Reprogramming mouse embryo fibroblasts (MEF)

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Method Article

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Abstract

MEFs can be reprogramed to pluripotent stem cells by defined reprogramming factors1. However, the reprograming efficiency is influenced by kinds of conditions, such as the combination of reprogramming factors, culture medium, types of the virus and the cell density etc2. This protocol described the optimized condition for reprogramming MEFs by different reprogramming system, especially the combination of different reprogramming factors.

Reagents

DMEM high glucose \(HyClone; #SH30022.01B) □ FBS\(MEF culture) \(PAA; #A15-101) □ FBS \(ES qualify) \(GIBCO; #10099141) □ Glutamax \(GIBCO ;#35050079)□ □ non-essential amino acids \(GIBCO; #11140076) □ sodium pyruvate \(GIBCO ;#11360070)□ □ β-mercaptoethanol\(GIBCO;#21985023)□ □ LIF \(Enzo Life Science ;#ALX-201-242) □ Ascorbic acid\(sigma ;#A4403) □ Gelatin\(sigma;#119k0062) □ Trypsin-EDTA \(Life Technologies; 25300054)□ □ Recombinant human fibroblast growth factor \(FGF2) \(Peprotech; 100-18B)□ □ Recombinant human epidermal growth factor \(EGF) \(Peprotech; AF-100-15)□ □ Recombinant human BMP4\(R&D;#314-Bp) □ DMSO\(Sigma;SHBC4445V) □ Y27632 \(Calbiochem; 688001) □ TTNPB\(Selleck; S4627) □ DZNep\(Selleck; S7120) □ RepSox\(Sigma; R0158) □ CHIR99021\(MCE; HY-10182) □ PD0325901\(MCE;HY-10254) □ VPA – Valproic acid sodium salt \(Sigma; P4543)

Procedure

pMXs-Based Retrovirus induced reprogramming **Preparation of the Virus** 1) Plate 8x10^6 Plat-E cells per 10-cm dish uniformly. Cells were cultured in 10%FBS medium for 16 hours to reach a 70-80% confluent. 2) Transfection was carried out with the modified calcium phosphate transfection method[3] as follows: Replace the medium of the Plan-E cells in 10-cm dish with 7.5 ml fresh 10% FBS medium. For each factor, 1068μl ddH_2O, 25μg plasmid, 156.25μl 2M $CaCl_2$, 1.25ml 2XHBS\(total 2.5ml) were added to a 15ml tube successively. Mix vigorously after adding the 2XHBS, and then incubate for 2 min at Room temperature. Transfer the 2.5 ml mixture into the dish that had replaced with 7.5ml fresh medium above. 3) 11-14 hours after transfection, replace the medium of the transfected 10 cm plat-E cell dishes with 10ml fresh medium. Continue to incubate the cells. 4) 48 hours after transfection, the Supernatant contained the virus was collected by a syringe and filter though a 0.45 μm filter as the first virus stock. A 10ml fresh Plan-E medium was added to the transfected Plat-E cells and harvest 24 hours later as the second virus stock. **Preparation of the OG2 MEFs** Thawing the frozen Passage 1 OG2 MEF into a 6 cm dish with MEF Medium, and cultured in the CO2 incubator to reach a 100% confluence. Then split the MEFs to P12 or P24 plate at different cell density. NOTE: As the cell plated density have a significant influence to the reprogramming efficiency2. We adjusted the plated density according to the combination of reprogramming factors. In summary, a higher density was used for a lower reprogramming efficiency system and a higher density was used for a lower reprogramming system. For detail: □ O/K/S/M: 1.2-1.5X10^4 cells per well of 12-well plate □ O/K/S:1.5-2 X10^4 cells per well of 12-well plate □

