Video Article

An Alternative Culture Method to Maintain Genomic Hypomethylation of Mouse Embryonic Stem Cells Using MEK Inhibitor PD0325901 and Vitamin C

Cuiping Li[1], Weiyi Lai[1], Hailin Wang[1]

[1]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences

Correspondence to: Hailin Wang at hlwang@rcees.ac.cn

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Abstract

Embryonic stem (ES) cells have the potential to differentiate into any of the three germ layers (endoderm, mesoderm, or ectoderm), and can generate many lineages for regenerative medicine. ES cell culture *in vitro* has long been the subject of widespread concerns. Classically, mouse ES cells are maintained in serum and leukemia inhibitory factor (LIF)-containing medium. However, under serum/LIF conditions, cells show heterogeneity in morphology and the expression profile of pluripotency-related genes, and are mostly in a metastable state. Moreover, cultured ES cells exhibit global hypermethylation, but naïve ES cells of the inner cell mass (ICM) and primordial germ cells (PGCs) are in a state of global hypomethylation. The hypomethylated state of ICM and PGCs is closely associated with their pluripotency. To improve mouse ES cell culture methods, we have recently developed a new method based on the selectively combined utilization of two small-molecule compounds to maintain the DNA hypomethylated and pluripotent state. Here, we present that the co-treatment of vitamin C (Vc) and PD0325901 can erase about 90% of 5-methylcytosine (5mC) at 5 days in mouse ES cells. The generated 5mC content is comparable to that in PGCs. The mechanistic investigation shows that PD0325901 up-regulates Prdm14 expression to suppress Dnmt3b (*de novo* DNA methyltransferase) and Dnmt3l (the cofactor of Dnmt3b), by reducing *de novo* 5mC synthesis. Vc facilitates the conversion of 5mC to 5-hydroxymethylcytosine (5hmC) catalyzed mainly by Tet1 and Tet2, indicating the involvement of both passive and active DNA demethylations. Moreover, under Vc/PD0325901 conditions, mouse ES cells show homogeneous morphology and pluripotent state. Collectively, we propose a novel and chemical-synergy culture method for achieving DNA hypomethylation and maintenance of pluripotency in mouse ES cells. The small-molecule chemical-dependent method overcomes the major shortcomings of serum culture, and holds promise to generate homogeneous ES cells for further clinical applications and researches.

Video Link

The video component of this article can be found at https://www.jove.com/video/56391/

Introduction

ES cells are originated from the ICM of a blastocyst[1]. The cells are in a pluripotency state and can form all somatic lineages and germline cells[2]. Establishment of ES cells provides the opportunity to investigate the development processes *in vitro* and can generate cells of medical relevance for regenerative medicine based on their pluripotency[3].

Two groups seminally established the mouse ES cell lines in 1981 and when cells derived from the early mouse embryo were cultured in fetal bovine serum (FBS)-containing medium with mouse embryonic fibroblasts (MEFs) as the feeder layer[1,4]. MEFs were inactivated mitotically and were pre-grown in dishes prior to culturing ES cells. MEFs provide support for mouse ES cell attachment and produce growth factors to promote propagation and repress the differentiation[5], while FBS offers essential trophic factors and hormones for cell proliferation. Subsequent studies indicated that LIF produced by feeder cells was the key cytokine for self-renewal and maintenance of pluripotency in mouse ES cells, and the addition of LIF into the medium could substitute for feeder cells[6]. Currently, the sustainment of mouse ES cells in FBS/LIF medium on feeders is still the standard method adopted by many researchers. However, some problems arise with this classical culture approach. Firstly, feeder cells secrete excess and uncontrolled factors and may cause pathogenic contamination[7]. To avoid this interference, coating the surface of dishes with gelatin and the addition of LIF in serum-containing medium are alternative methods for maintaining mouse ES cells without feeder-layer cells. Additionally, mouse ES cells grown under serum/LIF conditions exhibit morphological heterogeneity in cell populations and even in the expression level of pluripotency-related factors[8]. Recent studies suggest that under serum/LIF conditions, the pluripotency-related core transcription factors (including SOX2, Nanog, and OCT4) can sustain the pluripotency through LIF and WNT signaling; however, notably, they also activate a fibroblast growth factor (FGF) signal to trigger differentiation[8]. Due to the ambivalent dual action of the core transcription factors, mouse ES cells cultured in serum present heterogeneity, consisting of two interchangeable populations, one similar to ICM and another resembling the primed epiblast state[8]. Moreover, mouse ES cells in serum often exhibit global hypermethylation[9], whereas ICM and PGCs are in a global hypomethylated state, which is closely linked with their pluripotency[9,10].

There is a considerable demand to develop new methods for culturing mouse ES cells. Several improved protocols have been established since 2003[11] but there continues to be some limitations and shortcomings[7]. Since 2008, the combined utilization of two small-molecule kinase

inhibitors, PD0325901 (the inhibitor of the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) (MEK)) and CHIR99021 (the inhibitor of glycogen synthase kinase 3 (GSK3)), in N_2B_{27} medium with LIF and without serum has opened new perspectives for culturing mouse ES cells[12]. This new defined medium is characterized by the use of two inhibitors (2i). Mouse ES cells cultured in 2i/LIF medium are more homogeneous in cell populations and the expression of pluripotency factors. In addition, 2i/LIF-cultured mouse ES cells exhibit DNA hypomethylation globally, which is closer to ICM-like cells[9,13]. Even so, 2i culture has its disadvantages. PD0325901 and CHIR99021 are insoluble in water and generally are dissolved in dimethyl sulfoxide (DMSO)-based stock solution to add them in culture medium. Studies have showed that long-term and low-dose exposure of cells to DMSO can lead to cytotoxicity[14].

