Culturing of human pluripotent stem cells in a mesoderm biased state

Dylan Stavish (d.stavish@sheffield.ac.uk)
The University of Sheffield https://orcid.org/0000-0002-1441-2028
Christopher Price
The University of Sheffield

Method Article

Keywords: state, bias, pluripotency, lineage-bias, mesoderm, differentiation, antagonism

Posted Date: March 25th, 2021

DOI: https://doi.org/10.21203/rs.3.pex-1100/v1

Abstract

Here we present a new culturing system, PRIMO Plus, to generate mesoderm biased human pluripotent stem cells by cross antagonism of pro-differentiation and pro-pluripotency factors in a fully defined medium.

Introduction

The differentiation of human pluripotent stem cells (hPSC) towards clinically relevant cells for regenerative medicine has been hampered by poor differentiation efficiencies. This may be a result, in part, of the heterogeneity that exists within stem cell cultures. We have generated a new stem cell medium PRIMO Plus, which biases cells towards mesodermal differentiation without losing pluripotency. The cells can be maintained over multiple passaged and when cultured in this medium appear to be trapped at a relevant development stage on a mesoderm differentiation trajectory that impact upon their differentiation outcome. We hope that this medium could be used as a tool in both improving the differentiation of hPSCs towards mesodermal derivatives but also to investigate the basic biological principles behind biased sub-states, the exit from pluripotency and the commitment to differentiation.

Reagents

Cells:

Human Pluripotent stem cells (PSC)

e.g.

a) HES3 (Cooper et al., 2002)

b) H9 (Thomson et al., 1998)

Reagents:

DMEM/F12 (Sigma #D6421)

L-ascorbic acid (Sigma #A8960)

Sodium selenium (Sigma #S5261)

Insulin (ThermoFisher #A11382IJ)

NaHCO3 (Sigma #S5761)

Transferrin (Sigma #T0665)

Glutamax (ThermoFisher #35050038)

FGF2 (Peprotech #100-18B)

TGFB1 (Peprotech #100-21)

CHIR99021 (Tocris, #4423)

IWP-2 (Tocris, #3533)

DMSO(Sigma, #D2650)

Oleoyl-L-α-**lysophosphatidic acid** sodium salt (LPA) (Sigma, #L7260)

Fatty Acid free BSA (Probumin, Millipore, #810664)

ReLeSR (Stem Cell Technologies, # 05873)

PBS without Ca+, Mg++ (Sigma, #D8537)

Absolute (100%) Ethanol

Equipment

T25 flask or T12.5 flask.

CytoOne Bottle Top Filtration Unit (StarLabs #CC6032-8233) 0.2μM filter.

Procedure

Important note: Perform every step under sterile conditions as no antibiotics are used in the medium.

Medium preparation:

Basal Medium:

The basal medium of PRIMO Plus is E8. Our E8 was made in house with a recipe adapted from Chen et al, 2011, although commercially available version can be used instead. One key difference in the in-house media from the recipe published by Chen et al, 2011 is the replacement of standard glutamine with GlutaMax (Thermofisher). Glutamax is a thermostable form of glutamine, which is bound to alanine to increase stability. Large batches of 50X E8 supplements were prepared and frozen as 10ml aliquots at -20°C. Defrosted 10ml aliquots were added to 490ml of DMEM/F12 without glutamine and filtered using a CytoOne Bottle Top Filtration Unit 0.2μM filter.

E8 Composition:

1 litre of 50X concentrate: DMEM/F12 (Used neat to make mixture up to 1 litre), L-ascorbic acid (3200mg/L), Sodium selenium (700µg/L), Insulin (970mg/L), $NaHCO_3$ (27.15g/L), Transferrin (535mg/L), Glutamax (50X, 500ml), FGF2 (5mg/L) and TGFB1 (100µg/L).

1 litre of E8: DMEM/F12 (Used neat to make mixture up to 1 litre), L-ascorbic acid (64mg/L), Sodium selenium (14µg/L), Insulin (19.4mg/L), $NaHCO_3$ (543mg/L), Transferrin (10.7mg/L), Glutamax (1X, 10ml/L), FGF2 (100µg/L) and TGFB1 (2µg/L).

PRIMO Plus medium:

Preparations:

1. CHIR99021: resuspend the powder in DMSO at 10mM.
2. IWP-2: resuspend the powder in DMSO at 5mM.
3. Cholesterol: resuspend the powder in 100% ethanol at 20mM.
4. LPA: resuspend the powder in PBS with 0.1% Fatty acid free BSA at 381µM.

Preparation of 40ml 5X stock

1. Dissolve 400mg of BSA (1% in final volume) in 5ml of E8 media at 37˚c.
2. Add ~0.5ml (amount for 4.8 µM in 40ml) of LPA and incubate for 5 minutes.
3. Add 40µl of 20mM Cholesterol (20µM in 40ml) and incubate for a further 5 minutes.
4. Add ~34.5ml of E8.
5. Filter sterilise with a 0.22µM filter.
6. Freeze or make smaller aliquots and freeze at -20˚c

Preparation of PRIMO Plus:

1. Dilute 5X stock 1 in 5 with E8 media, ie 10ml of stock with 40ml of E8.
2. Add CHIR99021 at 3μM.
3. Add IWP-2 at 1μM.

PRIMO Plus Composition:

5x stock: Desired mL of E8 as basal, Fatty Acid Free BSA (1%), Cholesterol (20μM), and LPA (4.8μM).

PRIMO Plus complete: Desired mL of E8 as basal, Fatty Acid Free BSA (0.2%), Cholesterol (4μM), LPA (0.96μM), CHIR99021 (3μM) and IWP-2 (1μM).

