Reduced MEK inhibition preserves genomic stability in naïve human ES cells

Abstract

Here we provide a simple modification of the 5i/LAF protocol to enable robust growth and reduced genomic instability in naïve hESCs. We also introduce a new set of MEK inhibitors replacing PD0325901 to grow naïve hESCs and a short protocol to adapt 5i/LAF cells to t2iLGöY medium.

Introduction

Human embryonic stem cells (hESCs) self-renew indefinitely while retaining the capacity for multilineage differentiation, providing a valuable tool for basic research and potential therapeutic applications. Conventional hESC culture conditions include Activin A and basic FGF (abbreviated as A/F) and capture pluripotent cells in a developmentally advanced or "primed" pluripotent state that resembles the postimplantation epiblast[1,2]. In addition, several laboratories have recently developed protocols to capture pluripotent cells in a more primitive or "naïve" state that resemble the preimplantation epiblast[3-5]. For example, naïve stem cells upregulate gene expression programs associated with cleavage stage embryos, show global DNA hypomethylation and a lack of X chromosome inactivation[6,7], offering a useful system to study transcriptional and epigenetic mechanisms of preimplantation development. In addition, naïve hESCs are reportedly more efficient at producing certain specialized cell types, such as primordial germ cells[8]. Culture conditions to convert primed hESCs to a naïve state typically rely on a combination of growth factors and small molecules that suppress specific protein kinases involved in differentiation, cell adhesion, and survival[3-5]. Two culture methods appear to be particularly effective in endowing primed hESCs with a naïve-like transcriptional state[9]. The first protocol, termed "t2iLGöY", involves transient overexpression of the transcription factors KLF2 and NANOG in the presence of the MEK inhibitor (MEKi) PD0325901 and titrated amounts of GSK3ß inhibitor (CHIR99021), supplemented with the PKC inhibitor Gö6983 and human LIF (hLIF)[4,10]. The second protocol, termed "5i/LAF", requires treatment of primed hESCs with inhibitors targeting the GSK3 ß, ROCK, BRAF, MEK, and SRC kinases in addition to hLIF and A/F[5,7]. Despite differences in media components, inhibitors of the mitogen-activated protein kinase (MAPK/ERK) pathway are common to all currently available protocols.

We uncovered an unexpected sensitivity of naïve hESCs to MAPK signal inhibition, which affects their optimal proliferation rate, survival and genome integrity. We thus propose that minimal MAPK activity is beneficial to preserve both a robust growth potential and genomic stability in naïve human pluripotent stem cells maintained in 5i/LAF. Our data also suggest that different hESC lines respond differently to MEK inhibition. Thus, we suggest that MEK inhibitors should ideally be titrated in a cell line dependent fashion.

Reagents

Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Cat#11960044)
Dulbecco's phosphate-buffered saline (DPBS) (Life Technologies, Cat#14190144)
TeSR-E8 Medium (Stem Cell Technologies, Cat#05990)