Here, we utilized two small-molecule compounds and developed a new culture method of mouse ES cells. The novel culture method combines Vc and MEK inhibitor PD0325901 to promote DNA hypomethylation rapidly and effectively to a comparable level of PGC, named as the Vc/PD0325901 culturing protocol. Mouse ES cells in Vc/PD0325901-added serum-containing medium exhibit homogeneity in morphology and are sustained in a ground state. Compared to 2i culture, mouse ES cells cultured under Vc/PD0325901 conditions exhibit faster kinetics of DNA demethylation and can reach the hypomethylation level comparable to that of PGC. In addition, the use of a single inhibitor (PD0325901) decreases the input amount of DMSO into medium in comparison to that used in 2i (PD0325901/CHIR99021) and reduces the damage to cells.

Protocol

1. Preparations

1. **Prepare a solution of 1.0 mM PD0325901 (MEK inhibitor) and 3.0 mM CHIR99021 (GSK3 inhibitor).**
    1. Weigh 2 mg of PD0325901 and add 4.15 mL of DMSO in an amber glass vial.
    2. Weigh 2 mg of CHIR99021 and add 1.43 mL of DMSO in an amber glass vial.
    3. Following reconstitution, store aliquots (50 µL/tube in 200 µL PCR tubes) at -20 °C and protected from light. Remove a tube containing the frozen stock from a freezer and thaw at room temperature before use.

2. Weigh 0.0176 g of Vc into 1 mL of a centrifuge tube and reconstitute it with 1 mL of sterile water to a final concentration of 100 mM prior to use.
3. Inactivate FBS: incubate 500 mL of FBS in a water bath at 56 °C for 30 min.
4. **Prepare 600 mL of basic medium for culturing mouse ES cells.**
    1. Use a bottle of DMEM/high glucose medium (500 mL) and remove 40 mL.
    2. Supplement 460 mL of DMEM/high glucose medium with 20% inactivated FBS (120 mL), 1,000 U/mL LIF (60 µL of 10^7 U/mL stock solution), 0.1 mM non-essential amino acids (NEAA) (6 mL of 10 mM stock solution), 1 mM sodium pyruvate (6 mL of 100 mM stock solution), 2 mM L-glutamine (6 mL of 200 mM stock solution), 0.1 mM β-mercaptoethanol (600 µL of 100 mM stock solution), and 33 IU/mL penicillin and 33 µg/mL streptomycin (2 mL of penicillin-streptomycin mixed solution (10,000 IU/mL penicillin and 10,000 µg/mL streptomycin)). Define the basic medium as serum medium.
    3. Pipette 50 µL of stock solution of PD0325901 (1.0 mM) and Vc (100 mM), respectively, into 50 mL of the serum medium (final concentration: 1.0 µM PD0325901 and 100 µM Vc), and define the medium as Vc/PD0325901 medium.
    NOTE: Prepare the Vc/PD0325901 medium fresh before use due to the instability of Vc.
    4. Using a pipette, transfer 50 µL of stock solution of PD0325901 (1.0 mM) and CHIR99021 (3.0 mM), respectively, into 50 mL of the serum medium (final concentration: PD0325901 1.0 µM and CHIR99021 3.0 µM), and define the medium as 2i medium.

5. **Prepare the gelatin-coated dishes.**
    1. Prepare the 0.1% gelatin solution: dissolve 0.3 g of gelatin into 300 mL of ultrapure water in a glass reagent bottle with a cap and sterilize in an autoclave at 121 °C for 20 min. Store the sterile solution at room temperature.
    2. Incubate the cell culture dishes (6 cm diameter) with 2 mL of 0.1% gelatin solution for 15 min at room temperature and then aspirate the gelatin. Dry the dishes in air and use them within 12 h.

6. Prepare 10 mL of mouse ES cell freezing medium in a 15 mL centrifuge tube: 90% FBS (9 mL) and 10% DMSO (1 mL). Use the medium within 1 day.
7. Prepare a freezing container: add 100% isopropyl alcohol to the fill line of the freezing container, and discard the old isopropyl alcohol. Add new isopropyl alcohol directly after every fifth use.
8. Make the enzymatic digestion buffer: weigh 1.2114 g of Tris powder into 100 mL of ultrapure water to prepare 100 mM Tris solution and add concentrated HCl solution (about 12 M) into the Tris solution to adjust the pH to 7.6 (about 0.6 mL of concentrated HCl solution).
9. Prepare 50 mL of radio immunoprecipitation assay buffer (RIPA buffer): 20 mM Tris, pH 7.4, 1 mM $MgCl_2$, 150 mM NaCl, 20% glycerol, 0.5% NP40, 1 mM EDTA, 1 mM EGTA. Supplement with 1 mM DTT, 1X protease inhibitor cocktail, and 1 mM PMSF prior to use. Once the PMSF is added, use the buffer within 30 min.

