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Reprogram Murine Epiblast Stem Cells by Epigenetic Inhibitors

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[Abstract] Pluripotent stem cells in the naïve state are highly useful in regenerative medicine and tissue engineering. A robust reprogramming of the primed murine Epiblast Stem Cells (EpiSCs) to naïve pluripotency is feasible via chemical-only approach. This protocol described a method to reprogram murine EpiSCs by MM-401 treatment, which blocks histone H3K4 methylation by MLL1/KMT2A.
Keywords: Naïve pluripotency, EpiSCs, MLL1, H3K4 methylation, Chemical inhibitor

[Background] Previous protocols on EpiSC reprogramming depended on the genetic manipulations of transcriptional factors or chemical inhibition of the signaling pathways, albeit with varying efficiency and duration. Based on a recent mechanistic study that links the MLL1 complex to the naïve state, this protocol provides a straightforward and robust method to restore naïve pluripotency from EpiSCs via targeting MLL1 mediated H3K4 methylation and subsequent transcription regulation. The reprogramming efficiency is significantly higher than previous published method, achieving 50% conversion rate in two weeks.

Materials and Reagents

1. Tissue culture plate, 24 well (Corning, Falcon®, catalog number: 353047)
2. 15 ml conical tube (Corning, Falcon®, catalog number: 352196)
3. MEF feeder cells (University of Michigan, Transgenic Animal Model Core, https://www.med.umich.edu/tamc/list.html#reagents)
4. 0.2% gelatin solution (Sigma-Aldrich, catalog number: G1890)
5. PBS
6. Collagenase type IV (Thermo Fisher Scientific, Gibco™, catalog number: 17104-019)
7. 0.05% trypsin (Thermo Fisher Scientific, Gibco™, catalog number: 25300-054)
8. The Vector™ alkaline phosphatase (AP) staining kit (Vector Laboratories, catalog number: SK-5100)
9. Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Gibco™, catalog number: 11995-065)
10. Fetal bovine serum (FBS) (Atlas Biologicals, catalog number: F-0500-D)
11. L-glutamine (200 mM) (Thermo Fisher Scientific, Gibco™, catalog number: 25030-081)
12. 100x non-essential amino acids (Thermo Fisher Scientific, Gibco™, catalog number: 11140-050)

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13. 100x sodium pyruvate (Thermo Fisher Scientific, Gibco™, catalog number: 11360-070)
14. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M7522)
    Note: This product has been discontinued.
15. KnockOut™ D-MEM (Thermo Fisher Scientific, Gibco™, catalog number: 10829018)
16. KnockOut™ serum replacement (KSR) (Thermo Fisher Scientific, Gibco™, catalog number: 10828028)
17. Glutamax (Thermo Fisher Scientific, Gibco™, catalog number: 35050061)
18. Fibroblast growth factor basic (FGF2), human recombinant (R&D Systems, catalog number: 233-FB)
19. ESGRO® leukemia inhibitory factor (LIF) (EMD Millipore, catalog number: ESG1107)
20. MM-401
21. DMSO
22. Mouse embryonic fibroblasts (MEFs) culture medium (see Recipes)
23. EpiSCs culture medium (see Recipes)
24. Embryonic stem cells (ESCs) culture medium (see Recipes)
25. 100 mM MM-401 (see Recipes)
26. MM-401 reversion medium (see Recipes)
27. MM-401 maintenance medium (see Recipes)

Equipment

1. 1 ml pipette
2. Tissue culture incubator (Isotemp™ Microbiological Incubator) (Fisher Scientific, model: Fisher Scientific™ Isotempcatalog™ Microbiological Incubator, catalog number: S28670), humidified, 37 °C and 5% CO_2 in air
3. Stereomicroscope (Olympus, model: DP73)
4. Centrifuge (Eppendorf, model: 5810 R; Swing-bucket rotor: Eppendorf, model: A-4-81)

Procedure

A schematic summary of the reprogramming procedure described in this protocol can be found in Figure 1.