KSM/OSM/OKM: 1.5-2 X10^4 cells per well of 12-well plate c-JunDN/K/S: 2 X10^4 cells per well of 12-well plate Jdp2/K/S: 2 X10^4 cells per well of 12-well plate O/SI/d1: 3 X104 cells per well of 12-well plate Oct4/Id1/Jhdm1b/Lrh1/Glis1/Sall4: 3 X104 cells per well of 12-well plate **Infect the MEFs with the virus stock** 1) Mix the virus stock with equal volume and add one volume fresh MEF medium. For example: OKSM induced reprogramming: 0.5ml Oct4, 0.5ml Klf4, 0.5ml Sox2, 0.5ml Myc, and 0.5ml fresh MEF medium \(Total 2.5ml) for one well MEFs of 12-well plate. OKS induced reprogramming: 0.5ml Oct4, 0.5ml Klf4, 0.5ml Sox2, and 0.5ml fresh MEF medium \(Total 2ml) for one well MEFs of 12-well plate. OKS induced reprogramming: 0.25ml Oct4, 0.25ml Klf4, 0.25ml Sox2, and 0.25ml fresh MEF medium \(Total 2ml) for one well MEFs of 24-well plate. Oct4/Jhdm1b/Id1 induced reprogramming: 0.5ml Oct4, 0.5ml Jhdm1b, 0.5ml Id1, and 0.5ml fresh MEF medium \(Total 2ml) for one well MEFs of 12-well plate. Jdp2/Id1/Jhdm1b/Lrh1/Glis1/Sall4: 0.5ml JDP2, 0.5ml Jhdm1b, 0.5ml Id1, 0.5ml Lrh1, 0.5ml Id1, 0.5ml Sall4 and 0.5ml fresh MEF medium \(Total 3.5ml) for one well MEFs of 12-well plate. 2) Add polybrene to a final concentration of 4ug/ml. 3) Repeat the infection with the second virus stock 24 hours after the first infection. The Day after the second infection noted as post-infection Day0. **Generation of the iPSCs** 1) At post-infection Day0, replace the virus contained medium with fresh reprogramming medium such as mES , mES+Vc4 or iCD15 medium., according to the design of the experiment. \(1ml per 12-plate well) 2) Change the medium everyday and observe the morphology change. 3) Count the GFP+ colonies at proper days. 4) Pick the GFP+ colonies and passage. **pSuper bashed shRNA induced reprogramming** All of the protocols are same with the pMXs-Based Retrovirus except that, the puromycin \(2μg/ml) was added to the reprogramming medium at post-infection Day2 to root out the uninfected cells. **pW-TRE based Doxycycline\(Dox) induced reprogramming.** **Preparation of the Virus** 1) Plate 6x106 HEK93T cells per 10-cm dish uniformly. Cells were cultured in 10%FBS medium for 16 hours to reach a 70-80% confluent. 2) Transfection was carried out with the modified calcium phosphate transfection method as follows: Replace the medium of the HEK93T cells in 10-cm dish with 7.5 ml fresh 10% FBS medium. For each factor, 1070μl ddH2O, 12.5μg pW-TRE-c-Jun/ pW-rtTA, 7.5μg psPAX2, 5μg psMD2.G, 156.25μl 2M CaCl2, and 1.25ml 2XHBS\(total 2.5ml), were added to a 15ml tube successively. Mix vigorously after adding the 2XHBS, and then incubate for 2 min at Room temperature. Transfer the 2.5 ml mixture into the dish that had replaced with 7.5ml fresh medium above. 3) 11-14 hours after transfection, replace the medium of the transfected 10 cm HEK93T cell dishes with 10ml fresh medium. Continue to incubate the cells. 4) 48 hours after transfection, the Supernatant contained the virus was collected by a syringe and filter though a 0.45 μm filter as the first virus stock. A 10ml fresh HEK93T medium was added to the transfected HEK93T cells and harvest 24 hours later as the second virus stock. **Preparation of the MEFs** Same with relative section of “pMXs-Based Retrovirus induced reprogramming” Infect the MEFs with virus stock Infect the MEFs with virus carrying the Oct4, Klf4, Sox2 and c-Jun coding sequences. To reprogram cells in a P12 well: 1) mix the virus stock as following: 0.5ml Oct4 \(pMXs-Based Retrovirus), 0.5ml Sox2 \(pMXs-Based Retrovirus), 0.5ml Klf4 \(pMXs-Based Retrovirus), 0.125ml c-Jun \(pW-TRE-Based Lentivirus), 0.125ml rtTA \(pW-Based Lentivirus), and 0.5ml fresh MEF medium. 2) Add polybrene to a final concentration of 4ug/ml. 3) Repeat the infection with the second virus stock 24 hours after the first infection. The Day after the second infection noted as post-infection Day0. **Generation of the iPSCs** 1) At post-infection Day0, replace the virus contained