2. Grow Mouse ES Cells on Gelatin-coated Dishes

1. Pre-warm mouse ES cells (wild type, 129 SvEv) culture medium, trypsin, and phosphate buffered solution (PBS) in a water bath at 37 °C.
2. **Passage the cells. Aspirate the medium completely from the dish and pipette 2 mL of PBS to the dishes to rinse the cells.**
    1. Remove the PBS with a pipette and wash the cells again with 2 mL of PBS.
    2. Remove the PBS and add 0.3 mL of trypsin-EDTA (0.25%) to each dish.
    3. Cover the whole dish with the trypsin quickly and then remove it immediately.
    4. Put the dish in an incubator at 37 °C for 1 min to detach the cells from the dish. Take out the dishes from the incubator and add 2 mL of pre-warmed serum medium to terminate the action of trypsin.
    5. Dissociate the cell colonies into a single cell suspension by resuspending with a pipette (5 mL pipette tip) 10 times.

3. Transfer 150 µL of 2 mL of the cell solution (about 190,000 cells by counting with a hemocytometer) into a new gelatin-coated dish, and supplement with 3 mL of freshly made Vc/PD0325901 medium per 6 cm culture dish. Culture the cells in a 37 °C incubator with 5% CO_2.
4. Every 24 h while incubating, remove the old culture medium and add 3 mL of fresh Vc/PD0325901-medium into the culture dish due to the instability of Vc. Expect 70–80% confluency after 2–3 days.
5. After reaching 70-80% confluency, passage the cells and continue to culture them as described in steps 2.2–2.4.

3. Freeze and Thaw Mouse ES Cells

1. **Freeze mouse ES cells.**
    1. Detach the cells from the dishes with trypsin by following step 2.2.
    2. Transfer the cells into a 15 mL plastic tube and centrifuge at 800 x g and room temperature for 3 min.
    3. Dump the supernatant gently into a beaker and resuspend the cell pellet with 1 mL of freshly made freezing medium. Prepare the freezing medium 30 min prior to this step to ensure that it is room temperature before use.
    4. Transfer the cells in freezing medium to a 1.8 mL microcentrifuge tube with a 1 mL pipette and place the microcentrifuge tube into a freezing container. Add isopropyl alcohol to the fill line of the freezing container and keep it at room temperature before using.
    5. Leave the freezing containers overnight in a -80 °C freezer.
    6. Transfer the microcentrifuge tubes to a liquid nitrogen container for up to 2–3 years.
    NOTE: Do not keep cells in the freezing medium for a long time and quickly transfer the cells to a -80 °C freezer due to the toxicity of DMSO.

2. **Thaw mouse ES cells.**
    1. Pre-warm 50 mL of serum medium in a 37 °C water bath.
    2. Take out a cell-containing vial from the liquid nitrogen container and immerse the capped vial in a 37 °C water bath. Shake it quickly in the water bath to thaw. Transfer the cells into a 15 mL tube with a 1 mL pipette. Add 2 mL of pre-warmed serum culture medium into the tube to dilute the freezing medium and then centrifuge at 800 x g and room temperature for 3 min.
    3. Remove the supernatant and gently resuspend the cell pellets with 3 mL of fresh Vc/PD0325901 medium using a 5 mL pipette.
    4. Transfer the cells from the 15 mL tube to a gelatin-coated dish and culture the cells for 24 h. Culture the cells again as described in Step 2.

4. Extract Total Protein from Cells

1. Rinse the collected cells with 1 mL of PBS and then centrifuge at 800 x g and room temperature for 3 min.
2. Aspirate the supernatant and resuspend the cell pellet thoroughly with RIPA buffer supplemented with DTT, protease inhibitor cocktail, and PMSF by pipetting gently. The volume of RIPA buffer is about 5x that of the cell pellet.
NOTE: Carry out the cell resuspension on ice.
3. Keep the cells in RIPA buffer for 30 min on ice and centrifuge at 13,000 x g and 4 °C for 5 min.
4. Transfer the supernatant to a new tube and store aliquots at -80 °C.
5. Determine the concentration of the protein with a Bradford protein assay kit.

5. Extract DNA from Cells and Digest DNA into a Single Nucleoside Using Enzymes

1. Collect the cells with trypsin as described in steps 3.1.1 and 3.1.2.
2. Perform the DNA extraction with a genomic DNA purification kit following the manufacturer's instructions.
3. Store the extracted DNA in 1-mL centrifuge tubes. Add 100 µL of ultrapure water into the centrifuge tube, and dissolve the DNA by pipetting approximately 15 times. Determine the concentration and evaluate the quality of DNA by the measurement of the absorbance at 260 nm and 280 nm[15]. The DNA concentration is about 500 ng/µL.
4. Digest 5 µg of DNA in a 50 µL solution system: add 5 µL of 100 mM Tris-HCl solution, pH 7.6 (final concentration: 10 mM), 2 U calf intestinal phosphatase, 1 U DNase I, 0.005 U snake venom phosphodiesterase I, and 5 µg of DNA (calculate the added volume according to the measured DNA concentration, ~10 µL) into a centrifuge tube and increase the volume of solution to 50 µL with ultrapure water. Incubate the mixture at 37 °C overnight. Centrifuge the digested DNA solution at 1,000 g and room temperature for 1 min to collect the solution to the bottom of tubes.
5. Transfer the digested DNA solution from the centrifuge tubes into ultra-filtration tubes (a molecule weight cutoff: 3 kDa) and centrifuge at 13,000 g and 4 °C for 30 min to remove the digestion enzymes.
6. **Prepare the samples for 5mC and 5hmC analysis.**
    1. For 5hmC analysis: pipet 36 µL of filter solution to a new centrifuge tube (1 mL) and add 4 µL of 5'-(hydroxymethyl-d_3)-2'-deoxycytidine ([D_3]-5hmC) with the final concentration of 3 nM.
    2. For 5mC analysis: add 196 µL of ultrapure water into a new centrifuge tube and pipet 4 µL of filter solution to the tube (50-fold dilution), due to the high density of 5mC in genomic DNA. Transfer 36 µL of the diluted solution to a new centrifuge tube and add 4 µL of (5'-(methyl-d_3)-2'-deoxycytidine ([D_3]-5mC) with the final concentration of 50 nM. Use [D_3]-5hmC and [D_3]-5mC as the internal standard to calibrate 5hmC and 5mC, respectively.

