Isolation, Culture, and Transfection of Melanocytes

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Located in the basal epidermis and hair follicles, melanocytes of the integument are responsible for its coloration through production of melanin pigments. Melanin is produced in a type of lysosome-related-organelle (LRO) called the melanosome. In humans, this skin pigmentation acts as an ultraviolet radiation filter. Abnormalities in the division of melanocytes are quite common, with potentially oncogenic growth usually followed by cell senescence producing benign naevi (moles), or occasionally, melanoma. Therefore, melanocytes are a useful model for studying both cellular senescence and melanoma, as well as many other aspects of biology such as pigmentation, organelle biogenesis and transport, and the diseases affecting these mechanisms. Melanocytes for use in basic research can be obtained from a range of sources, including surplus postoperative skin or from congenic murine skin. Here we describe methods to isolate and culture melanocytes from both human and murine skin (including the preparation of mitotically inactive keratinocytes for use as feeder cells). We also describe a high-throughput transfection protocol for human melanocytes and melanoma cells. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Primary explantation of human melanocytic cells
Basic Protocol 2: Preparation of keratinocyte feeder cells for use in the primary culture of mouse melanocytes
Basic Protocol 3: Primary culture of melanocytes from mouse skin
Basic Protocol 4: Transfection of human melanocytes and melanoma cells

Keywords: epidermis • keratinocytes • melanocytes • primary culture • transfection

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INTRODUCTION

Located in the basal epidermis and hair follicles, melanocytes of the integument are responsible for its coloration through the production of melanin pigments. Melanin is produced in a type of lysosome-related organelle (LRO) called the melanosome, which is transported along dendrites of the melanocyte and transferred into surrounding

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keratinocytes (reviewed by Wu & Hammer, 2014). In humans, the resulting skin pigmentation acts as an ultraviolet (UV) radiation filter, whereby melanin forms supranuclear caps within basal and suprabasal keratinocytes to reduce both UV irradiation of the nucleus and resulting DNA damage (Kobayashi et al., 1998; reviewed by Brenner & Hearing, 2008). Abnormalities in the division of melanocytes are quite common, with potentially oncogenic growth followed by cell senescence, producing benign naevi (moles) (Michaloglou et al., 2005). A number of signaling pathways regulating proliferation, cell senescence, and apoptosis are affected by common genetic changes in melanoma (reviewed by Bennett, 2016). Culture of melanocytes with and without such genetic changes is therefore useful for elucidating the pathways of the development of melanoma (Sviderskaya et al., 2003) and other cancers.

The differentiation of melanocytes and melanosomes and the development of skin pigmentation are tightly regulated by a number of processes, including signaling pathways and transcription factors. Mutations in key genes affecting these processes may result in a lack of melanocytes, causing hypopigmentation, or in defects or changes in melanin (reviewed by Lamoreux et al., 2010, and Yamaguchi & Hearing, 2014). With over 680 pigmentary mutations described (see Internet Resources), experimentation using melanocytes grown in culture is particularly useful for exploring the underlying genetics and mechanisms of normal variation of pigmentation as well as pigmentary disorders, such as the various forms of albinism (reviewed by Le et al., 2021). Additionally, eumelanin synthesis in the skin and eye are identical; therefore, skin melanocytes can serve as a useful model to understand pigmentation within the eye. We have established immortal murine melanocyte lines of over 40 pigmentary mutant genotypes, which have been widely used for some of the above studies (Sviderskaya et al., 2010; see Internet Resources).

Melanocytes are also useful for studying organelle dynamics as they are adherent cells with dendrites extending up to 100 μm and contain many melanosomes, which are easily visualized using bright-field as well as phase-contrast microscopy, owing to their color (reviewed by Hume & Seabra, 2011). Furthermore, the molecular mechanisms controlling the transport of melanosomes are comparable to those of other LROs; therefore, melanocytes can be used to elucidate the roles of other LROs in inflammation and the immune response (reviewed by Hume & Seabra, 2011). Melanocytes can also be used to provide insight into organelle transport and the further roles of molecular motors (Alzahofi et al., 2020).

For these valuable applications, the successful isolation and culture of melanocytes is paramount. Melanocytes may be derived from patients (for example from postoperative surplus skin) or laboratory mice with specific pigmentary mutations. The methods described here primarily focus on the isolation and culture of both human and murine melanocytes for use in downstream applications, such as transfection (also described). A key feature in the culture and maintenance of melanocytes is the use of a reduced pH compared to that used for most cell types; RPMI 1640 medium is used with 10% CO_2, resulting in a pH of 6.9-7.0. This reduces the level of melanin synthesis, allowing for better growth (believed to be because toxic melanin intermediates can escape from melanosomes at high rates of synthesis).

Human melanocytes can be isolated from a small biopsy, as described in Basic Protocol 1. Overnight treatment in dispase separates the dermis and epidermis and is followed by incubation in trypsin-EDTA to give a single-cell suspension. These melanocytes are easily grown in culture using four mitogens: 12-O-tetradecanoylphorbol 13-acetate (TPA), cholera toxin (CT), endothelin 1 (EDN1), and stem cell factor (SCF). More

information regarding these mitogens can be found in the Commentary. They do not require the inclusion of feeder cells for successful culture.

