma                | S5886      | Burlington, MA, USA |
| Mouse leukemia inhibitory factor (LIF)               | Millipore            | ESGE107    | Burlington, MA, USA |
| pMX-Oct4   | Addgene              | 13366      | Watertown, MA, USA  |
| pMX-Sox2   | Addgene              | 13367      | Watertown, MA, USA  |
| pMX-Klf4   | Addgene              | 13370      | Watertown, MA, USA  |
| pMX-DsRed  | Laboratory of D. Pei | N/A        | Guangzhou, China    |

**Table 3.** Detailed information on the media and solution formula.

| Name                           | Recipe   |
|--------------------------------|--|
| 2 × HBS (500 mL)               | NaCl 8.1816 g, KCl 0.8715 g, Na <sub>2</sub> HPO <sub>4</sub> 0.10647 g, Glucose 1.08096 g, HEPES 5.95775 g, adjust PH to 6.92–6.95 with NaOH, add ultrapure water to 500 mL   |
| 2 M CaCl <sub>2</sub> (500 mL) | CaCl <sub>2</sub> 147.02 g, add ultrapure water to 500 mL  |
| Fibroblast medium              | DMEM/high glucose 500 mL, FBS (NATOCOR) 10% (56 mL), NEAA 1/100 (5.6 mL), GlutaMAX 1/100 (5.6 mL)  |
| mES + Vitamin C                | Lonsera FBS 7.5 mL, NEAA 500 µL, GlutaMAX 500 µL, Sodium Pyruvate 500 µL, β-Mercaptoethanol (55 mM) 91 µL (Final concentration 0.1 µM), 2-Phospho-L-ascorbic acid trisodium salt (Vitamin C, final concentration 50 µg/mL) 50 µL, Mouse leukemia inhibitory factor (0.1 mg/mL, Final concentration 12.5 ng/mL) 6.25 µL, add DMEM/high glucose to make the volume 50 mL |
| iCD1                           | The recipe is shown in Table 4   |
| 3% GSA Blocking Buffer         | GSA 1.5 mL, DPBS 48.5 mL   |
| 0.2% Permeabilization Buffer   | Triton 0.1 mL, DPBS 49.9 mL  |