7. Transfer the sample solution to measuring tubes and store at 4 °C. Analyze 5mC and 5hmC with ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry with multiple-reaction monitoring (UHPLC-MS/MS (MRM)) as described in a previous report[15].

6. Analyze 5mC and 5hmC Using UHPLC-MS/MS[15]

1. **Set up various parameters for analyzing 5mC and 5hmC using the UHPLC-MS software.**
    1. Establish a new method. Open the software and click "File | New | Method". Exhibit the message box of "Method Editor" with the content "Do you want to save the changes for the current method" and click "No".
    2. Build the parameters of the sampler. Click "Sampler | Setup | Injection". Select "Standard injection" and input "5 µL at Injection volume".
    3. Build the parameters of the pump in UHPLC.
        1. Click "BinPump2 | Setup" to build the parameters of the pump. Set up "Flow" at "0.25 mL/min" and "Stop time" at "15 min".
        2. Set up "Solvent B at 5%" and "Pressure limits" at "Max 600 bar".
        3. Click "BinPump2 | Timetable" to build the isocratic elution parameters for 5mC analysis. Click "Append" to add a row. Input "0.00" at "Time" and "5.0" at "B%". Click "Append" once more to add a new row. Input "15.00" at "Time" and "5.0" at "B%".
        4. Click "BinPump2 | Timetable" to build the gradient elution parameters for 5hmC analysis. Click "Append to add a row. Input 0.00 at Time and 5.0 at "B%". Click "Append" once more to add a new row. Input "3.00" at "Time" and "5.0" at "B%". Click "Append" for another 6 times to add 6 rows. Input "3.01" at "Time" and "15.0" at "B%", "6.00" at "Time" and "15.0" at "B%", "6.01" at "Time" and "100" at "B%", "10.00" at "Time" and "100.0" at "B%", "10.01" at "Time" and "5.0" at "B%", "15.00" at "Time" and "5.0" at "B %" in sequence.
        NOTE: Perform the analysis of 5mC and 5hmC individually due to the different separation conditions of UHPLC.
    4. Build the parameters of the temperature of the column compartment (TCC) in UHPLC.
    Click "TCC | Setup | temperature (Left)" and input "30 °C".
    5. Establish the parameters of the QQQ-MS/MS.
        1. Click "MS QQQ | Stop time | No limit/As pump", "Ion Source | ESI", "Time filtering | Peak width: 0.07 min". Set up "Time segments: Start time: 0"; "Scan Type: MRM"; "Div Value: To MS"; "Delta EMV (+): 200" and "Delta EMV (-): 0".
        2. Build the parameters of monitoring the transitions of 5mC, D_3-5mC, dC, 5hmC, and D_3-5hmC
            1. Click "MS QQQ | Acquisition | Scan segments: Dwell: 90"; "Fragmentor: 90"; "Collision Energy: 5"; "Cell Accelerator Voltage: 4" and "Polarity: Positive".
            2. Input "5mC" at "Compound Name", "242" at "Precursor Ion", "126" at "Product Ion" for 5mC analysis. Click the right button and select "Add row" to add a new row. Input "D_3-5mC" at "Compound Name", "245" at "Precursor Ion", "129" at "Product Ion" for D_3-5mC analysis.
            3. Supplement another three rows using the same mode. Input "dC" at "Compound Name", "228" at "Precursor Ion", "112" at "Product Ion" for dC analysis. Input "5hmC" at "Compound Name", "258" at "Precursor Ion", "142" at "Product Ion" for 5hmC analysis. Input D_3-5hmC at Compound Name, "261" at "Precursor Ion", "145" at "Product Ion" for D_3-5hmC analysis.
        3. Build the parameters of the gas source. Click "MS QQQ | Source | Source parameters: Gas Temp: 300 °C"; "Gas Flow: 11 L/min"; "Nebulizer: 15 psi"; "Positive-Capillary: 3500 V".
        4. Save the proposed method. Click "MS QQQ | Instrument | Save method As". Select a pathway for the proposed method by "Directory" and give a name for the method by inputting the content in "Method", for example: 5mC and 5hmC analysis.