Basic Protocol 2 describes the preparation and treatment of mouse keratinocytes for use as feeder cells in Basic Protocol 3. Mouse keratinocytes must be rendered mitotically inactive by treatment with mitomycin C for use as feeder cells. Basic Protocol 3 describes isolation of murine melanocytes from sacrificed newborn mice, which are preferred to older or embryonic mice owing to ease of epidermal separation. The protocol differs slightly from that described for human melanocyte isolation. Overnight dispase treatment is replaced by digestion with concentrated trypsin. There is a reduced need for mitogens, with only TPA and CT being used, but keratinocyte feeder cells are used to allow sustained growth in culture. Melanocytes are readily amenable to genetic modification, and Basic Protocol 4 describes a process for the transfection of melanocytes.

NOTE: All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly.

NOTE: All cell culture incubations should be carried out in a 37°C, 10% CO_2 humidified incubator.

CAUTION: Follow relevant safety guidelines and regulations as some of the reagents used in this article are potentially harmful.

PRIMARY EXPLANTATION OF HUMAN MELANOCYTIC CELLS

BASIC PROTOCOL 1

This protocol describes the primary culture of melanocytes from human skin as well as the maintenance of growing cultures. This technique can be used on skin from around the whole body as well as from donors of different ages. However, skin from much older people may result in a lower yield than that from younger donors. Dispase, a neutral protease isolated from *Bacillus polymyxa*, is used to cleave the basement membrane between the epidermis and dermis as it is more specific than trypsin and its use results in a greater yield of primary cells. For a more detailed explanation regarding the theoretical basis of this protocol, see Commentary.

NOTE: This protocol involves working with human tissue and therefore all local biosafety procedures should be followed. Local ethical approval may be required, and relevant guidelines for using human tissue should be adhered to.

Materials

Iodine solution (see recipe)
70% ethanol
Dulbecco's phosphate-buffered saline without calcium or magnesium (CMF-DPBS; e.g., Thermo Fisher, cat. no. 14190094; see Current Protocols, 2006)
10 µg/ml gentamicin (Sigma, cat. no. G1272) in CMF-DPBS
Skin sample (surplus postoperative skin or obtained by punch biopsy; >5 mm diameter, best results are obtained with >1 cm^2 of skin)
10 mg/ml dispase II (Sigma, cat no. D4693) in Hank's balanced salt solution (Sigma, cat no. H6648)
500 µg/ml trypsin/EDTA solution (see recipe)
RPMI growth medium (see recipe)
Mitogen stocks:
  40 µM TPA (see recipe)
  40 nM CT (see recipe)
  5 µM EDN1 (see recipe)
  5 µg/ml SCF (see recipe)

125 µg/ml trypsin/EDTA solution (see recipe)
Dimethylsulfoxide (DMSO; Sigma, cat. no. D2650)
Phenylthiourea stock (PTU; see recipe)

Biological safety cabinet
6-well plates (e.g., Thermo Scientific, cat. no. 140675)
Automated pipettor with serological pipettes
Sterile fine forceps, blunt
37°C water bath
2.5-ml Combitips (Eppendorf, cat. no. 0030089650) and dispenser
Cell culture centrifuge, 4°C
Hemocytometer and cover glass
Cell culture microscope
P200 and P1000 pipettors with tips
Tissue culture flasks and dishes
10% CO_2 incubator, 37°C
Ungassed incubator, 37°C
30-ml universal tubes (i.e., skirted tube with a conical bottom) (e.g., Elkay Laboratory Products, cat. no. 500-1000-302)
2-ml cryotubes
Liquid nitrogen storage

Preparation

1. In a 6-well plate, add to 3 wells, respectively, 5 ml iodine solution, 5 ml 70% ethanol, and 5 ml CMF-DPBS. Add 5 ml 10 µg/ml gentamicin to each of two wells.
2. Briefly submerge the skin sample in iodine solution, 70% ethanol, then the CMF-DPBS for a few seconds each, and then submerge in each of the two 10 µg/ml gentamicin solutions, each for 10 min.

   If skin sample is too large, before submerging, cut into smaller pieces (∼1 cm wide) with a surgical scalpel and also remove subcutaneous fat by scraping with the scalpel.

   For small or thin samples, the iodine and ethanol steps may be too stringent; an alternative is to soak for 10 min in RPMI growth medium containing ten times the normal concentrations of penicillin and streptomycin (i.e., add 1 part penicillin/streptomycin solution (stock solution contains 10,000 U/ml of penicillin and 10,000 µg/ml streptomycin) to 9 parts RPMI medium).

3. Add 5 ml 10 mg/ml dispase II in Hank's solution to one well of a new 6-well plate, and submerge skin epidermis-side-down overnight at 4°C.