**Table 4.** Components of the iCD1 medium.

| Substance                        | mg/L                    | Substance                       | mg/L                    |
|----------------------------------|-------------------------|---------------------------------|-------------------------|
| L-Arginine·HCl                   | 8.40 × 10 <sup>1</sup>  | Arachidonic acid                | 2.00 × 10 <sup>-2</sup> |
| L-Alanine                        | 8.90                    | Cholesterol                     | 2.20                    |
| L-Asparagine                     | 1.32 × 10 <sup>1</sup>  | Linoleic acid                   | 1.00 × 10 <sup>-1</sup> |
| L-Aspartic acid                  | 1.33 × 10 <sup>1</sup>  | Linolenic acid                  | 1.00 × 10 <sup>-1</sup> |
| L-Cystine·2HCl                   | 6.30 × 10 <sup>1</sup>  | Myristic acid                   | 1.00 × 10 <sup>-1</sup> |
| L-Glutamic acid                  | 1.47 × 10 <sup>1</sup>  | Oleic acid                      | 1.00 × 10 <sup>-1</sup> |
| L-Histidine HCl·H <sub>2</sub> O | 4.20 × 10 <sup>1</sup>  | Palmitoleic acid                | 1.00 × 10 <sup>-1</sup> |
| L-Isoleucine                     | 1.05 × 10 <sup>2</sup>  | Palmitic acid                   | 1.00 × 10 <sup>-1</sup> |
| L-Leucine                        | 1.05 × 10 <sup>2</sup>  | Pluronic F-18                   | 1.00 × 10 <sup>3</sup>  |
| L-Lystine HCl                    | 1.46 × 10 <sup>2</sup>  | Stearic acid                    | 1.00 × 10 <sup>-1</sup> |
| L-Methionine                     | 3.00 × 10 <sup>1</sup>  | Tween 80                        | 2.20 × 10 <sup>1</sup>  |
| L-Phenylalanine                  | 6.60 × 10 <sup>1</sup>  | 2-Phospho-L-ascorbic acid       | 5.00 × 10 <sup>1</sup>  |
| L-Proline                        | 1.15 × 10 <sup>1</sup>  | D,L-alpha-tocopherol(Vitamin E) | 1.00                    |
| L-Serine                         | 6.30 × 10 <sup>1</sup>  | D,L-alpha-tocopherol acetate    | 1.00                    |
| L-Threonine                      | 9.50 × 10 <sup>1</sup>  | Biotin                          | 1.00 × 10 <sup>-1</sup> |
| L-Tryptophan                     | 1.60 × 10 <sup>1</sup>  | D-Ca pantothenate               | 4.00                    |
| L-Tyrosine·2Na·2H <sub>2</sub> O | 1.04 × 10 <sup>2</sup>  | Choline chloride                | 4.00                    |
| L-Valine                         | 9.40 × 10 <sup>1</sup>  | Folic acid                      | 4.00                    |
| Glycine                          | 4.50 × 10 <sup>1</sup>  | i-Inositol                      | 7.20                    |
| D-Glucose                        | 4.50 × 10 <sup>3</sup>  | Niacinamide                     | 4.00                    |
| Sodium pyruvate                  | 1.10 × 10 <sup>2</sup>  | Pyridoxine HCl                  | 4.00                    |
| D(+)-Galactose                   | 1.50 × 10 <sup>1</sup>  | Riboflavin                      | 4.00 × 10 <sup>-1</sup> |
| Insulin(Bovine, Recombinant)     | 5.00 × 10 <sup>1</sup>  | Thiamine HCl                    | 4.00                    |
| Transferrin(Human)               | 1.00 × 10 <sup>2</sup>  | Retinol, all trans(Vitamin A)   | 1.00 × 10 <sup>-1</sup> |
| Recombinant BSA Fraction V       | 1.00 × 10 <sup>3</sup>  | Vitamin B12                     | 1.40                    |
| Catalase                         | 2.50                    | Putrescine·2HCl                 | 3.22 × 10 <sup>1</sup>  |
| Glutathione(Reduced)             | 1.50                    | L-Carnitine HCl                 | 2.00                    |
| Superoxide dismutase             | 2.50                    | Ethanolamine HCl                | 1.00                    |
| T-3/Albumin Complex              | 2.00 × 10 <sup>-3</sup> | Lipoic acid                     | 4.70 × 10 <sup>-2</sup> |
| Corticosterone                   | 2.00 × 10 <sup>-2</sup> | Phenol red                      | 1.50 × 10 <sup>1</sup>  |
| Progesterone                     | 1.26 × 10 <sup>-2</sup> | CHIR99021                       | 1.40                    |
| basic FGF                        | 5.00 × 10 <sup>-3</sup> | 2-mercaptoethanol               | 8.17                    |
| Leukemia inhibitory factor       | 1.00 × 10 <sup>-2</sup> |                                 |                         |
| LiCl                             | 2.12 × 10 <sup>2</sup>  |                                 |                         |
| CaCl <sub>2</sub> (anhyd.)       | 2.00 × 10 <sup>2</sup>  |                                 |                         |

Table 4. Cont.

| Substance  | mg/L                    | Substance | mg/L |
|--|-------------------------|-----------|------|
| Fe(NO <sub>3</sub> ) <sub>3</sub> ·9H <sub>2</sub> O | 1.00 × 10 <sup>-1</sup> |           |      |
| KCl  | 4.00 × 10 <sup>2</sup>  |           |      |
| MgSO <sub>4</sub>                                    | 9.77 × 10 <sup>1</sup>  |           |      |
| NaCl   | 6.40 × 10 <sup>3</sup>  |           |      |
| NaHCO <sub>3</sub>                                   | 3.70 × 10 <sup>3</sup>  |           |      |
| NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O   | 1.25 × 10 <sup>2</sup>  |           |      |
| Sodium selenite                                      | 1.86 × 10 <sup>-2</sup> |           |      |

