Differentiation of Dendritic Cells from Human Pluripotent Stem Cells in the Absence of Animal Products

Fairchild Laboratory

Sir William Dunn School of Pathology, South Parks Road, Oxford, OX1 3RE

1. Materials

1.1 Coating tissue culture plates with matrigel matrix

1. Matrigel (BD Biosciences) thawed on ice
2. Ice-cold knock-out Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen)
3. 50 ml centrifuge tubes on ice
4. Culture vessels to be coated with matrigel

1.2 Culture of human ES cells and routine passage/harvesting

1. Warm XVIVO-10 medium without gentamycin or phenol red (Lonza)
2. Medium for culture of hESC: XVIVO-10 supplemented with 80 ng/ml recombinant human basic fibroblast growth factor (rhbFGF) (R&D Systems) and 0.5 ng/ml recombinant human transforming growth factor-β (rhTGF-β) (R&D Systems)
3. Warm collagenase IV (Invitrogen)

4. Room temperature tissue culture grade PBS (Invitrogen)
5. Cell scrapers or 5 ml pipettes, depending on the culture vessels used
6. Culture vessels coated with matrigel

1.3 Counting hPSC

1. Warm collagenase
2. PBS at room temperature
3. Tryple Express (Gibco) at room temperature
4. Medium containing 10% FCS (any medium suitable for cell culture may be used)

1.4 Differentiation of PSC into dendritic cells and feeding of differentiation cultures

1. Culture medium for the differentiation of hPSC into DC consists of room temperature XVIVO-15 (with phenol red and gentamycin) (Lonza) and is supplemented with the following:

    a) 1 mM Sodium Pyruvate (PAA Laboratories GmbH)

    b) 1 x non-essential amino acids (PAA Laboratories GmbH)

    c) 2 mM L-glutamine (PAA Laboratories GmbH)

    d) 5 x 10^{-5} M 2-mercaptoethanol (Sigma)

    e) Recombinant human bone morphogenetic protein-4 (rhBMP-4) (R&D Systems) to give a final concentration of 50 ng/ml; 50 ng/ml recombinant human vascular endothelial growth factor (rhVEGF) (R&D Systems); 20 ng/ml recombinant human stem cell factor (rhSCF) (R&D Systems); and 50 ng/ml recombinant human granulocyte macrophage-colony stimulating factor

(rhGM-CSF) (R&D Systems). On day 5, BMP-4 is removed from feeding medium, followed by VEGF on day 10 and SCF on day 15.

2. XVIVO-10 medium at room temperature
3. Tissue culture-grade PBS at room temperature
4. Warm collagenase
5. Cell scrapers or 5 ml pipettes

1.5 Differentiation of monocytes into immature DCs (iDCs)

1. Room temperature XVIVO-15 supplemented with 50 ng/ml rhGM-CSF and 100 ng/ml recombinant human interleukin-4 (rhIL-4) (R&D Systems).
2. Trypan blue
3. PBS
4. 70 μm cell strainers

1.6 Maturation of DC differentiated from hPSC

1. Recombinant human interferon-γ (rhIFN-γ) (R&D Systems)
2. Prostaglandin E_2 (PGE_2) (Sigma)
3. Recombinant human tumour necrosis factor-α (rhTNF-α) (R&D Systems)
4. Recombinant human interleukin-1β (rhIL-1β) (R&D Systems)
5. rhGM-CSF

2. Methods

2.1 Culture of human PSC

1. Human PSC lines can be cultured using different methods. We have adopted feeder and serum-free conditions for their culture ***(1, 2)*** (*see* **fig 1**). Defined

culture conditions are more reliable, have important implications in down-stream clinical studies, facilitate scale-up of cultures and avoid the time required to maintain feeders.

2. hPSC are cultured in XVIVO-10 medium supplemented with 80 ng/ml bFGF and 0.5 ng/ml TGF-β on a matrigel matrix. XVIVO-10 medium is first warmed before adding bFGF and TGF-β (*see* **Note 1**).
3. A complete change of medium is performed daily except on the day immediately following either passage of the cells or thawing.