Separation of epidermis and dermis

4. Using suitable forceps (blunt, not to damage the tissue), gently peel away the epidermis from the dermis and place the epidermis in 5 ml 500 µg/ml trypsin/EDTA solution.

   Use one pair of forceps to hold the dermis and one to peel the epidermis. Do not mix up the forceps, as this may increase fibroblast contamination from the dermis. Epidermis is more brown and less shiny than dermis. If large sheets of epidermis come off, cut gently into smaller pieces. If parts of the epidermis stay firmly attached, do not attempt to pull, as this will increase the risk of fibroblast contamination. Discard such parts.

Culturing melanocytes

5. Incubate epidermis in 500 µg/ml trypsin/EDTA solution in a 37°C water bath for ∼10-15 min.
6. Disperse the epidermis into a cell suspension by pipetting up and down using a 2.5-ml Combitip attached to a dispenser.

7. Add 5 ml RPMI growth medium and centrifuge 15 min at 450 × *g*, 4°C.
8. Remove supernatant and resuspend the pellet in 5 ml fresh medium.
9. Count cells using a hemocytometer.
10. Plate cells at 1.5 × 10^5 cells/ml (plate 5 ml total volume for a 25-cm^2 flask or in general ∼1 ml/5 cm^2, depending on the number of cells available), adding 5 µl/ml 40 µM TPA, 5 µl/ml 40 nM CT, 2 µl/ml 5 µM EDN1, and 2 µl/ml 5 µg/ml SCF.

    Final concentrations are 200 nM TPA, 200 pM CT, 10 nM EDN1, and 10 ng/ml SCF.

11. Place the culture in a humidified, 37°C, 10% CO_2 incubator.

Propagating melanocyte cultures

12. Change RPMI growth medium every 3-4 days, adding the same concentrations of mitogens as in step 10.

    Keratinocytes are alive initially but fail to grow in this medium. If cells become sparse in the early stages of melanocyte growth, the volume of medium can be reduced, e.g., by half. This is thought to concentrate autocrine growth factors released by the cells. When enough melanocytes are present, the volume of medium can be increased to normal.

13. When cells are confluent, for subculture, remove medium and wash cells in 0.5 vol CMF-DPBS, where 1 vol is the normal culture volume for the flask or plate. Tilt the container gently around to rinse away serum.
14. Add 0.5 vol 125 µg/ml trypsin/EDTA solution. Tilt again briefly then remove most of it to leave 0.1 vol. Place in a 37°C ungassed incubator.
15. When cells are detached, resuspend into fresh medium to give the normal culture volume, with two rinses to remove all cells.

    For example, if cells were grown in a 25-cm^2 (5-ml) flask, resuspend in 2.5 ml RPMI growth medium and transfer to a universal tube, then rinse with 2 ml medium and add this to the universal tube. With the 0.5 ml trypsin-EDTA, this adds up to the 5 ml.

16. Count cells in triplicate using a hemocytometer. Keep the cell suspension on ice to prevent loss, as melanocytes can quickly start to reattach at room temperature.
17. Dilute, mix, and replate cells at the required density in fresh medium with mitogens at the same concentration as in step 10.

    3–6 × 10^4 cells/ml can be used according to growth rate etc.

Freezing and thawing melanocytes

18. Freeze cells using cultures approaching confluence as follows:
    a. Trypsinize as in step 14, and count cells using a hemocytometer.
    b. Centrifuge cells 7 min at 200 × *g*, 4°C.
    c. Label cryotubes before proceeding to minimize the time cells are in DMSO.
    d. Resuspend cells in previously mixed 7.5% (v/v) DMSO in RPMI growth medium at a total density of 0.5–1 × 10^6 cells/ml.
    e. Dispense 1-ml aliquots into 2-ml cryotubes and freeze at –80°C in an insulated container before moving into liquid nitrogen for long-term storage.

    For heavily pigmented cells, add 100-300 µM PTU for a whole passage before freezing, to depigment them.

    Keep cells at least overnight at –80°C before placing in liquid nitrogen but do not keep at –80°C for more than a few days. PTU acts to inhibit melanin synthesis by inhibiting tyrosinase. This can greatly improve viability, as DMSO causes release of toxic melanin intermediates into the cytosol.

19. Thaw cells quickly as follows:

    a. Place the cryotube in a 37°C water bath.
    b. Resuspend by pipetting and transfer cells to a 30-ml universal tube.
    c. Add 1 ml RPMI growth medium dropwise with swirling over ~30 s, then add another 1 ml dropwise over 15 s, and finally 18 ml dropwise over 30 s.
    d. Centrifuge cells 5 min at 200 × *g*, 4°C.
    e. Resuspend cells in fresh medium at the desired density (usually 3×10^4 cells/ml in 5 ml total volume per 25 cm^2 culture flask, or in general ~1 ml/5 cm^2) with the same concentration of mitogens as in step 10.

    For well-pigmented cells, also add 100-300 μM PTU for a few days after thawing cells.