Neurobasal Medium (Life Technologies, Cat#21103049)
KnockOut Serum Replacement (KSR) (Life Technologies, Cat#10828028)
N-2 Supplement (Life Technologies, Cat#17502048)
B-27 Supplement (Life Technologies, Cat#17504044)
Fetal Bovine Serum (FBS) (HyClone, Cat#SH30071.03)
Sodium Pyruvate (Life Technologies, Cat#11360070)
GlutaMAX Supplement (Life Technologies, Cat#35050061)
L-Glutamine (Life Technologies, Cat#25030081)
MEM Non-Essential Amino Acids Solution (NEAA) (Life Technologies, Cat#11140050)
beta-mercaptoethanol (Life Technologies, Cat#21985023)
Penicillin-Streptomycin (Pen-Strep) (Life Technologies, Cat#15140122)
Bovine Albumin Fraction V (7.5%) (BSA) (Life Technologies, Cat#15260037)
mFreSR (Stem Cell Technologies, Cat#05854)
Dimethyl Sulfoxide (DMSO) (Sigma, Cat# D2650)
Accutase (Stem cell technologies, Cat#07920)
TrypLE Express Enzyme (Life Technologies, Cat#12604013)
Recombinant Human bFGF (154 a.a.) (Peprotech, Cat# 100-18B)
Recombinant Human Activin A (Peprotech, Cat#120-14E)
Recombinant Human LIF (Peprotech, Cat#300-05)
Y-27632 (Axon Medchem, Cat#1683)
PD0325901 (Axon Medchem, Cat#1408)
Gö6983 (Axon Medchem, Cat#2466)
IM-12(Axon Medchem, Cat#2511)
SB590885 (Axon Medchem, Cat#2504)
WH-4-023 (Axon Medchem, Cat#2381)
Selumetinib (Selleck Chemicals, Cat#S1008)
Binimetinib (Selleck Chemicals, Cat#S7007)
Trametinib (Selleck Chemicals, Cat#S2673)
Pimasertib (Selleck Chemicals, Cat#S1475)
Refametinib (Selleck Chemicals, Cat#S1089)
TAK-733 (Selleck Chemicals, Cat#S2617)
RO5126766 (Selleck Chemicals, Cat#S7170)
Cobimetinib (Selleck Chemicals, Cat#S8041)
AZD8330 (Selleck Chemicals, Cat#S2134)
CHIR99021(Axon Medchem, Cat#1386)
Versene Solution (Life Technologies, Cat#15040066)
Corning™ Matrigel™ hESC-Qualified Matrix (Fisher Scientific, cat# 08-774-552)

Equipment
Biological safety cabinet
Incubator at 37oC, with 5% O2 and 5% CO2
Benchtop Centrifuge
Water bath set at 37°C
Electric Pipetman, Pipet filler, plastic serological pipettes, pipette tips and pasteur pipettes
15 ml polystyrene conical tubes (Corning, Cat#430052)
50 ml polypropylene conical tubes (Corning, Cat#430290)

6-well tissue-culture treated plate (Corning, Cat#3516)
T25 flask (Corning, Cat#430639)
T75 flask (Corning, Cat#430725U)
T175 flask (Corning, Cat#431080)
1.8 ml cryovials (Nunc, Cat#377267)
Cell strainer 40 µM (Corning, Cat#CLS431750)
Mr. Frosty (Thermo Fisher, Cat#51000001)

Procedure

MEF medium

DMEM (Life Technologies), 10% FBS (Hyclone), 1% nonessential amino acids (Life Technologies), 1mM GlutaMAX (Life Technologies), 1% Pen-strep (Life Technologies), 0.1mM beta-mercaptoethanol (Life Technologies) and 1mM sodium pyruvate (Life Technologies).

TeSR-E8 Medium

Add 20ml of TeSR-E8 supplement (Stem Cell Technologies) to 500ml basal medium (Stem Cell Technologies), supplement with 1% Pen-strep (Life Technologies).

5i/LAF medium

A 50:50 mixture of DMEM/F-12 (Life Technologies) and Neurobasal medium (Life Technologies) containing 1x N2 supplement (Life Technologies), 1x B27 supplement (Life Technologies), 10 ng/mL bFGF (Peprotech), 1% nonessential amino acids (Life Technologies), 1mM GlutaMAX (Life Technologies), penicillin-streptomycin (Life Technologies), 0.1 mM beta-mercaptoethanol (Life Technologies), 50 µg/mL BSA (Life Technologies), 0.5 µM IM-12 (Axon Medchem), 0.5 µM SB590885 (Axon Medchem), 1 µM WH-4-023 (Axon Medchem), 10 µM Y-27632 (Axon Medchem), 20 ng/mL Activin A (Peprotech), 20 ng/mL rhLIF (Peprotech), 0.5% KSR (Life Technologies) and 1 µM PD0325901 (Axon Medchem).