2.2 Coating tissue culture plates with matrigel matrix

1. Culture vessels coated with matrigel matrix need to be prepared in advance of passaging hPSC. To a 10ml vial of phenol red-free, growth factor-reduced matrigel, add 10 ml ice cold KO-DMEM. Keep matrigel on ice and work quickly. Be careful not to introduce excess bubbles. The diluted matrigel can be aliquoted and stored at -20ºC.
2. It is important to avoid the generation of bubbles when handling matrigel to prevent uneven coating of the tissue culture surface. If matrigel starts to warm, it gels very quickly; for this reason it is best to work with matrigel on ice and keep KO-DMEM ice-cold.
3. Thaw aliquotes of matrigel on ice. Transfer matrigel from aliquots into sterile 50ml centrifuge tubes. To each 1-3ml of matrigel, add 5 ml KO-DMEM medium using a 5 ml pipette and mix thoroughly. Top up with medium to give a final volume of 15 ml per 1 ml of thawed matrigel (includes initial volume of thawed matrigel and volume of medium used to mix). The final dilution of matrigel is 1:30.

4. A 6-well tissue culture plate can be coated with 1 ml per well of diluted matrigel, a T25 with 3 ml and a T75 with 10ml. Calculate the volume required according to the surface area of other culture vessels using these volumes as a guide. Tap the sides of culture vessels to distribute the matrigel evenly over the surface.
5. Vessels can be coated by leaving them at room temperature for at least one hour or stored immediately at 4°C. Use parafilm over the tops of vented TC flasks and seal TC plates with micropore tape and wrap in cling-film to prevent evaporation. Discard culture vessels if medium is no longer covering the entire surface.

2.3 Routine passage of hPSC/harvesting hPSC

1. In our hands, using xeno-free culture conditions, hPSC can be routinely passaged every 4-6 days. hPSC are passaged as clusters of cells using collagenase to lift colonies off the tissue culture surface and scraping with a cell scraper or 5 ml pipette.
2. Using the same counting method as that used to estimate the number of hPSCs (*see* **2.4**), cells can be seeded at a density of approximately 1×10^5 cells per cm^2. In practice, once culture vessels reach 50% confluency, cells can be passaged at a 1:5 dilution (*see* **Notes 2 and 3**).
3. If hPSC are being expanded, the volume of culture medium required can be prepared in advance and hPSC passaged using supplemented XVIVO-10 medium. If hPSC are being maintained then the volume of unsupplemented room temperature XVIVO-10 used to passage hPSC can be subtracted from the final volume required; TGF-β and bFGF can be added to this amount to give the correct final concentration for culture and used to top up the suspension of harvested hPSC clusters.

4. Remove culture medium from hPSC cultures and incubate with pre-warmed collagenase at 37ºC for the time period calculated while counting hPSC. Alternatively, if using a pre-determined dilution, observe cultures after 4-7 minutes: when the majority of stromal cells have lifted off the tissue culture surface and hPSC colonies are beginning to round at the edges, immediately remove collagenase. Wash gently with PBS, being careful not to scrape off colonies with the pipette.
5. With XVIVO-10 medium, cover the tissue culture surface and gently scrape off hPSC colonies. A 5 ml pipette can be used to scrape the surface of wells from a 6-well plate or a cell scraper for flasks. It is critical to maintain clusters of hPSC and prevent generation of a single cell suspension that will result in loss of viability.
6. Using a 5 ml pipette, generate a suspension of hPSC clusters.
7. Top up the suspension of hPSC clusters to give the correct final volume and concentration of bFGF and TGF-β necessary for culture and pipette into matrigel coated culture vessels (remove matrigel immediately before adding hPSC, there is no need to rinse the culture vessel first).
8. Gently rock the culture vessel backwards and forwards and side to side to distribute clusters of hPSC evenly over the tissue culture surface. Incubate at 37ºC, 5% CO_2 in a humidified atmosphere (*see* **Note 5**).

2.4 Counting hPSC

1. In order to plate hPSC at the correct density in differentiation cultures, it is important to first count the number of hPSC.

