Video Article

# Feeder-free Derivation of Melanocytes from Human Pluripotent Stem Cells

Scott J. Callahan[1,2], Yvonne Mica[3], Lorenz Studer[1]

[1]The Center for Stem Cell Biology, Developmental Biology Program, Memorial Sloan-Kettering Cancer Center

[2]Cancer Biology and Genetics Program, Gerstner Sloan-Kettering Graduate School, Sloan-Kettering Institute for Cancer Research

[3]Thermo Fisher Scientific

Correspondence to: Lorenz Studer at studerl@MSKCC.ORG

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## Abstract

Human pluripotent stem cells (hPSCs) represent a platform to study human development *in vitro* under both normal and disease conditions. Researchers can direct the differentiation of hPSCs into the cell type of interest by manipulating the culture conditions to recapitulate signals seen during development. One such cell type is the melanocyte, a pigment-producing cell of neural crest (NC) origin responsible for protecting the skin against UV irradiation. This protocol presents an extension of a currently available *in vitro* Neural Crest differentiation protocol from hPSCs to further differentiate NC into fully pigmented melanocytes. Melanocyte precursors can be enriched from the Neural Crest protocol via a timed exposure to activators of WNT, BMP, and EDN3 signaling under dual-SMAD-inhibition conditions. The resultant melanocyte precursors are then purified and matured into fully pigmented melanocytes by culture in a selective medium. The resultant melanocytes are fully pigmented and stain appropriately for proteins characteristic of mature melanocytes.

## Video Link

The video component of this article can be found at https://www.jove.com/video/53806/

## Introduction

Human pluripotent stem cells (hPSCs) provide a platform to mimic normal differentiation in a scalable fashion for disease modeling, drug screening, and cell replacement therapies [1-6]. Of particular interest, hPSCs open up avenues for studying difficult to isolate or rare/transient cell types where patient samples are scarce. Furthermore, induced pluripotent stem cells (iPSCs) enable researchers to study development and disease modeling in a patient specific manner to unravel unique mechanisms [1,2,7-11]. The previously published protocol for differentiation of melanocytes from hPSCs requires up to 6 weeks of differentiations and involves culturing cells with conditioned medium from L-Wnt3a cells [12]. The protocol first presented by Mica *et al.* and described here produces pigmented cells in three weeks and removes the ambiguity and inconsistencies associated with conditioned medium.

Melanocytes are derived from the neural crest, a migratory population of cells unique to vertebrates. The neural crest is defined during gastrulation and represents a population of cells at the edge of the neural plate, bordering between the neural and non-neural ectoderm. During neurulation, the nervous tissue evolves from a neural plate to form neural folds, which converge at the dorsal midline resulting in the neural tube [13,14].

The neural crest cells emerge from the roof plate of the neural tube, opposite the notochord, and undergo an epithelial to mesenchymal transition before migrating away to give rise to a diverse population of differentiated cells. The fates of the crest cells are defined in part by the anatomic location of the roof plate along the body axis of the embryo. Neural crest cell derivatives include lineages characteristic of both mesoderm (smooth muscle cells, osteoblasts, adipocytes, chondrocytes) and ectoderm cells (melanocytes, Schwann cells, neurons) [14]. Neural crest stem cells upregulate the transcription factor SOX10 and can be isolated by fluorescence-activated cell sorting with antibodies to p75 and HNK1.

The neural crest cells fated to become melanocytes pass through a melanoblast stage and upregulate KIT and MITF (microphthalmia-associated transcription factor) [6,21] MITF is a master regulator of melanocyte development and is a transcription factor responsible for controlling much of melanocyte development [22-24]. Human melanoblasts migrate to the basal layer of the epidermis where they reside either in the hair bulge or surrounded by keratinocytes in the epidermis (forming pigmentation units) to serve as precursors to the mature, pigmented melanocytes. The differentiation and maturation of melanoblasts into pigmented melanocytes occurs concomitant with colonization of the hair bulb and expression of the melanin production pathway (TYRP1, TYR, OCA2 and PMEL) [25,26].