BASIC PROTOCOL 2

PREPARATION OF KERATINOCYTE FEEDER CELLS FOR USE IN THE PRIMARY CULTURE OF MOUSE MELANOCYTES

This protocol describes the subculture and preparation of mouse keratinocyte feeder cells. The first technique describes the subculture of XB2 mouse keratinocytes, which can be difficult to subculture as they tend to attach very firmly. The XB2 keratinocytes can be grown and subcultured with a view to treating them with mitomycin C to render them mitotically inactive for use as feeder cells. For a more detailed explanation regarding the theoretical basis of this protocol, see Commentary.

Additional Materials *(also see Basic Protocol 1)*

XB2 mouse keratinocytes (available from the Functional Genomics Cell Bank at St. George's, University of London; see Internet Resources) growing in culture
500 μM EDTA solution in CMF-DPBS (Sigma, cat. no. E8008)
250 μg/ml trypsin/EDTA solution (see recipe)
Dulbecco's Modified Eagle Medium (DMEM) growth medium (see recipe)
Mitomycin C stock (see recipe)

Maintenance and subculture of XB2 mouse keratinocytes

1. Thaw XB2 keratinocytes as described in step 19 of Basic Protocol 1 except use DMEM rather than RPMI growth medium.
2. Change DMEM growth medium every 3-4 days.
3. Once ready to subculture, remove medium and wash the cells twice with 500 μM EDTA solution in CMF-DPBS. Use 0.5 vol per wash, where 1 vol is the normal culture volume for the flask or plate, e.g., use 2.5 ml in a 5-ml (25-cm^2) flask. Tilt the container gently around to rinse away serum, which inhibits trypsin.

   XB2 cells should be around 80% confluent (i.e., cover 80% of the area of the culture vessel) and still growing. They become very hard to detach at saturation density.

4. Wash the cells once with 0.5 vol of 250 μg/ml trypsin/EDTA solution and tilt around again. Remove most of this to leave 0.1 vol of solution.
5. Incubate at 37°C (in an ungassed incubator rather than with CO_2) until the cells have completely detached.
6. Resuspend the cells by pipetting in two rinses of fresh DMEM growth medium into a suitable container (e.g., 30-ml universal tube), to make 1 vol of cell suspension, and count using a hemocytometer.

   For example, if cells were grown in a 25-cm^2 flask, resuspend in 2 ml DMEM growth medium and then 2.5 ml DMEM growth medium. The 0.5 ml of trypsin solution makes the total up to the 5 ml volume.

7. Dilute the required amount of suspension with fresh DMEM growth medium and replate the cells at 3×10^4/ml (plate 5 ml total volume per 25-cm^2 flask or in general ~1 ml/5 cm^2 depending on the number of cells available and your requirements, e.g., if preparing feeder cells, plate 35 ml total volume per 175-cm^2 flask).

Preparation of feeder cells

In order to prepare feeder cells, the XB2 cells should be ready to subculture, but still growing. They should not be confluent, as this may lead to ineffective treatment with mitomycin C and lead to some of the cells still retaining the capacity to divide. Plate the required volume of cultures (e.g., if you require a large stock of feeder cells, plate cells into 175-cm^2 flasks as previously described). It is recommended to make large amounts of vials to allow batch testing. There is no specific limit on passage number for the XB2, but the growing cells should form patches of continuous epithelial pavement. If they start to show a disrupted layer with spindly-looking cells, they should be discarded in favor of a lower passage level.

8. Remove medium and add 0.5 vol fresh DMEM growth medium containing 8 μg/ml mitomycin C. Swirl to mix this with residual old medium.

9. Incubate for 3-3.5 hr in a 37°C, 10% CO_2 incubator.

10. Remove medium containing mitomycin C, wash once in 0.5 vol DMEM growth medium and subsequently incubate in DMEM growth medium (no mitomycin C) for 10 min in a 37°C, 10% CO_2 incubator.

    This elutes any remaining mitomycin C present.

11. Subculture as previously described (steps 3-7 of this protocol) and either replate 2 ml per 35-mm dish at 3×10^4/ml for use immediately or freeze 1 ml aliquots of 1×10^6 cells/ml and store in liquid nitrogen for future use. For freezing cells, resuspend as detailed above in steps 3-7 of this protocol, then follow step 18 of Basic Protocol 1, except use DMEM rather than RPMI growth medium and use 250 μg/ml trypsin/EDTA instead of 125 μg/ml.

    It is advisable to check each batch to ensure that the mitomycin C treatment has worked by plating 10^6 treated XB2 cells at 3-5 $\times$ 10^4 cells/ml in 5 ml per 25-cm^2 flask and checking for at least 2 weeks to make sure no proliferating cell colonies appear.

PRIMARY CULTURE OF MELANOCYTES FROM MOUSE SKIN

BASIC PROTOCOL 3

This protocol describes the primary culture of melanocytes from mouse skin and their subsequent subculture (Sviderskaya et al., 1997). There is a requirement for feeder cells, and so Basic Protocol 2 should be followed prior to this Protocol. To generate immortal melanocyte lines efficiently, it is recommended to cross the required genotype of mouse with *Cdkn2a* (*Ink4a-Arf*) null mice, as *Cdkn2a* null melanocytes do not senesce at all (Sviderskaya et al., 2002).