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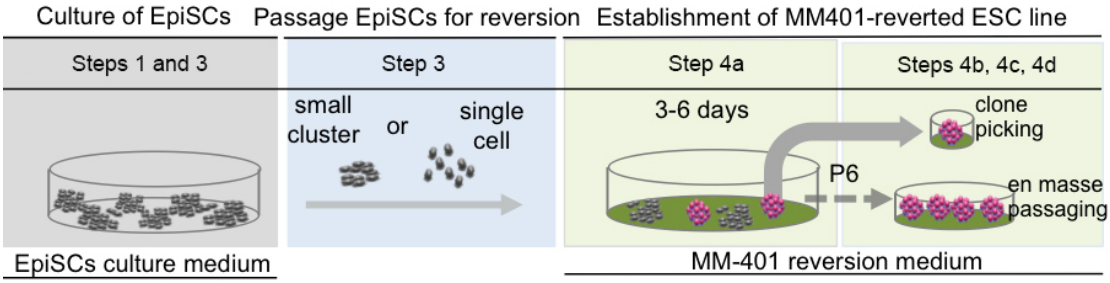

Figure 1. The schematic view of general procedures for the reversion of EpiSCs by MM-401 treatment

1. Preparation of MEF feeder cells: Irradiated MEF feeder cells are seeded at 1 x 10^5 cell/well in MEF medium in a 24-well plate (pre-coated with 0.2% gelatin solution).
2. EpiSC culture: EpiSCs are routinely cultured following previously described protocol (Chenoweth and Tesar, 2010). For chemical treatment experiments, EpiSCs are cultured in the 24-well plate and enzymatically passaged every third day (see Figure 2).

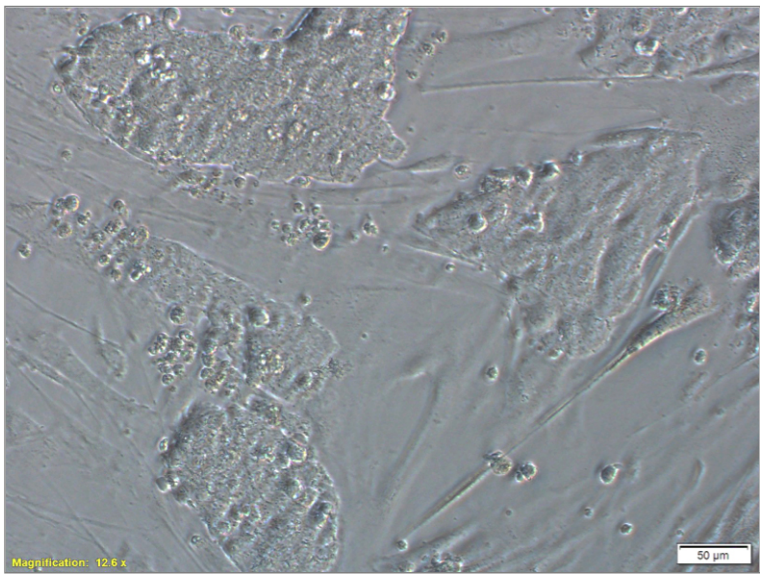

Figure 2. Representative phase image of EpiSCs at the third day of culture before splitting. Scale bar = 50 µm.

To passage,

a. Remove EpiSC culture medium from each well and wash briefly with PBS. Discard PBS and add 150 µl of collagenase type IV or 0.05% trypsin to each well, and incubate at room temperature for up to 4 min.
b. Add 1 ml of EpiSC culture medium (without FGF2) to each well and gently pipet with a 1-ml pipette. Combine suspensions from 1 to 2 wells into a 15-ml conical tube. Add EpiSC culture medium (without FGF2) to a final volume of 5 ml to each tube for centrifugation.
c. Separate EpiSCs from MEFs (Optional):

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i. For EpiSCs passaging in small cluster, separate clusters by centrifugation at 8 *x g* for 15 sec (25 °C). The EpiSC clusters will loosely pellet while the individual MEFs remain in the supernatant.
ii. For EpiSCs passaging in single cell, separate EpiSCs by centrifugation at 8 *x g* for 3 min (25 °C). A mixture of the MEFs and EpiSCs will loosely pallet while the individual single EpiSC will remain in the supernatant.
iii. Examine the separated EpiSCs after centrifugation under the microscope. Single EpiSCs and remaining MEF feeder cells can be distinguished by size (see Figure 3).