Isolating human melanocytes and melanoblasts from patients is expensive, difficult and limiting in quantity. This protocol enables researchers to differentiate hPSCs (induced or embryonic) into melanocytes or melanocyte precursors in a well defined, rapid, reproducible, scalable, and

 

inexpensive method without cell sorting. The protocol was used previously to identify disease-specific defects when differentiating iPSCs from patients with pigmentation disorders .

## Protocol

NOTE: The melanocyte protocol outlined here was first demonstrated by Mica et al.

# 1. Preparation of Culture Medium, Coated Dishes and Maintenance of hPSCs

1. Medium Preparation
   Note: Store all medium at 4 °C in the dark for up to 2 weeks. Filter all medium for sterilization.
   1. Prepare DMEM/10% FBS. Mix 885 ml DMEM, 100 ml FBS, 10 ml Pen/Strep and 5 ml L-Glutamine. Filter for sterilization.
   2. Prepare hESC-medium. Mix 800 ml DMEM/F12, 200 ml KSR, 5 ml L-Glutamine, 10 ml MEM minimum essential amino acids solution, 1 ml β-Mercaptoethanol, and 5 ml Pen/Strep. After filtering add 10 ng/ml FGF-2.
   3. Prepare KSR-differentiation medium: Mix 820 ml Knockout DMEM, 150 ml KSR, 10 ml L-Glutamine, 10 ml Pen/Strep, 10 ml MEM minimum essential amino acids solution and 1 ml β-Mercaptoethanol. Filter for sterilization.
   4. Prepare N2-differentiation medium. Dissolve 12 g DMEM/F12 powder in 980 ml $dH_2O$. Add 1.55 g glucose, 2 g sodium bicarbonate and 100 mg apo human transferrin. Mix 2 ml $dH_2O$ with 25 mg human insulin and 40 µl 1 N NaOH; once dissolved, add the mixture to the medium. Add 100 µl putrescine dihydrochloride, 60 µl selenite, 100 µl progesterone. Bring the final volume to 1 l with $dH_2O$ before filtering.
   5. Prepare Full melanocyte medium. Combine 50% Neurobasal medium, 30% Low glucose DMEM, and 20% MCDB201. To this add: 0.8% ITS+, 250 nM L-glutamine, 100 µM Ascorbic Acid (L-AA), 50 ng/ml Cholera toxin, 50 ng/ml SCF, 0.05 µM Dexamethasone, 100 nM EDN3, 4 ng/ml FGF2. Sterile filter then add remaining reagents: 2% B27 Supplement, 25 ng/ml BMP4, 3 µM CHIR99021, 500 µM cAMP.

2. Coating of Culture Dishes
   1. Carry out coating using gelatinous protein such as Matrigel. Upon opening, aliquot and freeze Matrigel into 1 ml parts to avoid repetitive freeze thaw cycles. Thaw and re-suspend a 1 ml frozen aliquot with 19 ml DMEM/F12. Plate 5 ml onto a 10 cm dish. Incubate the dishes for 1 hr at RT. Aspirate the gelatinous protein immediately before plating the cells and wash with DMEM/F12.
   2. Carry out coating using Poly-L ornithin hydrobromide/ mouse Laminin-I/ fibronectin (PO/Lam/FN). Coat dish with PBS containing 15 µg/ml Poly-L ornithin hydrobromide. Incubate O/N at 37 °C in a humidified incubator. Aspirate the solution and wash the plates with PBS three times before coating with PBS containing 1 µg/ml mouse Laminin-I and 2 µg/ml fibronectin. Incubate the dishes O/N at 37 °C in a humidified incubator.
      1. Prior to plating cells, aspirate the solution and let the plates dry thoroughly without the lid in the tissue culture hood. Allow the plates to dry for approximately 10-15 min.
         Note: The dishes are dry and ready for cell plating when crystal structures appear on the surface by eye. The plates can be kept with PBS containing LAM/FN in the incubator for two weeks, as long as the liquid does not evaporate. The plates can be kept dried at RT for a few hr.