This technique can be carried out on either embryonic or newborn skin (of mice up to 3 days old). Older skin is very hard to split because of the hair and contains few melanocytes anyway. It is also hard to split embryonic skin (which is fragile), and this increases the risk of contamination with fibroblasts. Thus, it is preferable to use newborn skin. However, it may be necessary to work with embryonic skin, for example if a mutation you wish to study is lethal to postnatal mice.

In contrast to human melanocytes (which require 4 mitogens; TPA, CT, EDN1, and SCF), successful culture of murine melanocytes only requires TPA and CT. For a more detailed explanation regarding the theoretical basis of this protocol, see Commentary.

NOTE: This protocol involves working with animals and therefore local ethical approval may be required and relevant guidelines for working with animals should be adhered to.

Additional Materials *(also see Basic Protocol 1)*

Keratinocyte feeder cells (see Basic Protocol 2)
Mouse pups (up to 3 days old) or pregnant mouse
5 mg/ml trypsin (see recipe)
250 µg/ml trypsin/EDTA solution (see recipe)
1 mg/ml soybean trypsin inhibitor (see recipe)

35-mm culture dishes (e.g., Thermo Scientific, cat. no. 153066)
Reagents and equipment for dissection of pregnant mouse to obtain embryos or to sacrifice the mouse pups (Limaye et al., 2009)
10-cm culture dishes (e.g., Thermo Scientific, cat. no. 150350)
Two pairs of fine forceps (for each skin to be processed)
Laminar flow hood
Dissecting microscope in culture hood (need sterile atmosphere)
60-mm (5-ml) culture dishes (e.g., Thermo Scientific, cat. no. 150288)
Two curved scalpels (for each skin to be processed)

Skin preparation

1. Plate out keratinocyte feeder cells on a 35-mm dish at 5×10^4/ml in RPMI growth medium (2 ml total volume per 35-mm dish), preferably 1 day before carrying out the remainder of this protocol.

   If you have more than one skin sample, plate out a dish for every skin sample you have.

2. Sacrifice the mouse pups (or the pregnant mouse in the case of embryos) by a legal method (depending on the country). If using embryos, dissect the pregnant mouse to obtain the embryos (Limaye et al., 2009).

   Newborn skin is sterilized by immersing the pup in 70% ethanol for 5-10 s (no more).

   Do not sterilize embryo skin in ethanol; it may kill the cells, and embryos are already sterile, so they can be kept sterile during removal from the uterus. Every step from here on should be performed under sterile conditions.

3. Wash off the ethanol (or wash the embryos) in CMF-DPBS.
4. Dissect off the trunk skin in one piece and keep wet in CMF-DPBS.
5. Transfer to a 10-cm culture dish with enough CMF-DPBS to cover the skin and remove any remaining pieces of muscle using two pairs of fine forceps and a dissecting microscope in a sterile environment.

   If you have more than one skin sample, use different forceps and dish for every skin to avoid any cross-contamination. Make sure you use the same forceps for the same skin throughout this protocol.

Splitting the epidermis from the dermis

6. Transfer the skin(s) to individual 30-ml universal tubes containing 5 ml 5 mg/ml trypsin.

   The 5 ml volume is a guideline. With a particularly large skin sample, make sure there is enough trypsin to cover it.

7. Incubate at 37°C for ∼1 hr for newborn skin.

   For embryonic skin, trypsinize on ice for 15 min to 3 hr—it needs to be trypsinized until it will split, and the time for this can be quite variable, so it is best to keep checking to avoid leaving the skin in trypsin for too long.

8. After the allotted time, transfer the skin to a 10-cm dish containing enough CMF-DPBS to cover the skin.

9. Using a dissecting microscope and two pairs of fine forceps split off the epidermis from the dermis. If it does not come off easily, do not force but place the skin back into 5 mg/ml trypsin and continue incubation. Remove any pieces of dermis still attached to the epidermis.

   The epidermis is thin and silvery/white and the dermis is a more whitish layer. Do not force, as this can lead to contamination with fibroblasts.

Culturing melanocytes

10. Wash the epidermis in fresh CMF-DPBS and transfer the 10-cm dish to a laminar flow hood.

    All steps from here on are performed in a flow hood.

11. Add 100 µl 250 µg/ml trypsin/EDTA solution to the middle of a 60-mm dish.

12. Prepare 5 ml RPMI growth medium with 5 µg/ml soybean trypsin inhibitor (add from 1 mg/ml stock).

13. Transfer the epidermis from the CMF-DPBS onto the drop of trypsin/EDTA solution.

    Try to remove as much of the CMF-DPBS from the epidermis as possible before placing onto the drop.

14. Using two curved scalpels, chop the epidermis finely.

    If you have more than one skin sample, use new scalpel blades and a new dish for every sample. Make sure that you remove all pieces of epidermis from the scalpel blades before discarding, to ensure that the majority of the epidermis goes into culture.