2. Remove culture medium from one representative flask or well. Add warm collagenase so that the surface is covered and incubate at 37°C for 4-7 minutes. When the majority of stromal cells have lifted off the surface and colonies of hPSC are beginning to round up at the edges, immediately remove collagenase, and gently wash with PBS (*see* **2.3.4**). Record the time of collagenase incubation as this will be required later.
3. Add room temperature tryple express and incubate at 37°C. After 5 minutes, shake the culture vessel in quick, sharp motions to dislodge all the hPSC colonies. Pipette to create a single cell suspension and wash any remaining cells from the tissue culture surface. Quickly add the cell suspension to the same volume of medium containing 10% FCS to give a 1:2 dilution and pipette further if necessary.
4. The cells are counted without trypan blue exclusion to monitor viability, as trypsin treatment and producing a single cell suspension will generate significant cell death.
5. Trypsin treated hPSC can be stained for Oct-4, SSEA-4 and Tra-1-60 and analysed by flow cytometry to monitor their pluripotency.

2.5 Directed differentiation of PSC into dendritic cells

1. After counting hPSC, it is now possible to calculate how many wells can be set up for differentiation culture and the volume of medium required (*see* **Note 5**). Cells are plated at 3 x 10^6 cells per well of a 6-well plate. Prepare XVIVO-15 medium supplemented with BMP-4, VEGF, SCF and GM-CSF. These growth factors are successively removed from the differentiation culture leaving only GM-CSF in the final feed. Prepare sufficient medium to set up the differentiation culture and

for the first feed (6 ml per well). XVIVO-10, XVIVO-15 and PBS should be at room temperature.

2. Harvest hPSC cultures using collagenase treatment for the time period calculated when counting hPSC.
3. Wash cells gently using PBS as before. Add sufficient XVIVO-10 medium to cover the culture vessel surface and gently scrape off colonies, being careful not to create a single cell suspension (*see* **2.3.5**).
4. Wash the surface of culture vessels with XVIVO-10 medium to ensure all hPSC colonies have been removed.
5. Pool colonies and allow them to settle for 10-20 mins at the bottom of a 50 ml centrifuge tube or other suitable tissue culture tube.
6. Gently remove medium without disturbing the loose pellet of colonies. Add some of the prepared differentiation medium, a similar volume or less than that used to harvest the colonies, is ideal. Using a 5 ml pipette, create a suspension of cell clusters.
7. Calculate the volume of differentiation medium required to set up the determined number of wells. Use 4 ml of medium per well of a 6-well plate. Dilute cell clusters to give this final volume.
8. Due to the nature of differentiation, variation is often observed between cultures. For this reason, it is important to be as accurate as possible when pipetting the correct number of cells per well. Use a 10 ml pipette to prevent further breakdown of cell clusters and keep mixing the suspension as you pipette. It is best to aspirate and dispense 4 ml per well of the suspension for each individual well, in order to distribute cell clusters as accurately as possible. Pipette into ultra low attachment 6-well plates.

9. Seal 6-well plates with micropore tape and incubate in a humidified 37ºC incubator at 5% CO_2.

2.6 Feeding differentiation cultures

1. Cultures of hPSC differentiating into DC need to be fed every 2-3 days. This is particularly important during the early stages of differentiation. In practice, cells can be fed on Mondays, Wednesdays and Fridays. Every 5 days, a growth factor is removed from the differentiation medium until only GM-CSF remains. Concentrations of the added growth factors are therefore effectively diluted throughout the course of the experiment. BMP-4 is removed from the differentiation culture first, followed by VEGF and then SCF.
2. For the first feed, warm XVIVO-15 containing BMP-4, VEGF, SCF and GM-CSF and top-up wells with an extra 2 ml, giving a final volume of 6 ml per well. For successive feeds, gently remove either 2 ml or 3 ml of culture medium using a 10ml pipette, being careful not to remove cells or at later stages of culture the embryoid bodies (EBs) that spontaneously form (*see* **Note 6**). Replace with warm medium containing the appropriate growth factors.
3. Differentiation cultures contain significant debris during the early stages of differentiation due to high levels of cell death, a normal process during differentiation, and due to the inability of intermediates to adhere to the ultra-low attachment (ULA) surface. Around days 14-19 of culture, small, round, non-adherent haematopoietic cells should start to appear and later to accumulate. From day 19 onwards, “monocyte-like” cells should become apparent. These cells look morphologically like human blood monocytes and express high levels

of CD14 as well as other myeloid markers, such as CD11b. Cells with monocytic morphology build up in number towards later stages of culture.