3. Maintenance of hPSCs
   Note: hPSCs are maintained on 0.1% gelatin and mitotically inactivated mouse embryonic fibroblasts (MEFs) in hESC-medium. The cells should be split every 6-8 days.
   1. Coat a 10 cm dish with 0.1% gelatin in PBS at RT for 5 min.
   2. Thaw frozen MEFs quickly in a 37 °C water bath.
   3. Aspirate the gelatin and plate the cells. Plate MEFs at a density of ~50,000 cells/$cm^2$ in DMEM/10% FBS. Incubate MEFs at 37 °C O/N prior to adding hPSCs.
   4. Aspirate the DMEM/10% FBS from the prepared MEF plate, wash plate with PBS and add 10 ml hESC-medium with hPSCs. Passage the hPSCs at a ratio of 1:5/10 depending on density prior to passaging.
      Note: If the hPSCs are being thawed or passaged as single cells, supplement with 10 µM Y-27632 dihydrochloride (ROCKi) until the first passage.
   5. Feed cells daily with fresh 10 ml hESC-medium.
   6. Prior to starting a differentiation remove any pluripotent colonies that appear to contain differentiated cells, irregular borders, or transparent centers [28]. Mechanically dislodge and aspirate the irregular colonies with a pipette under a laminar flow hood with a dissecting microscope.

# 2. Plating of hPSCs for Differentiation

Note: Differentiation conditions are described for 10 cm dishes.

1. Prepare 10 cm Matrigel dishes before starting the differentiation as described in Step 1.3.1.
2. When plating hPSCs for a differentiation start with a plate of hPSCs at a density ready for passage (~80% confluent) aspirate the hESC-medium, wash with PBS and add 3 ml of 0.05% trypsin-EDTA to the cells.
3. Vigorously shake the dish horizontally for 2 min while visualizing under the microscope until the MEFs lift off as single cells. The hPSC colonies should remain attached as colonies.
4. Aspirate the trypsin after the MEFs have lifted but before the hPSCs colonies detach.

5. Add 10 ml of hESC-medium containing 10 µM Y-27632 dihydrochloride to the plate and detach the cells by pipetting up and down over the colonies.
6. Aspirate the Matrigel solution from the 10 cm dish prepared in step 2.1 and wash with DMEM/F12 to remove clumps. Plate the hPSCs at a 1:2 ratio onto the Matrigel plate. Add hESC-medium containing 10 µM Y-27632 dihydrochloride up to 10ml. Incubate at 37 °C O/N.
   Note: This plating should result in approximately 100,000 cells/$cm^2$.

## 3. Induction of Neural Differentiation

Note: The differentiation should be initiated (day 0) when the hPSCs are 80% confluent. The cells can be fed daily with hESC-medium containing 10 µM Y-27632 dihydrochloride until beginning the differentiation.

1. On day 0 and day 1, feed the cells with 10 ml of KSR-differentiation medium containing 100 nM LDN193189 and 10 µM SB431542.
2. On day 2, feed the cells with 10 ml of KSR-differentiation medium containing 100 nM LDN193189, 10 µM SB431542 and 3 µM CHIR99021.
3. On day 3, feed the cells with 10 ml of KSR-differentiation medium containing 10 µM SB431542 and 3 µM CHIR99021.
4. On days 4 and 5 feed the cells with 15 ml of 75% KSR-differentiation medium and 25% N2-differentiation medium containing 3 µM CHIR99021.
   Note: Do not be alarmed to see a large amount of cell death in terms of floating cells. This is normal and to be expected.
5. On days 6 and 7 feed the cells with 15 ml of 50% KSR-differentiation medium and 50% N2-differentiation medium both containing 3 µM CHIR99021, 25 ng/ml BMP4, and 100 nM EDN3.
6. On days 8 and 9 feed the cells with 20 ml of 25% KSR-differentiation medium and 75% N2-differentiation medium both containing 3 µM CHIR99021, 25 ng/ml BMP4, and 100 nM EDN3.
7. On days 9 and 10 prepare PO/Lam/FN dishes as indicated in 1.2.2 for re-plating of the cells on day 11.
8. On day 10 feed cells with 20 ml of N2-differentiation medium containing 3 µM CHIR99021, 25 ng/ml BMP4, and 100 nM EDN3.