15. Using a serological pipette and the RPMI growth medium with soybean trypsin inhibitor prepared in step 12, suspend and rinse the chopped pieces of epidermis into a 30-ml universal tube.

16. Mix vigorously three to five times using a 2.5-ml Combitip.

17. Add 5 µl/ml 40 µM TPA and 5 µl/ml 40 nM CT and mix thoroughly.

    Final concentrations are 200 nM TPA and 200 pM CT.

18. Remove the medium from the mitomycin C-treated XB2 keratinocytes (from step 1), plate the full 5 ml epidermal suspension onto these cells, and place in 10% CO_2 incubator at 37°C.

    Take care when transferring the cultures to the incubator, as the dish will be very full.

19. Reduce the amount of medium to 2 ml after 1-2 days (or when changing medium). Initially change medium twice a week (using RPMI growth medium with 200 nM TPA and 200 pM CT).

Subculture of mouse melanocytes

The cells should be subcultured at least every 2 weeks (if sparse), and before reaching confluence, onto fresh mitomycin C-treated XB2 keratinocytes until immortal cells take over the culture. At this stage some lines may not require constant culture with CT; this can be determined empirically by the user, i.e., keep at least two separate cultures and remove the CT from one to compare proliferation and differentiation. The XB2 cells are thawed and plated as in Basic Protocol 3, step 1, on the required number of dishes or flasks.

20. Remove medium from the melanocyte culture and wash cells in 0.5 vol CMF-DPBS, where 1 vol is the normal culture volume for the flask or plate. Tilt the container gently around to rinse away serum.
21. Add 0.5 vol 250 µg/ml trypsin/EDTA solution. Tilt again briefly and then remove most of it to leave 0.1 vol. Place in a 37°C ungassed incubator.
22. When cells are detached, resuspend into fresh medium to give the normal culture volume, with two rinses to remove all cells.

    For example, if cells were in a 35-mm culture dish, resuspend in 0.8 ml RPMI growth medium, transfer to a sterile tube, then rinse with 1 ml growth medium and add to the tube. With the 0.2 ml trypsin-EDTA, this adds up to the 2 ml.
23. Count cells in triplicate using a hemocytometer. Keep the cell suspension on ice to prevent loss, as melanocytes can quickly start to reattach at room temperature.
24. Dilute, mix, and replate cells either at 3-5 $\times$ 10^4 cells/ml (5 ml total volume for 25-cm^2 flasks or in general $\sim$1 ml/5 cm^2, depending on the amount of cells available), or (for sparse cultures) at a ratio of 1:1, in fresh medium with mitogens (200 nM TPA and 200 pM CT), onto the mitomycin C-treated XB2 cells.
25. For freezing and thawing mouse melanocytes, use the method in Basic Protocol 1, steps 18-19, except use 250 µg/ml trypsin/EDTA solution instead of 125 µg/ml. When initially thawing established mouse cell lines, plate 5 ml total volume in a 25-cm^2 flask at 3-10 $\times$ 10^4 cells/ml (depending on viability), adding 200 nM TPA but no CT.

    Mouse melanocytes (unless null for Cdkn2a) will senesce (stop growing) after only a few weeks. If the senescent cultures are patiently kept, with medium changes, for a few more weeks, immortal cells will often eventually emerge as growing colonies and can be propagated to make a permanent cell line.

BASIC PROTOCOL 4

TRANSFECTION OF HUMAN MELANOCYTES AND MELANOMA CELLS

This protocol describes how to transfect human melanocytes and melanoma cells in 96-well plates. It uses "reverse transfection," where the nucleic acid is added to wells before the cells, which gives efficient transfection in these cell types. This protocol is based on a high-throughput system designed to screen the activity of tens to hundreds of different siRNAs. However, the volumes used could be scaled up or down depending on the nature of the experiments. Cell and nuclear staining are used to determine the effects of the transfected siRNA on the cells. A sophisticated plate reader, such as the Thermo Scientific Cellomics ArrayScan VTI HCS Reader, is recommended for capturing the outputs in this high-throughput scenario.

Additional Materials *(also see Basic Protocol 1)*

siRNAs:
- Negative control siRNA—Silencer Select negative control (siRNA #2; Invitrogen, cat. no. 4390846)
- Positive control siRNA—CDC2 siRNA gene solution (Qiagen, cat. no. 1027416 gene ID 983)
- Transfection positive control siRNA—AllStars Hs Cell Death Control siRNA (Qiagen, cat. no. 1027298)
- Test siRNAs—Silencer Select validated or predesigned (inventoried) siRNA (Ambion)

RNase-free distilled H_2O
RPMI serum-free medium (see recipe)
Lipofectamine 2000 (Invitrogen, cat. no. 11668027)
Human melanocyte or melanoma cell suspension (see Basic Protocol 1)

siRNA a	siRNA a	siRNA a	siRNA b	siRNA b	siRNA b	siRNA c	siRNA c	siRNA c	siRNA + control	siRNA + control	siRNA + control
siRNA d	siRNA d	siRNA d	siRNA e	siRNA e	siRNA e	siRNA f	siRNA f	siRNA f	siRNA - control	siRNA - control	siRNA - control

Figure 1 An example of a plate map showing six test siRNAs (siRNA a to f) and two control siRNAs. Each siRNA is transfected in triplicate.