m5i/LAF medium

A 50:50 mixture of DMEM/F-12 (Life Technologies) and Neurobasal medium (Life Technologies) containing 1x N2 supplement (Life Technologies), 1x B27 supplement (Life Technologies), 10 ng/mL bFGF (Peprotech), 1% nonessential amino acids (Life Technologies), 1mM GlutaMAX (Life Technologies), penicillin-streptomycin (Life Technologies), 0.1 mM beta-mercaptoethanol (Life Technologies), 50 µg/mL BSA (Life Technologies), 0.5 µM IM-12 (Axon Medchem), 0.5 µM SB590885 (Axon Medchem), 1 µM WH-4-023 (Axon Medchem), 10 µM Y-27632 (Axon Medchem), 20 ng/mL Activin A (Peprotech), 20 ng/mL rhLIF (Peprotech), 0.5% KSR (Life Technologies) and 0.5 µM PD0325901 (Axon Medchem).

4i/LAF medium with alternative MEK inhibitors

A 50:50 mixture of DMEM/F-12 (Life Technologies) and Neurobasal medium (Life Technologies) containing 1x N2 supplement (Life Technologies), 1x B27 supplement (Life Technologies), 10 ng/mL bFGF (Peprotech), 1% nonessential amino acids (Life Technologies), 1mM GlutaMAX (Life Technologies), penicillin-streptomycin (Life

Technologies), 0.1 mM beta-mercaptoethanol (Life Technologies), 50 µg/mL BSA (Life Technologies), 0.5 µM IM-12 (Axon Medchem), 0.5 µM SB590885 (Axon Medchem), 1 µM WH-4-023 (Axon Medchem), 10 µM Y-27632 (Axon Medchem), 20 ng/mL Activin A (Peprotech), 20 ng/mL rhLIF (Peprotech), 0.5% KSR (Life Technologies).
The medium can then be supplemented with one of the following inhibitors at 0.5 µM: Refametinib (Selleck Chemicals), TAK-733 (Selleck Chemicals), Cobimetinib (Selleck Chemicals).

t2iLGöY medium
t2iLGöY medium contained a 50:50 mixture of DMEM/F-12 (Life Technologies) and Neurobasal medium (Life Technologies) containing 1x N2 supplement (Life Technologies), 1x B27 supplement (Life Technologies), 10 ng/mL bFGF (Peprotech), 1% nonessential amino acids (Life Technologies), 1 mM GlutaMAX (Life Technologies), penicillin-streptomycin (Life Technologies), 0.1 mM beta-mercaptoethanol (Life Technologies), 50 µg/mL BSA (Life Technologies), 1 µM PD0325901 (Axon Medchem), 1 µM CHIR99021 (Axon Medchem), 2.5 µM Gö6983 (Axon Medchem), 10 µM Y-27632 (Axon Medchem), 20 ng/mL hLIF (Peprotech) and 50 µg/ml ascorbic acid (Sigma-Aldrich).

All culture media were filtered before use with a 0.22µm filter (Corning).

Culturing human embryonic stem cells in conventional primed condition
1. Conventional primed human ESCs (WIBR3 cell line, DPE-OCT4GFP[5], UCLA1, UCLA3, UCLA4, UCLA5, UCLA9 and UCLA17 cell lines) are maintained in a feeder-free TeSR-E8 medium and passaged every 4-5 days at 1:4 ratio.
2. Before passaging, coat culture wells with Matrigel (Corning) for 30 minutes at 37C.
3. For passaging, wash primed hESCs with PBS (Life Technologies) and then add Versene solution (Life Technologies) for ~5 minutes. Aspirate the Versene solution, wash with PBS (Life Technologies), add fresh TeSR-E8 (Stem Cell Technologies) and break the colonies into small clumps by gently pipetting. 10 µM Y- 27632 (Axon Medchem) can be added to the TeSR-E8 media to improve hESC survival rate.
4. Cells are cultured in a 37C, 5% O2 and 5% CO2 incubator and media are changed every day.
NOTE: Primed hESCs are frozen in mFreSR medium (Stem Cell Technologies) following the manufacturer instructions.

