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Pigmented human melanocyte differentiation, maintenance and BRAF V600E inhibition media

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Abstract

These are published or long-used protocols and media used in the culture of primary human melanocytes and their progenitors to favor melanogenesis. An example is provided of using a small molecule inhibitor of BRAF V600E. This is intended to be forked and improved by the community as needed.

Materials

- Neurobasal and Ham's F12 (ThermoFisher)
- B-27 without vitamin A
- N-2
- CHIR-99021 (TOCRIS)
- LDN-193189 (Miltenyi)
- SB 431542 (TOCRIS)
- EDN3 (American peptide)
- SCF (Peprotech)
- Human recombinant BMP4 (Peprotech)
- Ascorbic acid (Sigma-Aldrich)
- Gelatine 0.1% (Sigma-Aldrich)
- MGM-4 BulletKit (Lonza)
- Trypsin 0.05%
- Forskolin (Sigma-Aldrich)
- Penicillin-streptomycin
- Supplement mix
- Eppendorf tubes
- Falcon tube
- PLX4720 (BRAF V600E inhibitor)
- PBS
- ITS
- SVF
- Dexamethasone
- TPA
- bFGF
- MCDB201
- L-Acide Ascorbique
- DMEM
- F12
- P/S
- Wnt-3A
- Endothelin 3
- Matrigel (BD BioSciences)

Pour 100 ml de milieu :

- ITS: 1 ml de 100X (1X final)
- SVF: 1ml (1% final)
- Dexamethasone: 5µl de 1 mM (0.05µM)
- TPA: 5 µl de 1 mM (0.05 µM=50 nM)
- EDN3: 50 µl de 200 µM (100 nM)

- bFGF: 16 µl de 25 µg/ml (24 ng/ml final)
- MCDB201: 20 ml de 1X (20% final)
- L-Acide Ascorbique: 2 ml de 5 mM (0.1 mM)
- DMEM: 25 ml
- F12: 50 ml
- P/S: 1 ml

A ajouter extemporanément : pour 15 ml de milieu de différenciation

- 75.6 µl de SCF
- 7.5 µl de Wnt3A à 10 ng/µl (5 ng/ml final)

Préparation des composants du milieu :

- *L-acide ascorbique* (SIGMA)
  72.4 mg à reprendre dans 50 ml d'eau filtrée. Concentration : 5 mM. Conserver à + 4ºC.

- *Wnt-3A* (R26D SYSTEMS – 1324-WN)
  2 µg à reprendre dans 200 µl de PBS 1X. Concentration : 10 ng/µl. Conserver à – 20ºC.

- *Endothelin 3, human rat* (SIGMA E9137-1MG)
  Reprendre les 1 mg dans 1.89 ml d'eau filtrée. Concentration : 200 µM. Conserver à – 20ºC.

- *TPA (Phorbol 12-Myristate 13-acetate)* (SIGMA P8139-1MG)
  Reprendre les 1 mg dans 1.62 ml de DMSO. Concentration : 1mM. Conserver à – 20ºC.

- *MCDB201* (SIGMA)
  Reprendre les 1.77 mg dans 500 ml d'eau filtrée. Solution 2X. Le pH est acide. Ajuster ce pH de manière à ce qu'il soit semblable à celui du BGjb (pH=8) (ajouter environ 400 µl de NaOH 4M).Conserver à +4ºC.

- *SCF* (R26D SYSTEMS – 255-SC)

Safety warnings

- Be careful not to touch inside of lid when capping.
- Replace quickly on ice.
- Keep cold at all times (vortex 10 seconds, replace on ice to cool off again, repeat as necessary).

Pigmented melanocyte differentiation from multipotent progenitors

1 According to **Saidani et al. 2023**, neural crest induction can be performed by growing embryoid bodies from pluripotent stem cells (ES or human iPS) for four days in low-attachment dishes with Neural Medium (NM), which is:
- Neurobasal and Ham's F12 (ratio 1:1, ThermoFisher)
- 2% of [B-27 without vitamin A]
- 1% N-2

And supplemented with:
- CHIR-99021 (3 µM) (TOCRIS)
- LDN-193189 (0.2 µM) (Miltenyi)
- SB 431542 (40 µM) (TOCRIS)

2 On day 4, NM is supplemented with
- 3 µM CHIR99021
- 100 nM EDN3 (American peptide, Albuquerque, NM, USA)
- 50 ng/mL SCF (Peprotech, NJ, USA)
- 0.02 nM of human recombinant BMP4 (Peprotech)
- 0.3 mM ascorbic acid (Sigma-Aldrich).

3 From days 7 to 30, EBs are plated on fish or porcine skin gelatin 0.1% (Sigma-Aldrich). Medium changes every 2-3 days with MGM-4 BulletKit (Lonza), supplemented with
- 0.5 µM CHIR99021
- 100 nM EDN3
- 50 ng/mL SCF
- 0.02 nM of human recombinant BMP4
- 0.3 mM ascorbic acid.

4 Cells are dissociated using trypsin 0.05% and grown in the same medium supplemented with 20 µM forskolin (Sigma-Aldrich) during 2 passages to induce pigmentation.

5 For passage 2, MGM-4 medium can be used without any supplements to culture the pigmented melanocytes. Cells were passaged at least 20 times.

Alternate melanocyte culture media

6 Reconstitute M1 « Melanocyte Growth Medium » C-24010 Promocell and add 5 ml penicillin-streptomycin to bottle using sterile technique.
Cap bottle and swirl to mix. Do not get medium into the cap.
Remove 49 mL to a 50 mL Falcon tube.
Put the rest at 4°C for long-term storage in refrigerator.