Table 5. Information on the primers.

| Name                | Sequence (5' to 3')       |
|---------------------|---------------------------|
| <i>Gapdh</i> -F     | AACTTTGGCATTGTGGAAGGGCTCA |
| <i>Gapdh</i> -R     | TTGGCAGCACCAGTGGATGCAGGGA |
| <i>Nanog</i> -F     | CTCAAGTCCTGAGGCTGACA      |
| <i>Nanog</i> -R     | TGAAACCTGTCCTTGAGTGC      |
| <i>Endo-Oct4</i> -F | TAGGTGAGCCGTCTTCCAC       |
| <i>Endo-Oct4</i> -R | GCTTAGCCAGTTCGAGGAT       |
| <i>Dppa3</i> -F     | TGTGGAGAACAAGAGTGA        |
| <i>Dppa3</i> -R     | CTCAATCCGAACAAGTCTT       |

### 2.5. Cell Lines

Table 6 provides all the cell lines used in Section 3.

Table 6. Detailed information on the cell lines.

| Name                           | Source   |
|--------------------------------|--|
| Platinum-E (Plat-E)            | A gift from The Fourth Military Medical University   |
| OG2 Mouse Embryonic Fibroblast | E13.5 mouse embryos from crossing male Oct4-GFP transgenic mice (CBA/CaJ XC57BL/6J) to 129Sv/Jae female mice |

## 3. Procedure

### 3.1. Viral Production through Calcium Phosphate Transfection

1. Plate  $7.5 \times 10^6$  or  $4 \times 10^6$  Plat-E cells to 100 mm or 60 mm cell culture dishes, respectively, depending on experimental design. The cells were cultured in fibroblast medium.
2. Observe the cells one day before performing calcium phosphate transfection and make sure the cells do not overgrow. The optimal confluence would be 70~80%.
3. Refresh with 7.5 mL and 2.5 mL media for 100 mm and 60 mm cell culture dishes 2 h before transfection, respectively.
4. Mix the following reagents in 1.5 mL or 15 mL tubes to prepare the cell transfection solution.

|                   | Storage Con. | Usage Con. | 60 mm Dishes | 100 mm Dishes |
|-------------------|--------------|------------|--------------|---------------|
| DNA               | 1 µg/µL      | /          | 8 µg         | 25 µg         |
| H <sub>2</sub> O  | /            | /          | 429.5 µL     | 1068.75 µL    |
| CaCl <sub>2</sub> | 2 M          | 125 mM     | 62.5 µL      | 156.25 µL     |
| 2× HBS            | 2×           | 1×         | 500 µL       | 1250 µL       |
| Total             | /            | /          | 1000 µL      | 2500 µL       |

Note: Add water first, then add DNA and mix thoroughly. Add 2 M CaCl<sub>2</sub> and pipette 15 times. Finally, add 2× HBS and shake vigorously for 15 times to mix well (if the HBS is added multiple times, add it fast to avoid pH changes for the optimal transfection efficiency). Allow the mixed reagents to rest for 5 min at room temperature.

5. Uniformly add the mixed reagents to the cells and move back and forth and then side-to-side gently 1–3 times to ensure equal distribution. The calcium phosphate

precipitation appears as black fine particles and can be observed throughout the dishes under a microscope. Place the cells back in a 37 °C incubator.

6. Replace the transfection medium with 10 mL or 3.5 mL fresh fibroblast medium for a 100 mm or 60 mm dish, respectively, 12 h after transfection.
7. Collect the supernatants that contain the viruses for the first transduction 48 h after transfection, and refresh with 10 mL or 3.5 mL fibroblast medium for a 100 mm or 60 mm dish, respectively.
8. Collect the supernatants with viruses 72 h after transfection for the second transduction.
9. Filter the virus-containing media with 0.45 µm filter and use the viruses freshly.