2. **UHPLC separation and MS/MS analysis of mononucleosides**
    1. Prepare the mobile phase for 5mC and cytosine (C) analysis: form the mobile phase of solution A and B. Solution A: 500 mL of ultrapure water with 500 µL of formic acid (final concentration: 0.1%), and solution B: 500 mL of 100% methanol. Mix solution A and B at 95:5 (v:v) as the mobile phase to elute 5mC and C (set in step 6.1.3.3). Set the flow rate at 0.25 mL/min (set in step 6.1.3.1).
    2. Prepare the mobile phase by solvent A and B for 5hmC analysis. Solvent A is 2.0 mM NH_4HCO_3 aqueous solution (pH 9.0) (500 mL), and solvent B is 100% methanol (500 mL). Separate 5hmC by an optimized gradient elution: 0-3 min, 5.0% B and 95% A (v:v); 3-6 min, 15.0% B and 85% A; 6-10 min, 100% B; 10-15 min, 5.0% B and 95% A (set in step 6.1.3.4). Set the flow rate at 0.25 mL/min (set in step 6.1.3.1).

3. **Utilize electrospray MS/MS with MRM mode (set in step 6.1.5.1) to analyze the elution from the column and adopt the positive ion mode (set in step 6.1.5.2.1).**
    1. Monitor the transitions: 5mC, m/z 242→126 (collision energy, 5 eV); [D_3]-5mC, m/z 245→129 (5 eV); dC, m/z 228→112 (5 eV); 5hmC, m/z 258→142 (5 eV); [D_3]-5hmC, m/z 261→145 (5 eV) (set in step 6.1.5.2.2).
    2. Set the capillary and fragment voltages at +3,500 V (set in step 6.1.5.3) and 90 V (set in step 6.1.5.2.1), respectively.
    3. Set the injection volume at 5 µL and analyze each sample 3 times (set in step 6.1.2). Employ the corresponding standard curves to evaluate the amount of 5mC and 5hmC.
        1. Establish the standard curve of 5mC: transfer 1–50 nM (1, 5, 10, 20, 50 nM, final concentration) standard solution of 5mC into centrifuge tubes and add 50 nM [D_3]-5mC to tubes. Supplement the total volume to 50 µL. Perform the analysis of standard 5mC following the instructions for the 5mC sample (step 6).
        2. Build the standard curve of 5hmC: transfer 1–20 nM (1, 2, 5, 10, 20 nM, final concentration) standard solution of 5hmC to centrifuge tubes and add 3 nM [D_3]-5hmC to the tubes. Supplement the total volume to 50 µL. Perform the analysis of standard 5hmC following the instructions for the 5hmC sample (step 6).

7. Analyze the Statistical Significance

1. **Perform the statistical analysis using software.**
    1. Open the software and select "Column" in "New table & graph". Set the parameters as follows: "Sample data | Start with an empty data table"; "Choose a graph-the first mode"; "Graphing replicates or error bars | Plot | Mean with SEM".

2. Click "Create" to input the three replicates of Control and Vc/PD0325901-treated group in the "A" and "B" line, respectively. Select the six data and click "Analyze" in the "Analysis".
3. Select "*t* tests (and nonparametric tests)" in "Column Analyses" and click "OK" to enter the next interface.
4. Set the parameters as follows: "Choose test | Test name | Unpaired *t* test; Options | Two-tailed"; "Confidence Intervals: 95%"; "Significant digits | Show 4 significant digits". Click "OK" to acquire the *p* value between the Control and Vc/PD0325901 treatment.

2. Evaluate the statistical significance between the control and the experimental groups employing the two-tailed and unpaired Student's *t*-test[16]. NOTE: p <0.05 denotes the difference possessing statistical significance. *p <0.05, **p <0.01, ***p <0.001.

Representative Results

Vc/PD0325901 synergistically induced global erasure of mouse ES cells. Mouse ES cells in serum exhibit DNA hypermethylation, while pluripotent ICM cells and PGCs show global erasure of DNA methylation and the hypomethylated state is closely associated with their pluripotency[9,10].

Previously, we and others found that Vc may enhance Tet-mediated 5mC demethylation[15,17]. Meanwhile, 2i was also found to inhibit *de novo* synthesis of 5mC. In the context of these findings, we further proposed the synergistic manipulation of mouse ES cells using Vc and 2i together. After the analysis of UHPLC-MS/MS, we found that Vc and 2i supplemented in the serum-containing medium can dramatically reduce the 5mC content from 3.2 to 0.33 per 100 C (~90% 5mC loss) by day 11 in mouse ES cells (**Figure 1A**). In contrast, Vc or 2i-treatment alone only caused 58% erasure with the steady level at 1.4% 5mC or 61% reduction at the level of 1.3% 5mC.

The 2i medium is composed of MEK inhibitor PD0325901 and GSK3β inhibitor CHIR99021. Next, we investigate which component induces the global loss of 5mC during the co-treatment of Vc and 2i. Interesting, Vc/PD0325901 caused a faster decline in levels of DNA methylation than Vc/2i. Vc/PD0325901 achieved steady 5mC levels (~0.33% 5mC) after 5 days, while Vc/2i reached a comparable level at day 11. In contrast, the supplement of CHIR99021 partly suppressed Vc-triggered DNA demethylation (**Figure 1A**). These data clearly suggest that PD0325901 in 2i contributes to the global hypomethylation of genomic DNA in mouse ES cells, while CHIR99021 of 2i partially inhibits the erasure of 5mC. Therefore, we speculate that the faster DNA demethylation induced by Vc/PD0325901 relative to Vc/2i may be attributed to the partial inhibition of CHIR99021 on demethylation.