Cell culture:

Preparing Vitronectin Flasks (*can be substituted with Geltrex (Thermofisher #A1413201) following manufacturers instructions):

1. Remove Vitronectin (ThermoFisher, A14700) Aliquot from -80°C Freezer and allow to thaw at room temperature.

2. Dilute Vitronectin at 1:100 in 1X PBS.

3. Coat the surface of a Tissue Culture flask with Vitronectin (3ml per T25, 2ml per T12.5) and leave at room temperature for minimum of 1 hour.

Following incubation at room temperature, flasks are now ready for use or alternatively can be stored in the fridge and used within 2 weeks. If stored in the fridge prior to use flasks must be left to return to room temperature for a minimum of 1 hour.

Passaging hES cells using ReleSR (Stem Cell Technologies, # 05873):

4. Aspirate Vitronectin and replace with PRIMO PLUS; 3ml per T25/ 1.5ml per T12.5, place flasks in the incubator until cells harvested.

5. Remove media from confluent flask of hPSCs and wash gently with 1X PBS.

6. Add 1ml ReleSR to the bottom of flask, rock the flask back and forth to ensure all cells have been coated, incubate for ~20 seconds at RT.

7. Tilt the flask sufficiently that ReleSR pools in one of the bottom corners of the flask and aspirate away the ReleSR.

8. Incubate cells for 2-4 minutes at room temperature – **Incubation period is dependent on cells line and colony size and should be adjusted appropriately. For large colonies the incubation time will have to be increased. Cultures on Geltrex usually require longer incubation.**

9. Inactivate ReleSR by adding 2ml of **PRIMO PLUS medium** and slowly rocking back and forth, cells can be encouraged to detach by tapping the flask – *avoid very aggressive tapping of the flask which will encourage any differentiated cells to also detach.*

10. Check cells under the microscope to assess colony size

11. Resuspend pellet in appropriate volume of PRIMO Plus medium and distribute into prepared flasks as relevant for the desired splitting ratio. Normally passaged as small clumps using ReleSR at a ratio of 1:6, sometimes 1:12, every 3-4 days (optimal split ratios and growth periods will have to be established experimentally for different PSC lines).

12. Final volume of media in flasks or plates should be kept to a low volume, for a T25 this should be~ 3ml. Higher volumes result in poor plating efficiency.

13. Place cells into 37˚c 5% CO_2 Incubator for culture.

14. Medium must be replaced daily because of LPAs short half-life.

15. Cells should be passaged again before reaching high confluency (over 90%), dense areas will lead to differentiation.

Important Notes: The medium is delicately balanced, over feeding can push the cells towards differentiation but under feeding can do the same as the cells degrade the LPA. We suggest to have cells growing in E8 first and allow them to reach high confluency (~80%) then passage with standard ReleSR technique into PRIMO Plus medium. Feed so that you see the visible colour change in the media the following day. For T25s we would normally passage into 3-4ml, then over the days increasing the volume by 1-2mls up to 10ml the day before passage.

Troubleshooting

Increased Differentiation in cultures: Batches of reagents, particularly LPA and CHIR99021 may effect results, either increasing the concentration of LPA or lowering the concentration of CHIR99021 might alleviate these issues. In our hands the optimal concentrations worked well as long as cells were fed with the appropriate volume of medium daily, this might require optimisation per cell line.

Time Taken

Anticipated Results

After initial seeding in PRIMO Plus morphology changes might become apparent in cultures. This can appear as flat, spread and distended colonies, these should compact over the days of culture, with colonies appearing rounder and with shinier edges under the microscope. After the line has adapted to the medium, colonies favour the more compact morphology post-passage but the flat colonies can sometimes still be seen. When differentiation occurs, it has appeared as small, spiky, fibroblast like cells that do not maintain a colony morphology. This can normally be removed with accurate ReleSR passaging to leave the differentiated cells in the flask. Decreasing the incubation time of ReleSR can be helpful to achieve this clean-up.

Assessing cells in mesoderm biased state:

Surface marker analysis:

Cells can be harvested and assessed for pluripotency associated surface marker expression by flow cytometry. In our hands, the marker, SSEA-3 (Kannagi et al., 1983) , proved the most sensitive marker. Cultures should maintain high SSEA-3 expression between 80-100% and should be compared to the same line grown in E8 medium.

Gene expression analysis:

Gene expression for pluripotency and early mesendodermal markers can be assessed by qPCR. Example pluripotency genes to assess can include: *POU5F1, NANOG, SOX2*, and *DNMT3B.* Example mesendodermal genes to assess can include: *T (TBXT), EOMES, GATA4, GATA6* and *HAND1*. Samples should be compared to hPSC growing in E8, in general expression of pluripotency genes should not deviate too far from E8 expression levels but mesendodermal genes should demonstrate strong upregulation.

References

1. Cooper, S. *et al.* Biochemical properties of a keratan sulphate/chondroitin sulphate proteoglycan expressed in primate pluripotent stem cells. *Journal of anatomy* **200**, 259-265 (2002).

2. Thomson, J.A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science (New York, N.Y.)* **282**, 1145-1147 (1998).

3. Chen, G. *et al.* Chemically defined conditions for human iPSC derivation and culture. *Nature methods* **8**, 424-429 (2011).

4. Kannagi, R. *et al.* Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *The EMBO journal* **2**, 2355-2361 (1983).

Acknowledgements

This work was supported by grants from the European Community's Sixth and Seventh Framework Programs (LSHG-CT-2006-018739 and FP7/2007-2013-602423, PluriMes) and the MRC through the UK Regenerative Medicine Platform (grant no. MR/L012537/1).