### 3.2. Thawing, Culturing, and Proliferating OG2-MEFs

1. Before thawing OG2-MEFs, coat the dish with 0.1% gelatin and incubate in a 37 °C incubator for at least 30 min. Note: OG2-MEFs should be tested for mycoplasma contamination as this will substantially impact the efficiency of iPSC generation.
2. Remove the vials from the liquid nitrogen using the appropriate safety equipment. Note: When handling frozen vials, make sure to wear appropriate personal protective equipment including cryo-gloves and eye protection as vials that are stored in liquid nitrogen may explode when warmed.
3. Immerse the vial in a 37 °C water bath without submerging the cap and keep swirling the vial gently. Note: The bottom of the vial that contains the OG2-MEFs should be immersed in the water bath; otherwise it would lead to cell death.
4. Remove the vial from the water bath when only a small piece of ice crystal is left as it will thaw within seconds due to the remaining heat.
5. Ensure that the cap is tight and spray the vial with 75% ethanol to sterilize the surface of the vial. Air-dry the vial in the sterile biosafety cabinet to ensure the absence of residual ethanol.
6. Transfer the cells into the bottom of a sterile 15 mL tube gently using a 1-mL pipette tip and add 5 mL fresh fibroblast medium dropwise with a 10 mL pipette. Note: If the medium is not added dropwise to make the cells adapt to the osmotic pressure of the medium, cell survival may be impacted due to the rapid change of the environment.
7. Centrifuge the cells at 200× g for 5 min.
8. Aspirate the supernatant and resuspend the cells with 1 mL fresh fibroblast medium gently.
9. Remove the 0.1% gelatin and slowly add 1 mL of the cell suspension into the 60 mm dish, followed by another 2 mL fresh fibroblast medium. Generally, 1 × 10<sup>6</sup> OG2-MEFs are sufficient for three 60 mm dishes.
10. Feed 3 mL fibroblast medium to cells in one 60 mm dish every three days until ready to be passaged or harvested.

### 3.3. Passaging, Counting, and Plating OG2-MEFs

1. OG2-MEFs are split when they reach 80~90% confluence. Wash the cells with 3 mL DPBS, as the serum in the fibroblast medium contains a massive amount of trypsin inhibitors.
2. Aspirate DPBS, then add 0.5 mL pre-warmed 0.25% trypsin-EDTA, and incubate at 37 °C for 2 min. Note: The best working temperature for trypsin is 37 °C. Pre-warmed trypsin can fully disassociate OG2-MEFs in 2 min. Long and short incubation will lead to poor cell vitality and residual cells, respectively.
3. When OG2-MEFs are well detached as single cells, add an equal volume of fibroblast medium to terminate the trypsin reaction.
4. Transfer the cell suspension to a sterile 15 mL tube. Take 10 µL of the cell suspension for cell counting.
5. Centrifuge the cells at 200× g for 5 min at room temperature. Meanwhile, count the cells using a hemocytometer or an automated cell counter. Note: Cell concentrations between 4–6 × 10<sup>5</sup> would give a more accurate result when using a hemocytometer.

6. Aspirate the supernatants and resuspend the cells first with 1 mL medium. Pipette up and down to ensure a single-cell suspension. Then add a proper medium volume to obtain  $1.5 \times 10^4$  living cells per well in 12-well plates. Note: Gently shake the tube to ensure equal distribution of the cells and an equal number of cells in every well. Normally,  $1.5\text{--}2 \times 10^4$  cells are recommended for OKS reprogramming and  $3 \times 10^4$  cells are recommended for OK/OS/O reprogramming.
7. Gently move the plates back-and-forth to ensure uniform distribution of the cells before placing them back in the incubator. Note: Due to the edge effect of the plate, rotational movement should be avoided to ensure that cells do not aggregate in the middle of the well. Close the incubator gently to avoid disturbance.
8. OG2-MEFs will completely adhere to the well in 8 h which is the appropriate window for viral transduction, as poor adhesion of OG2-MEFs will lower the reprogramming efficiency.