Conversion of primed hESCs to a naive state

5i/LAF culture condition:
1. For conversion of primed hESCs to a naïve state, irradiated CF-1 MEFs ($2.5x10^6$ cells per 9.5 cm2) are plated the day before in MEF medium on Matrigel coated dishes.
2. Primed hESCs are treated with TrypLE expressed enzyme for 3-5 minutes, dissociated into a single-cell suspension and plated at a density of 30.000 cells per 9.5cm2 on irradiated CF-1 MEFs in TeSR-E8 medium supplemented with 10 µM Y-27632 (Axon Medchem).
3. The medium is replaced the day after with fresh TeSR-E8.
4. Two days after passaging, medium is changed to 5i/LAF and then replaced daily.

5. After 8-10 days naive-like hESCs are passaged onto a fresh layer of irradiated CF-1 MEFs at a 1:1-1:2 ratio. Dome-shaped colonies usually arise after 2-3 passages.
6. Naive hESCs are passaged every 6-7 days. For passaging, naive hESCs are treated with Accutase (Life technologies) for five minutes at 37C, dissociated into a single-cell suspension and resuspended in MEF medium. Cells are then filtered through a cell strainer (40 µM, Corning), centrifuged at 300*g* for 5 minutes and seeded on fresh irradiated MEFs in 5i/LAF medium.

m5i/LAF culture condition:
1. For conversion of primed hESCs to a naïve state, irradiated CF-1 MEFs ($2.5x10^6$ cells per 9.5 cm2) are plated the day before in MEF medium on Matrigel coated dishes.
2. Primed hESCs are treated with TrypLE expressed enzyme, dissociated into a single-cell suspension and plated at a density of 30.000 cells per 9.5cm2 on irradiated CF-1 MEFs in TeSR-E8 medium supplemented with 10 µM Y-27632 (Axon Medchem).
3. The medium is replaced the day after with fresh TeSR-E8.
4. Two days after passaging, medium is changed to m5i/LAF and then replaced daily.
5. After 8-10 days naive-like hESCs are passaged onto a fresh layer of irradiated CF-1 MEFs at a 1:1-1:2 ratio. Dome-shaped colonies usually arise after 3-5 passages.
NOTE: for some primed hESCs (i.e. UCLA1, UCLA9, UCLA17), the naïve conversion can be very inefficient in m5i/LAF medium. For this reason, it may be necessary to pick individual colonies to expand a homogenous population of naïve cells. We also note that the use of bFGF for expansion in m5i/LAF condition is not recommended.
6. Naive hESCs are passaged every 6-7 days. For passaging, hESCs are treated with Accutase (Life technologies) for five minutes at 37C, dissociated into a single-cell suspension and resuspended in MEF medium. Cells are then filtered through a cell strainer (40 µM, Corning), centrifuged at 300*g* for 5 minutes and seeded on fresh irradiated MEFs in m5i/LAF medium.
NOTE: The PD03 concentration for the m5i/LAF medium may vary depending on the starting cell line. Thus, we recommend doing a titration of PD03 for each individual hESC line.