RNase/DNase-free tubes (e.g., Invitrogen, cat. no. AM12400)
RNase-free filter tips for P20, P200, and P1000 pipettors
96-well black optical-bottom tissue culture plates (e.g., Greiner Bio-One, cat. no. 655090)
Multichannel pipettors and reservoirs

Preparation and storage of siRNA

1. Resuspend lyophilized siRNAs to 5 µM (5 pmol/µl) in RNase-free water.

   Use RNase-free tips and tubes. Work in an RNase-free environment when handling siRNA stocks.

2. Prior to storage, aliquot all siRNAs to avoid freeze-thaw cycles and store at −20°C (short-term) and −70°C (long-term).

3. Create an siRNA master plate.

   This will ease the transfer of siRNAs to the experimental plates. Reactions should be performed in triplicate. Create a map of the plate (electronic and hardcopy). See Figure 1 for an example of a plate map. Ensure that the plate is well labeled to reduce errors.

Medium preparation

4. Prepare complete medium (RPMI growth medium with appropriate mitogens).

   For human melanocytes: add mitogens as in Basic Protocol 1 (final concentrations 200 nM TPA, 200 pM CT, 10 nM EDN1, and 10 ng/ml SCF).

   For human melanoma cells: add no mitogens unless specified for the cell line.

Transfection

5. Grow cells to ∼80% confluency (i.e., 80% of the culture dish covered and not at saturation density). Trypsinize as for subculture (see Basic Protocol 1) and leave at room temperature.

6. Mix Lipofectamine 2000 with serum-free medium. Prepare a 100 × master mix in an RNase-DNase-free centrifuge tube containing 30 µl Lipofectamine 2000 in 470 µl serum-free medium for each 96-well plate (0.3 µl Lipofectamine 2000 in 4.7 µl serum-free medium per well). Incubate at room temperature for 5 min.

Ensure that the master mix is thoroughly mixed. Use a 1000 µl pipette tip and a pipettor (∼10 times).

7. Add 8.5 µl serum-free medium to each well of a 96-well black optical bottom plate using a multichannel pipettor.

   Add 40 ml serum-free medium to a solution reservoir. From this, use a multichannel pipettor to add the solution to the experimental plates.

8. Add 1.5 µl of the required siRNA to each well. Mix using pipettor.

9. Add 5 µl Lipofectamine 2000/serum-free medium to all wells. Mix using pipettor. Incubate for 15 min at room temperature from the last well.

   Use a fresh pipette tip for each column to avoid cross-contamination of siRNAs.

10. During the 15 min incubation, dilute the cell suspension in complete medium (2.22×10^4/ml in 15 ml complete medium (see step 4) per plate to give 3000 cells/well in 135 µl).

    Higher densities such as 6.66×10^4/ml may be better for normal melanocytes.

    Human melanoma cells are less sensitive than normal melanocytes, and therefore less cell death caused by transfection is observed with these cells.

11. After 15 min, add 135 µl cell suspension/well.

12. Incubate plates at 37°C, 10% CO_2 for the required period (typically around 3-7 days, depending on the research question and assay). For immunostaining, fix and permeabilize cells as required, depending on the assay to be used.

    For example, three fixation methods for cytochemistry or immunostaining of melanocytes and melanoma cells are given by Soo et al. (2011).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Bovine serum albumin (BSA), 0.1 mg/ml

Dissolve BSA (e.g., Sigma cat. no. A7906) in CMF-DPBS (e.g., Gibco, cat. no. 14190-094; see Current Protocols, 2006) at 0.1 mg/ml. Filter-sterilize using a 0.22-µm filter. Store for ≤1 month at 4°C.

This solution acts as a protein carrier for dilute mitogens and TPA, which can otherwise be lost by adhesion to plastic or glass.

Cholera toxin (CT)

Dissolve CT (Sigma, cat. no. C8052) in 0.1 mg/ml BSA (see recipe) to make a 2 µM stock solution. Filter-sterilize using a 0.22-µm filter. Aliquot and store indefinitely at –80°C. Thaw one aliquot at a time and dilute with 0.1 mg/ml to make a 40 nM working solution. Store aliquots indefinitely at –80°C or for ≤2 weeks at 4°C.

Do not refreeze a third time (the same applies to other peptides, EDN1, and SCF).