#### 3.4. Generation of iPSCs

1. Aspirate the spent medium and add 0.5 mL of each virus-containing media, which were collected and filtered from Section 3.1 followed by adding 0.5 mL fibroblast medium containing 4  $\mu\text{g}/\text{mL}$  polybrene. Normally, the volume of the virus-containing media depends on the transfection efficiency in Plat-E cells during viral production in Section 3.1. It is recommended to use 0.5 mL virus-containing media if the efficiency achieves 90%, and to use 0.75 mL virus-containing media if the efficiency is 80% or lower.
2. Perform the second transduction by repeating step 1 one day after the primary transduction.
3. The virus-containing media are removed 24 h after the second transduction and 1 mL of iCD1 is added to the cells. The day on which virus-containing media are removed is denoted as day 0 post-transduction.
4. Feed the cells daily with 1 mL fresh medium per well of a 12-well plate, 2 mL at day 5 and hereafter, as the robust proliferation of cells will lead to a deficiency of nutrition during reprogramming. Note: Medium should be pre-warmed at room temperature in the dark before use, as the small molecules in the medium are photolytic. Use the medium freshly (within a week) as it contains reducing substances and many unstable growth factors.
5. Oct4-GFP positive and dome-shaped colonies are considered as reprogrammed iPSC colonies.
6. The iPSC colonies are picked at day 7 post-transduction based on Oct4-GFP expression and characteristic ESC-like morphology. The picked colonies are subsequently expanded and maintained the same way as ESCs.

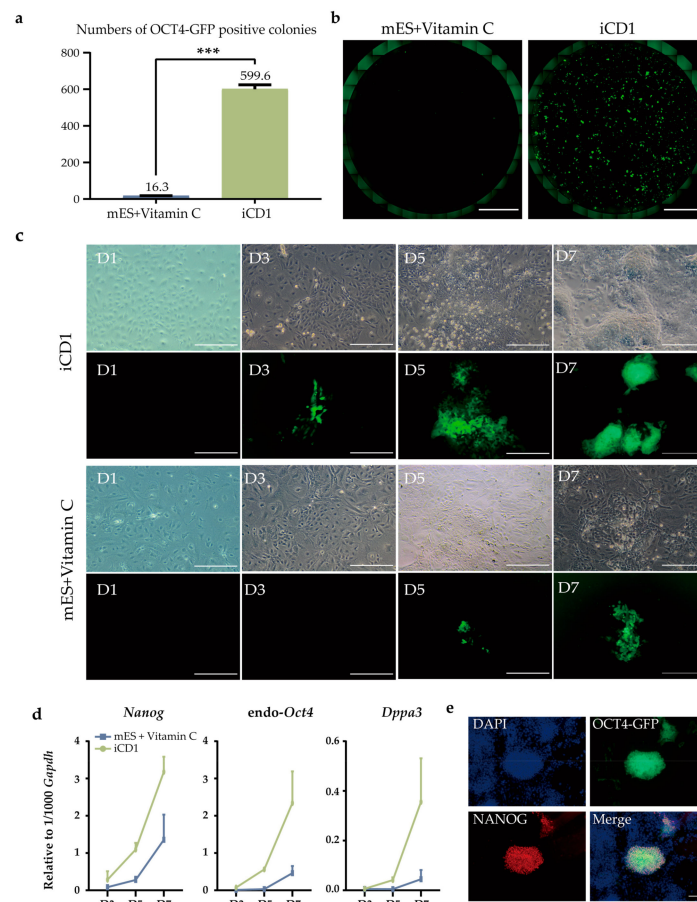
#### 3.5. Characterization of iPSCs by Immunostaining (in a Cell Culture Plate)