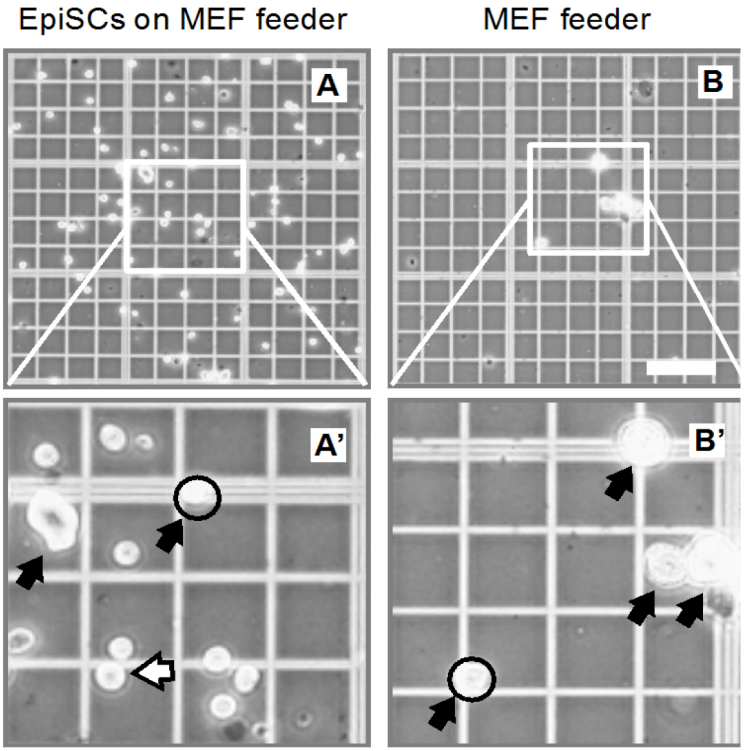

Figure 3. Representative image shows the EpiSCs and MEF feeders (collected from 2 well of 24-well plate) before and after centrifugation under the microscope. A and A'. EpiSCs and MEF feeder before separation; B and B'. MEF feeder cells. Black arrow, MEF feeder; Open arrow, EpiSC cells; Open circle, MEF feeder cells, which are still 2-3-fold larger than single EpiSC cell. Scale bar = 100 μm.

3. Plating of EpiSCs for reversion: EpiSCs were passaged in single cell or small clumps on MEF feeder cells (as described in step 1) in MM-401 reversion medium.
   a. Centrifuge the EpiSCs (from step 2b or 2c), aspirate and discard the supernatant. Resuspend EpiSC pellet in MM-401 reversion medium. For the 24-well plate, add 400 μl MM-401 reversion medium to each well.
   b. For EpiSCs passaging in small cluster, replate cells to a new well with 1:4-1:5 dilutions. For EpiSCs passaging in single cells, plate the cells at 20,000/cm^2 (approximately 4 x 10^4 cell/well for the 24-well plate, see Figure 4).

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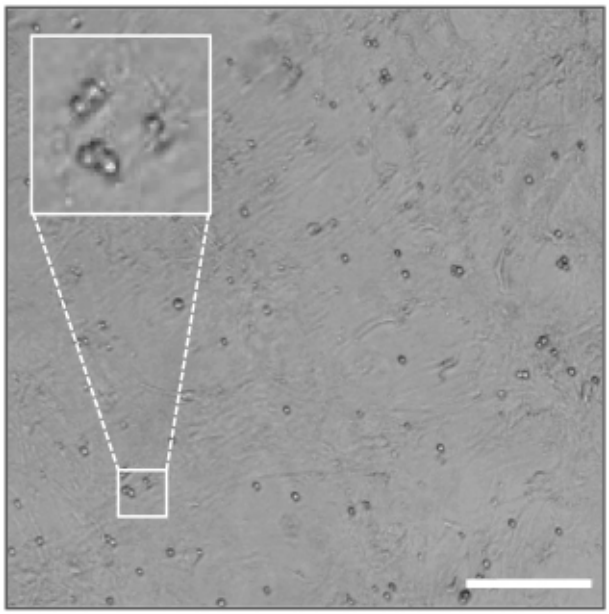

Figure 4. Representative image of single EpiSCs plated at the density of 20,000 cell/cm^2. Scale bar = 200 μm.

4. Establish MM-401-reverted ESC line
   a. Morphologically distinct reverted naïve colonies become evident over the next 3-6 days. AKP is used to stain the colonies to evaluate reversion efficiency. Strong AKP staining distinguishes the reverted naïve colonies from EpiSCs that have weak AKP staining (see Figure 5). AKP activity is detected using Vector™ alkaline phosphatase (AP) staining kit according to the manufacturer's instructions.