4. Monocytes are usually ready to be harvested for DC differentiation between days 30-35. The cultures can be monitored for the appearance of monocytes and their percentages assessed using CD14 expression as determined by flow cytometry.

2.7 Differentiation of monocytes into iDC

1. Monocytes are harvested by gently pipetting cultures using a pipetter set on slow and a 10 ml pipette. The aim is to remove monocytes that are non-adherent while leaving any adherent macrophages in the culture plate. Transfer cells into 50 ml centrifuge tubes. EBs can be left to settle at the bottom of the tube (approximately 2-5 mins) and removed using a pipette before cells also start to pellet.
2. Once EBs have been removed, the cell suspension can be passed through a 70 μm cell strainer. This excludes any large clumps of cell debris (mostly created by EBs breaking up). Rinse the cell strainer with PBS.
3. Monocytes can then be washed by centrifuging at 1200 rpm for 5 mins at 4ºC. Discard the cell supernatant and resuspend cells in XVIVO-15 medium supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4.
4. Pipette 1-1.5 x 10^6 monocytes per well of a 6-well ULA plate and incubate for 6-8 days to differentiate monocytes into iDC (*see* **Note 7**) (*see* **fig 2**).

2.8 Maturation of DC differentiated from hESC

1. iDC differentiated from hPSC can be matured using a cocktail of cytokines including TNFα, IFNγ, PGE_2 and IL-1β. This can be made up in medium already

supplemented with GM-CSF and IL-4 and added to cultures of iDC for the last 48 hours. Alternatively IL-4 can be removed by washing the cells and replacing media with the maturation cocktail (GM-CSF must be included throughout) (*see* **Notes 8 and 9**).

3. Notes

1. Human bFGF is extremely heat labile and therefore addition to pre-warmed media instead of warming media that has already been supplemented with bFGF prolongs its half-life in culture.
2. When cells are initially thawed and are therefore more fragile, dilutions of 1:3 or 1:4 can be used, depending on the appearance and recovery of the cells.
3. Under these culture conditions, fibroblast-like cells differentiated from hPSC are normally seen. Evidence has shown that these fibroblast-like cells support the pluripotent growth of hPSC however, it is also necessary to prevent them from overwhelming the cultures.
4. It is best not to move the cultures for 48 hours to allow hPSC to adhere to the matrigel surface.
5. It is important to note that in our experience it becomes increasingly difficult to direct cells along a haematopoietic route when hPSC have been cultured for more than 40 passages, although DCs have been generated from hPSC at higher passages.
6. EBs become cystic and therefore have a tendency to float, making it particularly difficult to avoid aspirating them with the pipette. Haematopoietic cells can often be found inside these cystic EBs when examining the cultures under a microscope, so they are highly likely to be a source of haematopoietic cells in the

differentiation cultures. It is, therefore, important to avoid losing these EBs as much as possible when routinely feeding cultures.

7. Some haematopoietic precursor cells may persist in cultures. By counting the large cells using a haemocytometer, it is possible to distinguish between monocytes and precursor cells when setting up cultures.
8. We have found that DC differentiated from hPSC express very low levels of TLR4, responding poorly to LPS and therefore the maturation cocktail is preferred for DC maturation.
9. In our hands, hPSC-derived DC already produce IL-6; for this reason IL-6 is not included in the maturation cocktail.

References

1. Li, Y., Powell, S., Brunette, E., Lebkowski, J., and Mandalam, R. (2005) Expansion of human embryonic stem cells in defined serum-free medium devoid of animal-derived products. *Biotechnol Bioeng* ***91***, 688-98.

2. Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., and Carpenter, M. K. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat Biotechnol* ***19***, 971-4.