1. Gently remove the medium and wash the cells with 1 mL of DPBS twice.
2. Add 1 mL of 4% (wt/vol) PFA to each well of a 12-well plate and let it stand at room temperature for 30 min. Gently replace with 1 mL of DPBS and shake for 5 min at room temperature. Repeat the DPBS wash twice.
3. Add 500  $\mu\text{L}$  blocking and permeabilization solution (one volume of 3% GSA and one volume of 0.2% triton mixed together). Incubate for 40 min at 25  $^{\circ}\text{C}$ .
4. Wash the well with 1 mL of DPBS, place it on a horizontal shaker at room temperature and shake for 5 min. Repeat the DPBS wash twice.
5. Incubate with the Anti-Nanog antibody (1:1000) at 4  $^{\circ}\text{C}$  overnight.
6. Wash the cells with DPBS thrice and incubate for 10 min each time at room temperature on a horizontal shaker.
7. Incubate with the secondary antibody (1:2000) for 1 h at room temperature and avoid light.
8. Wash the cells with DPBS thrice and incubate for 10 min each time at room temperature on a horizontal shaker (avoid light).

9. Add 1 mL DPBS into each well and use a fluorescence microscope to capture images.

#### 4. Expected Results

We treated OG2-MEFs with the modified medium iCD1 and traditional induction medium mES + Vitamin C and found that the number of Oct4-GFP-positive colonies was substantially increased in iCD1. The number of Oct4-GFP-positive colonies at day seven post-treatment was almost 40-fold higher than that in mES + Vitamin C medium (Figure 2a,b). We observed that mesenchymal-epithelial transition (MET) occurred the day after the second transduction (D1). The iCD1-treated cells activated Oct4 sooner than the mES + Vitamin C-treated ones and as reprogramming progressed. The iCD1-treated cells exhibited round shape, large nucleoli, and sparse cytoplasm similar to mouse ESCs (Figure 2c). We collected RNA samples of the cells that were treated by both conditions at day three, five, and seven, followed by quantitative real-time PCR to investigate the reprogramming kinetics using primers as stated in Table 4. We found that the iCD1-treated cells exhibited earlier activation of pluripotent markers, *Nanog*, endogenous *Oct4*, and *Dppa3*, than those by mES + Vitamin C, indicating the advantage of iCD1 in reprogramming kinetics (Figure 2d). Next, we examined the NANOG expression of iCD1-treated cells by immunostaining using NANOG antibody. The Oct4-GFP-positive cells were also found to be NANOG-positive, further confirming the pluripotency establishment (Figure 2e). Additional characterization of iCD1-induced iPSC can be found in our previous works [14]. Altogether, the upregulation of pluripotent markers indicates a successful cell fate transition and well-established pluripotency of the reprogrammed cells.



**Figure 2.** Characterization of generated iPSCs using the iCD1 medium. (a) Oct4-GFP-positive colonies were scored at day 7 post-treatment in indicated medium. \*\*\*  $p < 0.001$ , compared to the result of iCD1 ( $n = 2$ ); (b) Images of Oct4-GFP positive colonies generated by indicated medium at day 7. Scale



bar: 5mm; (c) The induction of pluripotency from MEFs by *Oct4* (O), *Klf4* (K), *Sox2* (S) in the indicated medium during reprogramming. The bright field and GFP images were shown on the top and bottom, respectively. Green colonies indicate successful reprogramming. Scale bar: 250  $\mu$ m; (d) Expression levels of *Nanog*, endogenous *Oct4* and *Dppa3* were analyzed by quantitative real-time PCR ( $n = 2$ ). Expression levels of pluripotent markers were relative to *Gapdh*; (e) Immunofluorescent staining of NANOG in Oct4-GFP positive iPSCs, showing a co-expression of OCT4 and NANOG proteins in GFP positive cells. Scale bar: 250  $\mu$ m.