We hypothesized that the global erasure of 5mC in mouse ES cells induced by Vc/PD0325901 may be partially pertinent to the enhancement of DNA demethylation. Hence, we examined genomic oxidation products of 5mC. It has been reported that 5mC can be converted to 5hmC by the catalytic oxidation of Tet family dioxygenases, which are dependent on Fe (II) and 2-oxoglutarate[18,19]. Genomic 5hmC may be diluted by DNA replication[20]. Furthermore, 5hmC can be further oxidized by Tet dioxygenases to produce 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be efficiently excised by thymine DNA glycosylase (TDG) to recover unmethylated cytosine, indicating an active DNA demethylation[21,22].

In line with previous work[15], Vc-containing treatment (Vc, Vc/2i, Vc/PD0325901, Vc/CHIR99021) dramatically increased 5hmC frequency (5hmC/10^6 C) after 1 day, and thereafter 5hmC levels declined gradually (**Figure 1B**) due to the progressively reduction in 5mC substrate. Notably, Vc/PD0325901 and Vc/2i showed faster kinetics of 5hmC loss in comparison to Vc and Vc/CHIR99021 owing to the faster reduction of 5mC. In addition, we also observed that at day 1, Vc-treatment stimulated more generation of 5hmC compared with Vc/CHIR99021, indicating that CHIR99021 partly suppressed the Vc-induced production of 5hmC and inhibited DNA demethylation. 5hmC loss in 2i treatment should be attributed to the decrease of the 5mC substrate. Taken together, these results showed that Vc-enhanced DNA demethylation activity partly contributed to the synergistically hypomethylation in co-treatment of Vc/PD0325901 and Vc/2i.

Both Habibi *et al.*[23] and von Meyenn *et al.*[24] also reported that mouse ES cells under 2i conditions showed global DNA hypomethylation and naïve pluripotency. 5mC levels showed decline gradually and reached a steady state (~1% 5mC) at 12–14 days after the reversion from serum to 2i. In the Vc/PD0325901 culture system, the co-treatment caused faster DNA demethylation and reached a stable level (0.33% 5mC) after supplementation of Vc/PD0325901 in serum medium for 5 days, due to the synergistic effect of Vc/PD0325901. The reached methylated level (0.33% 5mC) was pronouncedly lower than that under 2i conditions reported by Habibi *et al.* and von Meyenn *et al.*, while 5hmC levels were higher due to the promoted generation of Vc.

PD0325901 elevated Prdm14 expression to repress Dnmt3b and Dnmt3l, but not Dnmt3a[25]. To investigate the action of PD0325901 in inducing 5mC loss, a series of 5mC-related protein expression was examined, including the maintenance (Dnmt1) and *de novo* (Dnmt3a, and Dnmt3b) DNA methyltransferases and their cofactor (Uhrf1 and Dnmt3l) and regulation protein (Prdm14) [8,9,26]. After Western blot analysis, Prdm14 was pronouncedly elevated in PD0325901-including culture (**Figure 2**). In contrast, Dnmt3b and its cofactor Dnmt3l showed considerable down-regulation of protein expression in PD0325901-including treatment (**Figure 2**). CHIR99021 treatment showed no or slight effect on the level of Prdm14, Dnmt3b, and Dnmt3l. The expression of Dnmt3awas did not change and the cause is unclear. In addition, the maintenance DNA methyltransferase Dnmt1 and its targeting factor Uhrf1 also showed no alteration and the data were not shown.

Recent studies have indicated that Prdm14 can recruit polycomb repressive complex 2 (PRC2) to some gene promoters to repress their expression, such as *de novo* DNA methyltransferases (Dnmt3a and Dnmt3b) and their cofactor (Dnmt3l), and contributes to maintaining hypomethylation under 2i conditions[8,27]. Our data revealed that PD0325901 elevated the expression level of Prdm14 to inhibit Dnmt3b and Dnmt3l and reduced *de novo* genomic 5mC synthesis by the mechanism.

To explore the pluripotency maintenance of mouse ES cells under Vc/PD0325901 conditions, mRNA expression levels of several core pluripotency-linked factors were examined, and these factors have been confirmed to play vital roles in pluripotency[28]. Real-time PCR analysis found that Oct4, SOX2, Esrrb, and Sall4 showed uniform expression levels through 5 days of treatment with Vc/PD0325901 in contrast to serum culture (**Figure 3A**). Nanog and Tcf3 increased 1.3-fold and 1.6-fold, respectively. By contrast, Klf4 showed 42% down-regulation and Rex1 only decreased by about 12%. These results suggest that Vc/PD0325901 co-treatment induced diverse alterations in expression of pluripotency factors; however, Oct4 and SOX2, the most core factors, kept almost constant levels of expression.