medium with fresh reprogramming medium such as mES, mES+Vc or iCD1 medium. DOX was added in the indicated days as design of the experiment. 2) Change the medium everyday and observe the morphology change. 3) Count the GFP+ colonies at proper days.

Timing

Virus stock preparation: 3 Days MEFs preparation: 3 Days OKS induced reprogramming in iCD1 medium: 8 Days OKS/c-JunDN induced reprogramming in iCD1 medium: 8 Days OKS/c-Jun shRNA induced reprogramming in iCD1 medium: 10 Days OKS induced reprogramming in mES medium: 21days OKS induced reprogramming in mES+Vc medium: 16 days OKSM induced reprogramming in iCD1 medium: 6 Days OKSM induced reprogramming in mES+Vc medium: 12 Days c-JunDN/KSM, JDP2/KSM induced reprogramming in iCD1 medium medium: 8 Days c-Jun shRNA/KSM induced reprogramming in iCD1 medium medium: 8 Days c-JunDN/KS, JDP2/KS induced reprogramming in iCD1 medium medium: 16 Days Oct4/Sox induced reprogramming in iCD1+BMP medium: 16 Days. Oct4/Jhdm1b/Id1 induced reprogramming in iCD1+BMP medium: 14 Days. Jdp2/Id1/Jhdm1b/Lrh1/Glis1/Sall4 induced reprogramming in iCD1 medium: 16 Days.

Troubleshooting

Problem 1 Cells grow slowly. Reason: 1)MEFs are over grow before split; 2)The FBS is not qualified. Solution: 1)Use MEFs in passage number 2 and split the MEFs once the confluence reach to 100% after thawing; 2)Use the qualified FBS. Problem 2 The morphology change of the reprogramming cell is slowly Reason: 1) The titer of the virus is low; 2)The medium is out of date. Solution: 1)repare the virus following the protocol strictly; 2) Use fresh virus supernatant; 3) Use qualified reprogramming medium.

Anticipated Results

For OKS induced reprogramming in iCD1 medium, a significant mesenchyme to epithelial transition can be observed at post-infection Day2, and GFP+ cells will appear at post-infection Day4. More than 800 GFP+ colonies can be found at post-infection Day8 in a P12 well. c-Jun over expression can significantly inhibit the mesenchyme to epithelial transition induced by OKS, and few colonies can be found at post-infection Day8. For Jdp2/Id1/Jhdm1b/Lrh1/Glis1/Sall4 induced reprogramming in iCD1 medium, cells grow at a relative low rate, and mesenchyme to epithelial transition can be found at post-infection Day6, GFP+ colonies can be found at post-infection Day16.

References

1. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676 \(2006). 2. Chen, J. et al. Towards an optimized culture medium for the generation of mouse induced pluripotent stem cells. The Journal of biological chemistry 285, 31066-31072 \(2010). 3. Urabe, M., Kume, A., Tobita, K. & Ozawa, K. DNA/Calcium

phosphate precipitates mixed with medium are stable and maintain high transfection efficiency. Analytical biochemistry 278, 91-92 \(2000). 4. Esteban, M.A. et al. Vitamin C enhances the generation of mouse and human induced pluripotent stem cells. Cell stem cell 6, 71-79 \(2010). 5. Chen, J. et al. Rational optimization of reprogramming culture conditions for the generation of induced pluripotent stem cells with ultra-high efficiency and fast kinetics. Cell research 21, 884-894 \(2011).

Acknowledgements

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