7 Add 1.22 mL (twice 610 µL) of the Supplement mix into the 50 mL Falcon tube provided. Close.

8 Aliquot the remaining Supplement Mix by 1.22 mL aliquots into 9 sterile Eppendorf tubes .
Be careful not to touch inside of lid when capping.
Replace quickly on ice.
Add colored stickers “M1” to tops as well as the white stickers for the sides.
Bring these back to the laboratory when finished for long-term storage.

9 Now open the 50 mL M1 Falcon tube and remove 15 mL into each of the three tubes provided, keeping them upright so the edge is not wet.
Cap these "M1", "MSF" and "MSFB" tubes in the hood for now.
There will be 5 mL left in your 50 mL M1 tube that you will use to dilute other things and then to make up a medium later to inactivate your trypsin.

10 Reconstitute the 20 µg SCF with 0.4 mL (400 µL) from that 50 mL tube directly into tube of powder. Vortex to dissolve to a concentration of 50 ng/µL.

11 Keep cold at all times (vortex 10 seconds, replace on ice to cool off again, repeat as necessary).

12 When entirely dissolved and transparent, add 30 µL SCF stock to (only) the 15 mL "MSF" and "MSFBi" tubes for a final concentration of 100 ng/mL SCF (Kit ligand).

13 Aliquot the remaining 340 µL by 30 µL into sterile Eppendorf tubes.
Be careful not to touch inside of lid when capping.
Replace quickly on ice.
Add colored stickers “SCF” to top.
Bring these also back to the laboratory for long-term storage.

14 Add 15 µL forskolin stock (10 mM) to the "MSF" and "MSFBi" 15 mL tubes for a 10 µM final concentration. Cap them and vortex. Keep at 4°C.

15 90 µL M1 medium from your leftovers in the 50 mL tube to the PLX4720 (**BRAF V600E inhibitor**, 100 mM stock) aliquot, to dilute it 1/10. Mix with pipette.

16 Add to the "MSFBi" tube alone, 3 µL of this diluted PLX4720 aliquot to 2 µM final concentration. Cap and vortex. Keep the "MSFBi" medium at 4°C. Throw away the rest of the diluted inhibitor.

17 Filter-sterilize 10 mL of serum with a syringe into the 50 mL tube to make up a medium to inactivate the trypsin. Add PBS to complete to 50 mL total. Cap and keep at 4°C.

Alternate culture/differentiation protocol (2009)

18 **For 100 mL medium :**
ITS (insulin-transferrin-selenium supplement): 1 ml of 100X (1X final)
fetal calf serum : 1 ml (1% final)
Dexamethasone: 5 µl at 1 mM (0.05 µM final)
TPA (12-O-Tetradecanoylphorbol-13-acetate, a phorbol ester): 5 µl at 1 mM (0.05 µM = 50 nM final)
EDN3 (endothelin-3, EDNRB agonist) : 50 µl at 200 µM (100 nM final)
FGF2: 16 µl at 25 µg/ml (24 ng/ml final)
MCDB-201: 20 mL at 1X (20% final)
L-ascorbic acid, protected from light and oxygen: 2 ml at 5 mM (0.1 mM final)
DMEM: 25 mL
F12: 50 mL
penicillin/streptomycin 100X: 1 ml

19 Add at the last minute to 15 mL of the above medium
75.6 µl SCF at (50 ng/µL?) stock concentration
7.5 µl Wnt3A at 10 ng/µl stock concentration (5 ng/ml final)

20 Preparing adjuvants to above medium :
L-ascorbic acid (SIGMA) 72.4 mg into 50 ml sterile water. Concentration : 5 mM. Can keep at + 4ºC but better at -20°C in full tubes with little air and protected from light. 2-AAGP is more stable.
Wnt-3A (R&D SYSTEMS – 1324-WN) 2 µg to resuspend in 200 µl de PBS 1X. Concentration : 10 ng/µl. Keep at – 20ºC.
Endothelin 3, human or rat (SIGMA E9137-1MG) Resuspend 1 mg in 1.89 ml sterile water to a concentration : 200 µM. Keep aliquots at – 20ºC.
TPA (Phorbol 12-Myristate 13-acetate) (SIGMA P8139-1MG) Resuspend 1 mg in 1.62 ml de DMSO. Concentration : 1 mM. Keep aliquots at – 20ºC.
MCDB-201 (SIGMA) Resuspend 1.77 mg (all?) powder in 500 ml culture-grade sterile water for a 2X solution 2X, which is acid. Adjust pH to resemble BGjb (no more basic than pH=8, perhaps 7.4 is better) by adding about 400 µl NaOH 4M). Keep at +4ºC and complete to 1L before use.
SCF - stem cell factor or Kit ligand (R26D SYSTEMS – 255-SC)

21 Change medium every other day for three weeks. Use **TrypLE** or dilute trypsin in EDTA (0.05% trypsin rather than 0.25%) when splitting (1:2) is necessary, and restore cells to an acidic or at least neutral medium as soon as possible to maintain viability. Cells should touch or nearly, but not be crowded in dish.

Preparing Matrigel/Geltrex-conditioned substrate on plates

22 Matrigel: BD BioSciences. Work with solutions in an ice bucket in the hood. Dilute Matrigel in medium without serum. It is acid. Leave to coat wells at room temperature or better at 37°C for an hour. Rinse with serum-free medium or PBS. It is possible to leave in wells and store at +4°C, keeping for up to a week.