Next, an alkaline phosphatase (AP) assay was performed to assess whether mouse ES cells were in an undifferentiated or differentiated state. Photos of cells (**Figure 3B** and **3C**) were obtained from a camera connected to an inverted microscope with 10-fold amplification by an objective lens. After continuous cultivation of 26 days in Vc/PD0325901-added serum medium, the mouse ES cells showed homogeneous morphology. Furthermore, almost all cells were stained purple-black and exhibited a ball-like state without outgrowth (**Figure 3C**), showing an undifferentiated state. By contrast, under serum conditions, a portion of the mouse ES cells at day 26 were purple-black after staining and had a ball-like state in morphology, while others presented light color with outspreading marked by white dashed oval lines (**Figure 3B**); this indicated that the serum-cultured mouse ES cells were heterogeneous in morphology and in a pluripotent state, which is consistent with previous reports[13]. Collectively, the data supported that PD0325901 in combination with Vc can sustain excellent morphology and an undifferentiated state in the mouse ES cells.

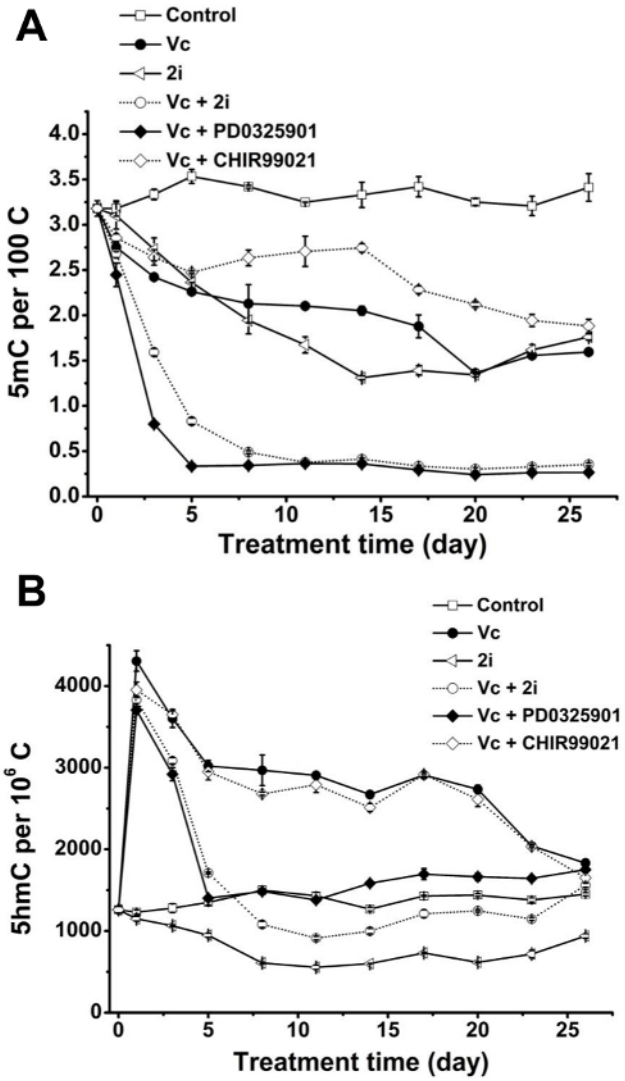

Figure 1: Vc/PD0325901 synergistically induced global erasure of genomic 5mCin mouse ES cells. (**A**) 5mC frequency (5mC/C) and (**B**) 5hmC frequency (5hmC/C) time-dependent change during a 26-day treatment of a continuous supplement with Vc, 2i, Vc/2i, Vc/PD0325901, or Vc/CHIR99021 in serum-containing medium. Error bar shows the standard deviation (SD) from three repeated analyses by UHPLC-MS/MS. C: cytosine. This figure has been modified from Li *et al.*[25]. Please click here to view a larger version of this figure.

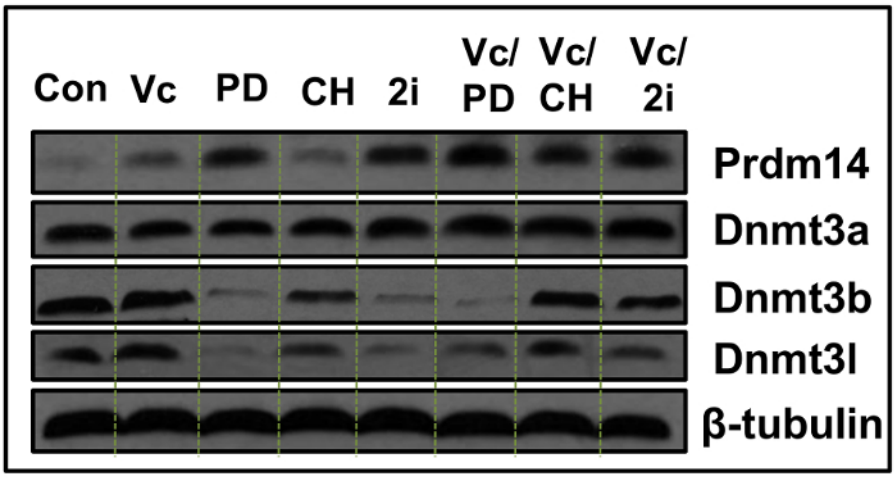

Figure 2: The protein expression alteration of Prdm14 and DNA methyltransferases (Dnmt3a, Dnmt3b, and Dnmt3l). The presented bands (from left to right) were obtained through a 5 day-treatment of serum culture (Control), Vc, PD0325901, CHIR99021, 2i, Vc/PD0325901, Vc/CHIR99021, and Vc/2i, respectively. The expression of Prdm 14 was up-regulated and Dnmt3b and Dnmt3l decreased after PD0325901-including culture. Dnmt3a exhibited no change. β-tubulin was set as the internal reference. Con: Control; PD: PD0325901; CH: CHIR99021. This figure has been modified from Li *et al.*[25]. Please click here to view a larger version of this figure.