## 4. Replating in Droplets for NC Specification

1. On day 11 aspirate Lam/FN from the prepared PO/LAM/FN plates and dry completely.
2. Remove medium from the Day 11 cells, wash with PBS and add 4 ml cell detachment solution such as Accutase per 10 cm dish. Incubate for 25 min at 37 °C.
3. Add 5 ml of Full Melanocyte medium to the dish and resuspend the cells by manually pipetting up and down with a 10 ml pipette until all the cells have lifted off the plate. Transfer the suspension to a 15 ml tube.
4. Spin the cells down for 5 min at 200 x g and resuspend the cells at 2 x $10^6$ cells per ml in Full Melanocyte medium. Count the cells using the trypan blue exclusion on a hemocytometer or equivalent technique.
5. Plate 10 µl droplets close to each other (without them touching) onto the dried PO/Lam/FN 10 cm dishes. If the plate has been sufficiently dried, the droplets should have well defined edges and not run.
   Note: This creates a high density local environment for the cells, which is important when replating the cells, while maintaining room within the dish for expansion.
6. Allow the droplets to stand at RT for 10-20 min to allow cells to adhere, then slowly (not to disturb the attached cells) add 10 ml of Full Melanocyte medium. Move dish to the incubator.

## 5. Expanding Melanocyte Progenitors

1. Continue feeding with Full Melanocyte medium every 2 to 3 days.
   Note: Pigmentation should start to be visible within clusters of cells by the end of the first week and become very clear by the second week. View the plate over a white background such as a sheet of paper to discern these early small, dark clusters. Cells will become progressively more pigmented over time, until the entire plate is uniformly pigmented (See **Figure 2B**).
2. Passage cells once a week at a ratio of ~1:6 (plating as droplets is no longer necessary at this stage). Use Accutase to dissociate cells and wash twice with plain Neurobasal medium before re-plating in Full Melanocyte medium. Maintain cells on PO/LAM/FN plates.
   Note: We have found that the Full Melanocyte medium is best suited for maintaining and expanding hESC and iPSC-derived melanocytes. However, cells can be briefly cultured in commercially available M254 medium but the simplified media increases the risk of cells dying and lifting off the plate.

## Representative Results

This protocol provides a method for deriving fully pigmented, mature melanocytes from hPSCs in an *in vitro* feeder-free, cost efficient, and reproducible manner. In contrast to the previously established Fang *et al.* protocol for hPSC-derived melanocytes, the outlined protocol does not require conditioned medium and decreases the time requirement. The Fang *et al.* protocol utilized conditioned medium from a WNT3A-producing murine cell line and took up to 6 weeks to visualize pigmentation [6,12]. To bias the neural crest stem cells towards a melanocyte fate (melanoblasts), we remove the BMP and TGFβ inhibitors (LDN and SB respectively) after an early pulse and subsequently introduce exogenous BMP4 and EDN3 signaling at day six while maintaining Wnt activation (CHIR) [6]. The resulting melanoblasts can be characterized by expression of KIT and MITF, as well as maintenance of SOX10 expression [6]. Expression of SOX10 can be visualized in areas in the culture dish that form ridges by day 11 culture (**Figure 1B**-**C**).

Following melanoblast induction, the cells are passaged onto PO/LAM/FN plates and fed with Full Melanocyte medium. This is an extremely rich medium that has the additional benefit of being selective for the melanocyte population, eliminating the need for any Fluorescent-Activated Cell Sorting [6]. During melanocyte maturation, the cell upregulates the melanin synthesis genes TYR and TYRP1 while maintaining many of the melanoblast genes, including TYRP2. Day25 stem cell derived melanocytes stain appropriately for the melanin production proteins TYRP1 and TYRP2 (**Figure 2**). This protocol is robust and has been used with the h9 hESC line and across several iPSC lines. Most recently, this protocol was used to faithfully reproduce the ultrastructural features of pigmentation diseases using patient-specific iPSC lines [6].