4i/LAF with Alternative MEKi:
1. For conversion of primed hESCs to a naïve state, irradiated CF-1 MEFs ($2.5x10^6$ cells per 9.5 cm2) are plated the day before in MEF medium on Matrigel coated dishes.
2. hESCs are treated with TrypLE expressed enzyme, dissociated into a single-cell suspension and plated at a density of 30.000 cells per 9.5cm2 on irradiated CF-1 MEFs in TeSR-E8 medium supplemented with 10 µM Y-27632 (Axon Medchem).
3. The medium is replaced the day after with fresh TeSR-E8.
4. Two days after passaging, medium is changed to 4i/LAF supplemented with 0.5 µM of one of the alternative MEK inhibitors and then replaced daily.
5. Cells are passaged onto a fresh layer of irradiated CF-1 MEFs at a 1:1-1:2 ratio. Dome-shaped colonies usually arise after 3-5 passages.
6. Naive hESCs are passaged every 6-7 days. For passaging, hESCs are treated with Accutase (Stem cell technologies) for five minutes at 37C, dissociated into a single-cell suspension and resuspended in MEF medium. Cells are then filtered through a cell strainer (40 µM, Corning), centrifuged at 300*g* for 5 minutes and seeded on fresh

irradiated MEFs in 4i/LAF medium supplemented with 0.5 μM of one of the alternative MEK inhibitors.

5i/LAF to m5i/LAF transition:
1. Some primed hESC lines can be resistant to naïve conversion in m5i/LAF conditions (i.e. UCLA3). In these cases, cells are converted in 5i/LAF medium and then switched to m5i/LAF medium after 5-6 passages in culture.
2. Cells at P5 in 5i/LAF medium are passaged onto a fresh layer of irradiated CF-1 MEFs at a 1:3.
3. The day after the cells are switched to m5i/LAF medium and passaged onto a fresh layer of irradiated CF-1 MEFs at a 1:2-1:3 ratio every 6-7 days.
4. For passaging, hESCs are treated with Accutase (Life technologies) for five minutes at 37C, dissociated into a single-cell suspension and resuspended in MEF medium. Cells are then filtered through a cell strainer (40 μM, Corning), centrifuged at 300*g* for 5 minutes and seeded on fresh irradiated MEFs in m5i/LAF medium.

5i/LAF to t2iLGöY transition:
1. For conversion of 5i/LAF adapted naïve cells in t2iLGöY conditions, naive hESCs grown in 5i/LAF medium at P>8 are passaged onto a fresh layer of irradiated CF-1 MEFs.
2. The day after the cells are switched to t2iLGöY medium and passaged onto a fresh layer of irradiated CF-1 MEFs at a 1:3 ratio every 6-7 days.
3. For passaging, hESCs are treated with Accutase (Life technologies) for five minutes at 37C, dissociated into a single-cell suspension and resuspended in MEF medium. Cells are then filtered through a cell strainer (40 mM, Corning), centrifuged at 300*g* for 5 minutes and seeded on fresh irradiated MEF in t2iLGöY medium.
NOTE: we have expanded the hESC in t2iLGöY for at least 4 passages. In addition, some hESC lines (i.e. UCLA17, UCLA3) cannot be adapted to the t2iLGöY while maintaining naïve pluripotency.

Timing
Depending on which cell line is used to generate naïve hESCs, it can take 2 to 4 months to have the cell lines established.

Troubleshooting
• Cell death during the conversion of primed hESCs to naive 5iLAF or m5i/LAF state is expected; dome- shaped colonies will arise after a few passages.
• If a large number of differentiated cells or primed-like cells are observed in the m5i/LAF condition, try to clean by picking undifferentiated colonies or by FACS sorting using a CD75 (naïve marker) and CD90 (primed marker) antibodies.
• If a large number of differentiated cells or primed-like cells are observed in the m5i/LAF condition, try to increase the concentration of PD03 for 1-2 passages.
• Make sure all media components are prepared and stored properly. Naive media are recommended to be stored at 4C for up to 7 days after preparation. We add the chemical compounds freshly every day.
• Change media on a daily basis and passage cultures as soon as they reach confluence.
• Do not passage the cells too densely or sparsely.

• Cells lines are usually stable after 5 passages in culture.
• We recommend to always coat the cell culture dishes with Matrigel before seeding the irradiated MEFs. This allow the irradiated MEFs to last for a longer time in culture in serum-free media.

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