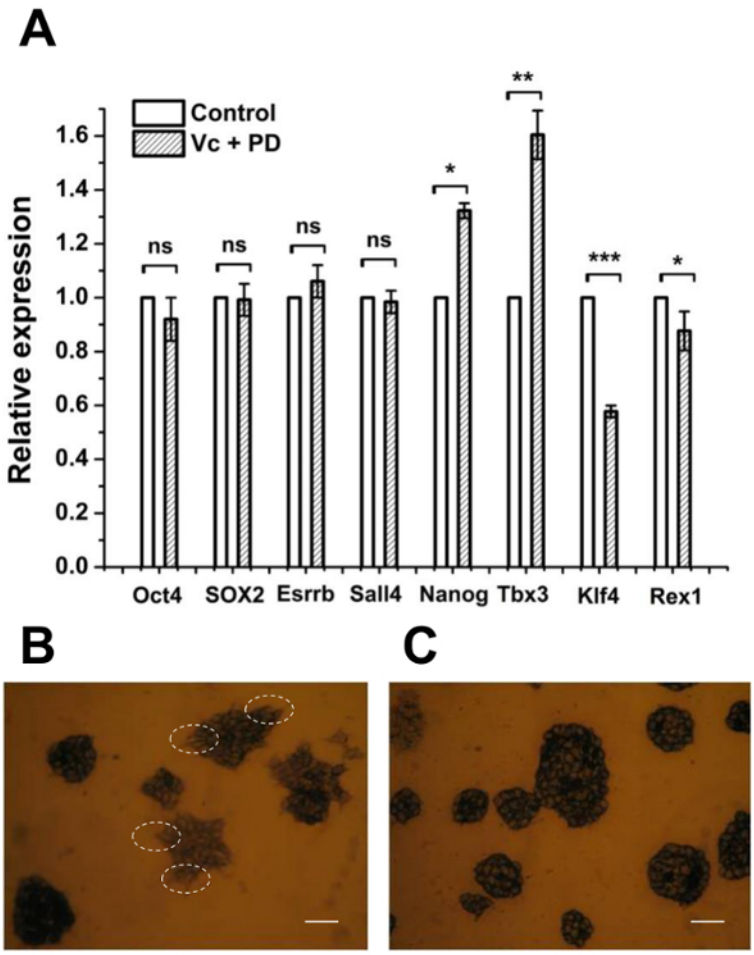

Figure 3: Vc/PD0325901-induced alteration in mRNA expression of pluripotent genes and in the activity of alkaline phosphatase. (**A**) The mRNA level of partial pluripotent genes was changed through a 5 day treatment of Vc/PD0325901 relative to that under serum conditions (Control). Data were represented as mean ± SD. The statistical significance was evaluated and p <0.05 was statistically significant. *p <0.05, **p <0.01, ***p <0.001, and ns represented non-significant. PD: PD0325901. Mouse ES cells in serum for 26 days (**B**) exhibited partial outgrowth marked by white dashed oval lines, while Vc/PD0325901-added serum medium maintained great morphology and an undifferentiated state (**C**). These images were acquired from an inverted microscope connected with a camera in the bright field. This figure has been modified from Li *et al.*[25]. Scale bars = 100 μm. Please click here to view a larger version of this figure.

Discussion

In the work, we demonstrated a novel method of combining Vc and PD0325901 to sustain mouse ES cells at an undifferentiated and hypomethylated state, which was achieved by a synergistic action of promoting DNA demethylation by Vc and suppressing *de novo* DNA methylation by PD0325901. Moreover, mouse ES cells showed great morphology under the Vc/PD0325901 culture system.

To better sustain the state of mouse ES cells in the Vc/PD0325901 culture system, there are some critical steps. Firstly, FBS batches need to be pre-screened to find and stock the best batches; also, frequent replacement of serum is detrimental to cell proliferation and to the maintenance of pluripotency. Secondly, avoid over pipetting cells during the dissociating of the cell colonies to a single cell suspension in the cell passage stage. Thirdly, to attenuate the adverse effect of trypsin during the cell cultivation, the trypsin should be removed immediately after it covers the cells in the dishes during cell passaging. Additionally, the Vc/PD0325901 culture medium should be replaced daily due to the instability of Vc.

In comparison with the classical serum culture, mouse ES cells under Vc/PD0325901 conditions show a more homogeneous cell population (**Figure 3B**, **C**) and a hypomethylated state, which resembles ICM and PGC. In addition, compared with the recently developed 2i culture, our method can realize the hypomethylation with faster kinetics, and the utilization of a single inhibitor (PD0325901) reduces the input amount of DMSO, thus decreasing the damage to cells. However, due to the addition of FBS in our culture system, the ambivalent dual actions of FBS may cause cell differentiation during long-term culture. Overall, this novel culture system is suitable for the maintenance and expansion in large scale of mouse ES cells.

Disclosures

The authors disclose no potential conflicts of interest.

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