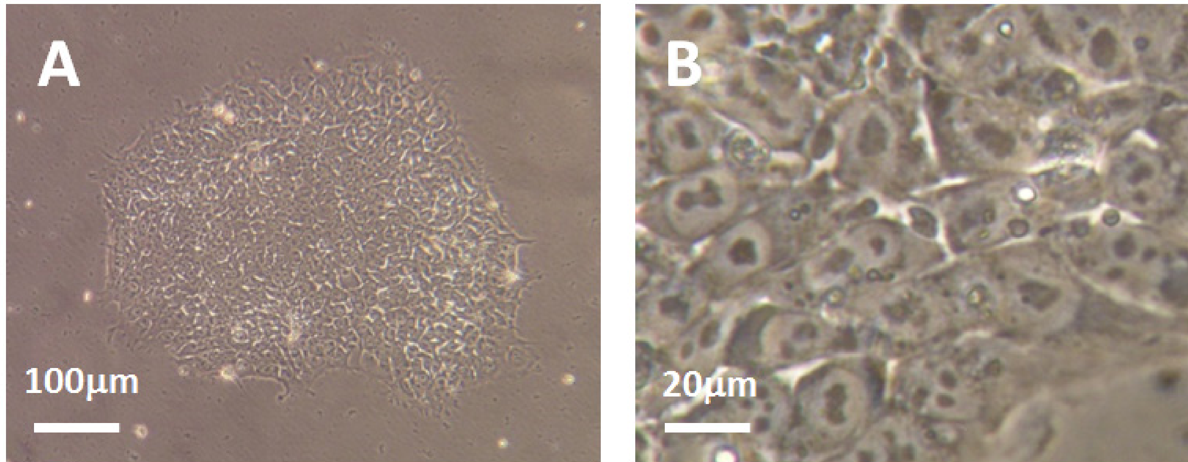

Figure 1. A typical hPSC colony cultured under the feeder and serum-free conditions (A). Human PSCs have clear borders and at higher magnifications the cells can be seen to have a high-nucleus to cytoplasm ratio (B).

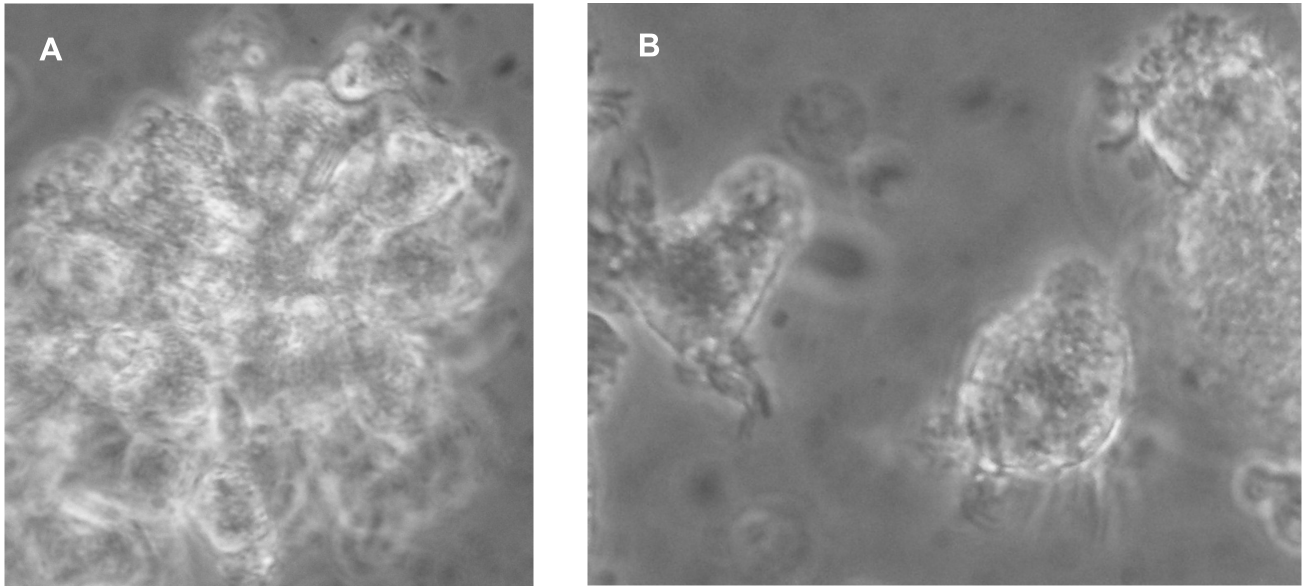

Figure 2. Immature DCs differentiated from hPSCs. a) immature DCs frequently form tight clusters of cells. Typical veils of cytoplasm can be seen on DCs at the edge of the cluster. b) Immature DC morphology showing veils of cytoplasm characteristic of DCs. Objective magnification: x 40.