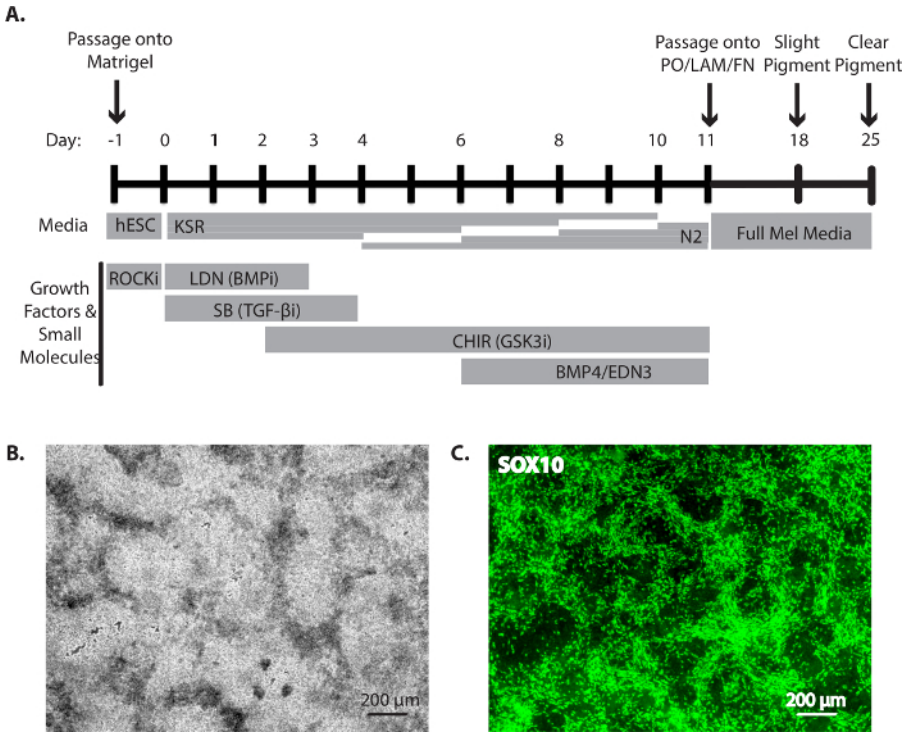


**Figure 1. Critical steps in the hESC-derived melanocyte differentiation protocol.** (**A**) The differentiation protocol scheme. KSR: KSR-differentiation medium, N2: N2-differentiation medium, LDN: LDN193189, SB: SB431542, CHIR: CHIR99021, BMP4: Bone morphogenetic protein 4, EDN3: endothelin-3, ROCKi: Y-27632 dihydrochloride **B**-**C**. Shows brightfield (**B**) and GFP fluorescence (**C**) image of differentiation on Day 11 prior to replating using a transgenic pSOX10:GFP hESC line. The dark ridges visible in the brightfield images are enriched for $SOX10^{+}$ cells that will give rise to melanoblasts and eventually melanocytes. Please click here to view a larger version of this figure.