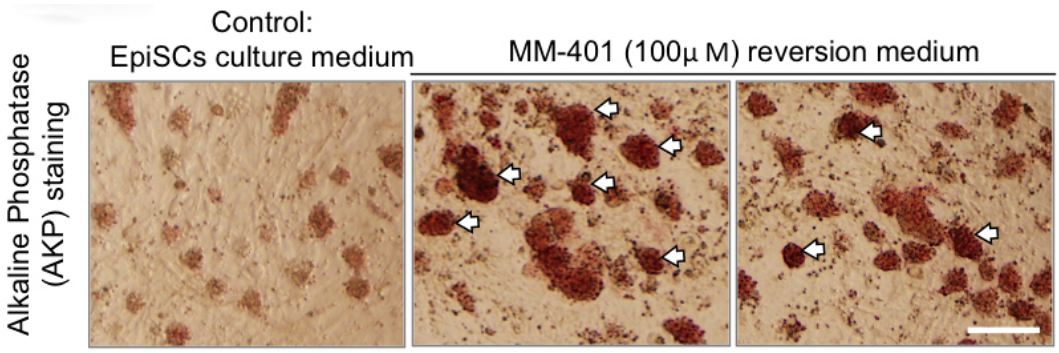

Figure 5. AKP staining of EpiSCs and reverted naïve colonies after MM-401 treatment as indicated on top. Left: Flat EpiSC clones with weak AKP staining before MM401 induction; Right: Two representative images of EpiSCs taken at 72 h of 100 μM MM-401 treatment. The reverted cells (indicated by arrows) are morphologically distinct from EpiSCs. The colonies are dome-shaped with intense AKP staining. Scale bar = 100 μm

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b. The reversion culture is passaged by a brief exposure (4-5 min) to 0.05% trypsin/EDTA with gentle pipetting into single cells. Cells can be re-seeded at 20,000-50,000/cm^2. MM-401 is replenished to reach 50-100 μM final concentrations in each passage.
c. Reverted ESC (rESC) lines can be established by clone picking or en masse passaging. The established rESC line can be maintained, passaged, cryopreserved and thawed by the standard protocol for conventional mESCs.
d. rESC line can be expanded in MM-401 maintenance medium or ESC culture medium without MM-401 after passage 6.

Notes

1. For reversion, MM-401 (50-100 μM final concentration) can be added to culture medium immediately or 2-3 days after EpiSCs forming clones.
2. Mediums containing MM-401 are recommended to be pre-warmed at room temperature (RT) for 15 min.
3. For MM-401 request: please contact valid@med.umich.edu.

Recipes

1. Mouse embryonic fibroblasts (MEFs) medium
   DMEM supplemented with:
   10% FBS
   2 mM 1x L-glutamine
   1x nonessential amino acids
   1x sodium pyruvate
   0.1 mM 2-mercaptoethanol
2. EpiSC culture medium
   KnockOut™ D-MEM supplemented with:
   20% KSR
   2 mM Glutamax
   1x nonessential amino acids
   1x sodium pyruvate
   0.1 mM 2-mercaptoethanol
   10 ng/ml FGF2
   Note: The medium can be stored at 4 °C for up to 2 weeks.
3. Embryonic stem cells (ESCs) culture medium
   KnockOut™ D-MEM supplemented with:
   20% FBS
   2 mM Glutamax

bio-protocol www.bio-protocol.org/e2168 Vol 7, Iss 05, Mar 05, 2017
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1x nonessential amino acids
1x sodium pyruvate
0.1 mM 2-mercaptoethanol
10^3 U/ml LIF
Note: The medium can be stored at 4 °C for up to 2 weeks.

4. 100 mM MM-401
   MM-401 (Molecular Mass: 700.75) is dissolved in DMSO at the final concentration of 100 mM
   Note: It is stored at -20 °C or -80 °C.
5. MM-401 reversion medium
   ESC culture medium supplemented with 100 μM MM-401
   Note: The medium can be stored at 4 °C for up to 2 weeks.
6. MM-401 maintenance medium
   ESC culture medium supplemented with 20 μM MM-401
   Note: The medium can be stored at 4 °C for up to 2 weeks.

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