## 5. Discussion

The technical improvement of iPSC reprogramming has mainly focused on reducing or replacing transcription factors (e.g., subtracting the oncogene c-Myc) [15,16]. Yet this strategy suffers from low efficiency and lengthy process compared to the iCD1 system that is described here. Alternatively, non-integrated delivery methods of reprogramming factors such as mRNA delivery and transient transfection make iPSCs more clinically applicable [17,18]. Subsequent studies have reported uses of many mouse cell types for reprogramming such as neural progenitor cells, pancreatic  $\beta$  cells, and stomach cells, thereby expanding the choices of the starting cell types [19–22]. Presently, more efforts are focused on adding small molecular compounds to replace transcription factors. For instance, Huangfu et al. reported that histone deacetylase (HDAC) inhibitors, particularly valproic acid (VPA), markedly improve the reprogramming efficiency [23]. Vitamin C is a dioxygenase agonist, and its remarkable role in promoting reprogramming efficiency is well documented [24]. It is noted that protocols using Knockout serum replacement (KSR)-based medium should be considered as Vitamin C-supplemented protocols, as KSR enriches Vitamin C. Hou et al. reported a successful induction of iPSC by replacing all the transcription factors with compound combinations [25]. As chemical-induced reprogramming is inefficient and requires a tedious process, optimized protocols have been developed for high efficiency and reproducibility [26,27].

However, these methods have their shortcomings. Transient transfection has delayed kinetics of reprogramming and short expression window of factors compared to the integrated expression [18]. Many reported chemicals such as 5'-azaC and BIX01294 are cytotoxic and not conducive to application [28,29]. Moreover, small molecules that replace transcription factors may have very low efficiency, a long induction time, and undefined side effects or toxicity on cells [23].

We have developed a user-friendly protocol allowing for fast and efficient reprogramming of MEFs into iPSCs in iCD1, an original reprogramming medium. Our lab has previously developed a serum-free medium containing multiple vitamins and engineered factors that improve reprogramming efficiency after testing candidate factors systematically [29,30]. The previously reported complex formulation can be substituted by iCD1, containing high glucose DMEM-supplemented N2, B27, and vitamin C. Moreover, the addition of small molecules GSK-3 $\beta$  inhibitor CHIR99021 and LiCl into our iCD1 medium maintains the undifferentiated state of already reprogrammed iPSCs while accelerating somatic cell reprogramming [31]. The growth factor, basic fibroblast growth factor (bFGF), is crucial to support OKS-transduced MEFs proliferation and later-stage reprogramming. Further, we used MEFs that were derived from E13.5 embryos carrying the Oct4-GFP transgenic allele [32], easy for tracing the kinetics of the reprogramming. We applied a retroviral delivery system as it is susceptible to transgene-silencing at the fully reprogrammed iPSC stage. We found that iCD1 supplemented with BMP factors can support Oct4-mediated or Oct4-Sox2-mediated reprogramming by promoting MET process, the lack of which is a major hindrance in reprogramming without Klf4 and c-Myc [33].

In general, iCD1 has extensive application in somatic reprogramming and has already been used and modified in many studies [34–36] to further decipher cell fate decision.

## 6. Conclusions

Here, we provide a detailed procedure of efficient and fast mouse iPSC generation using a chemically-defined medium that was developed in our lab previously. The resulting

iPSC colonies have morphological and molecular signatures as ES cells. Our protocol has provided a reliable and efficient way to comprehensively study molecular mechanisms of cell fate transition and facilitate the application of reprogramming technologies.

**Author Contributions:** J.C. and D.P. conceived the study. J.H. and X.Y. performed the experiments, collected the data, and did the analyses. J.H., H.W., J.W. and J.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by National Basic Research Program of China [2017YFA0105201]; Frontier Science Research Program of the CAS (ZDBS-LY-SM007); Science and Technology Planning Project of Guangdong Province, China (2020B1212060052); Science and Technology Projects in Guangzhou, China (202102021039); The National Natural Science Foundation of China (32000414, 32000501); GuangDong Basic and Applied Basic Research Foundation (2020A1515110096); and The Science and Technology Program of Guangzhou (202102020174).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data that are presented in this study are available within the figures.

**Acknowledgments:** We thank Ruhai Chen, Yuting Liu, Sa Li, Shaofei Cheng, Yujian Liu, Kaixin Wu, Jin Ming, and Yi Huang for technical support.

**Conflicts of Interest:** The authors declare no competing interest in this work.

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