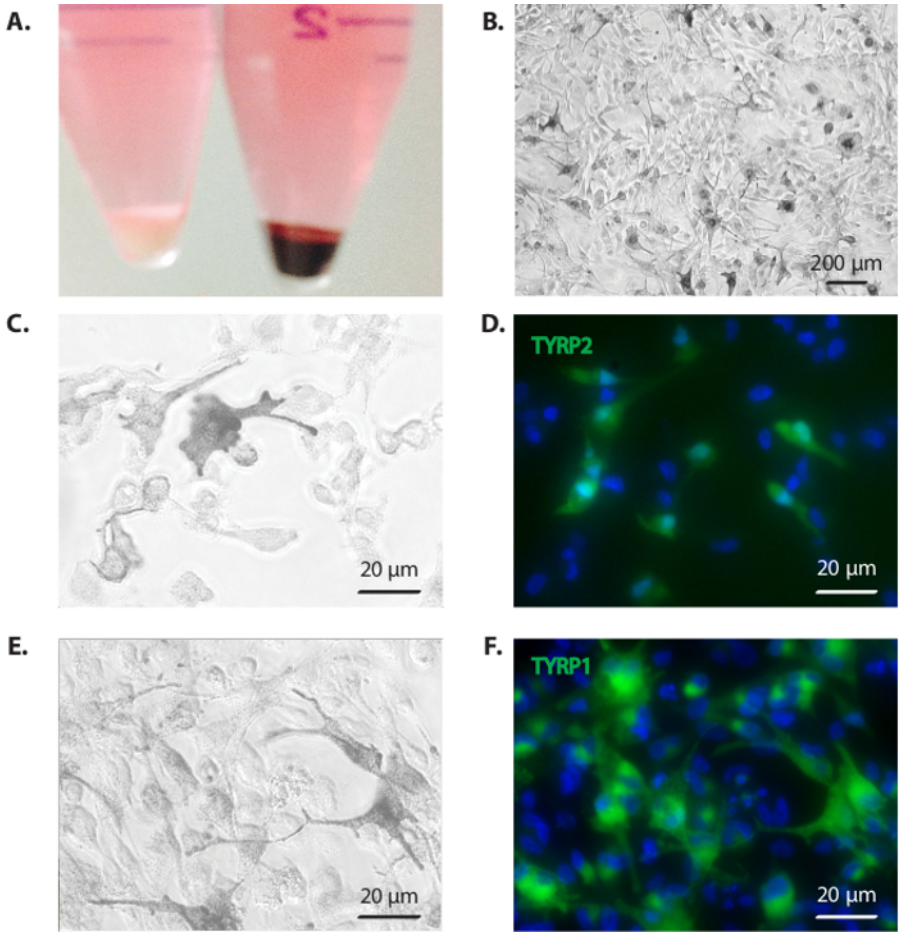


**Figure 2. Melanocyte and intermediate stages.** (**A**) The pigmented Day 25 cells (right) can be easily visualized and distinguished from Day 11 cells (left) when pelleted. (**B**) By day 20 of the protocol the culture will contain both unpigmented, maturing melanocytes and fully mature, pigmented melanocytes. (**C**-**D**) The melanocytes can be visualized under bright field and both the maturing and fully pigmented melanocytes stain for the melanoblast/melanocyte marker TYRP2. (**E**-**F**) Only the pigmented melanocytes stain for the late stage melanocyte marker TYRP1. Please click here to view a larger version of this figure.

## Discussion

For the successful differentiation of melanocytes from hPSCs the following suggestions should be taken into consideration. First and foremost, it is essential to work under sterile culture conditions at all times. Additionally, it is important to start with pluripotent, fully undifferentiated hPSCs; if the starting population contains differentiated cells the yield will invariably drop as the contaminants cannot be directed towards melanocytes and may even further disrupt the properly differentiating cells.

To ensure the cells remain pluripotent take care to adhere to the well-established rules for stem cell maintenance; feed and passage regularly and groom the cultures to remove differentiated cells before passaging. When passaging onto gelatinous protein for differentiation it is important that the dish is properly coated to prevent cells from lifting off during the differentiation.

The Melanoblast differentiation should be initiated around 80% cell density; too high densities will lead to increased cell death while too low densities will negatively affect differentiation. When passaging, the cells should be washed twice to remove any cell detachment solution before plating. To maximize survival on day 11, it is important to passage the cells into high-density, well-spaced droplets that are untouched for 20 min, so that the cells cluster and adhere.

This protocol is effective in generating a large numbers of melanocytes, and will be valuable when studying human patient samples of limited quantity. Furthermore, as the PSC-derived melanocyte population is selective and expandable the melanocyte population can be multiplied to very large numbers of cells even if the differentiation yields low numbers or percentages of melanocytes. Initiating the protocol with iPSCs opens up the possibility for studying developmental diseases and patient specific samples. One limitation of this protocol is the fact the melanocytes are derived as a monoculture without all other cell types present that form their normal niche *in vivo*. Furthermore the protocol requires expertise in hPSC culture and differentiation techniques. However, with recent developments in the iPSC field producing hPSCs has become increasingly manageable and methods for culturing hPSCs have become routine. To date, the protocol has been utilized in our lab to produce melanocytes from the h9 hES line, as well as from 14 iPS lines generated from 5 different donors.

Mica *et al.* used this protocol successfully for the derivation of melanocytes from human hESC and iPSCs [6]. The paper demonstrated that iPSCs derived from patients with pigmentation defects could be differentiated into melanocytes and the protocol faithfully produced melanosomes of the phenotypic size and quantity associated with the disease.

The work illustrated one of many possible applications for the protocol with the use of iPSC-derived melanocytes for studying disease mechanisms and introduced the possibility of scaling up production for drug screening [6,29]. Importantly, the paper demonstrated the robustness and reproducibility of the protocol to produce melanocytes from a large set of genetically distinct hiPSC lines.

## Disclosures

The authors have no conflicting interests to disclose.

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