DMEM growth medium (for keratinocytes)

500 ml DMEM (high glucose, 4500 mg/L) without L-glutamine (e.g., Sigma, cat. no. D6546)
5.6 ml penicillin/streptomycin stock solution (10,000 U/ml penicillin and 10,000 µg/ml streptomycin) (Gibco, cat. no. 15140-122)
5.6 ml 200 mM L-glutamine (Gibco, cat. no. 25030-024)
56 ml fetal bovine serum (FBS, Gibco, cat. no. 10270-106)
Store ≤1 month at 4°C

Endothelin 1 (EDN1)

Dissolve EDN1 (Bachem, cat. no 4040254) in 0.1 mg/ml BSA (see recipe) to make a 50 µM stock solution. Filter-sterilize using a 0.22-µm filter. Store indefinitely at –80°C. Thaw one aliquot at a time and dilute with 0.1 mg/ml BSA to make a 5 µM working solution. Store aliquots indefinitely at –80°C or ≤2 weeks at 4°C.

Iodine solution, 2.5 mg/ml

Dissolve 25 mg iodine crystals in 10 ml sterile distilled H_2O. Store indefinitely at 4°C.

Mitomycin C, 500 µg/ml stock

Dissolve 2 mg mitomycin C (Sigma, cat. no. M4287) in 4 ml distilled H_2O. Filter-sterilize using a 0.22-µm filter. Store wrapped in foil ≤1 month at 4°C.

If the vials are to be frozen, they should be wrapped in foil (or use brown tinted vials/tubes) and frozen rapidly to avoid precipitation (e.g., by dropping into liquid nitrogen). Frozen vials can be stored indefinitely at −70°C or in liquid nitrogen.

Phenol Red, 3 mg/ml

Dissolve 300 mg phenol red (e.g., Sigma, cat. no. P5530) in 100 ml distilled H_2O. Filter-sterilize using a 0.22-µm filter. Store indefinitely at room temperature.

Phenylthiourea (PTU), 100 mM

Dissolve PTU (also called phenylthiocarbamide, Sigma, cat. no. P7629) in 100% ethanol to make a 100 mM stock solution. Store indefinitely at −20°C or <1 month at 4°C.

The stock solution will precipitate when chilled so warm and mix before use.

RPMI growth medium

500 ml RPMI 1640 with phenol red and L-glutamine (Sigma, cat. no. R8758)
1.45 ml 3 mg/ml phenol red solution (see recipe)
5.6 ml penicillin/streptomycin stock solution (10,000 U/ml penicillin and 10,000 µg/ml streptomycin) (Gibco, cat. no. 15140-122)
Gas with 10% CO_2 for ∼1 min
Add 56 ml FBS (Gibco, cat. no. 10270-106)
Store ≤1 month at 4°C

Extra phenol red is added to the medium to help with monitoring of pH. Serum used for culturing melanocytes should not be heat-inactivated as this destroys its stimulatory activity for melanocytes.

RPMI serum-free medium

500 ml RPMI 1640 with phenol red and L-glutamine (Sigma, cat. no. R8758)
1.45 ml 3 mg/ml phenol red solution (see recipe)
5.6 ml penicillin/streptomycin stock solution (10,000 U/ml penicillin and 10,000 µg/ml streptomycin) (Gibco, cat. no. 15140-122)
Gas with 10% CO_2 for ∼1 min
Store <1 month at 4°C

Soybean trypsin inhibitor, 5 µg/ml

Dissolve soybean trypsin inhibitor (Sigma, cat. no. T9128) in CMF-DPBS (e.g., Gibco, cat. no. 14190-094; see Current Protocols, 2006) to a concentration of 5 µg/ml. Filter-sterilize using a 0.22-µm filter. Prepare small aliquots and store indefinitely at –20°C.

Stem cell factor (SCF)

Dissolve human SCF (Peprotech, cat. no. 300-07) in 0.1 mg/ml BSA (see recipe) to make a 20 µg/ml stock solution. Store indefinitely at –80°C. Dilute to 5 µg/ml with 0.1 mg/ml BSA (see recipe). Store indefinitely at –80°C or for ≤2 weeks at 4°C after first use.

12-O-Tetradecanoylphorbol 13-acetate (TPA)

Dissolve TPA (Sigma, cat. no. P8139) in 100% ethanol to make a 2 mM stock solution. Store aliquots indefinitely at –80°C. Dilute with 0.1 mg/ml BSA (see recipe) to make a 40 µM working solution. Store in aliquots indefinitely at –80°C or for ≤2 weeks at 4°C after first use.

CAUTION: *TPA is a tumor promoter.*

TPA is provided as a film and is hard to see; make sure it dissolves fully.

Handle in subdued light and keep vials covered (e.g., in foil or use brown tinted vials/tubes), as TPA is light-sensitive.

Trypsin/EDTA solution, 125 µg/ml

10 ml 1× trypsin-EDTA solution (Gibco, cat. no. 25300-054; contains 500 µg/ml trypsin and 500 µM EDTA in CMF-DPBS)
30 ml 500 µM EDTA solution in CMF-DPBS (Sigma, cat. no. E8008).
Store ≤2 weeks at 4°C

Trypsin/EDTA solution, 250 µg/ml

10 ml 1× trypsin-EDTA solution (Gibco, cat. no. 25300-054; contains 500 µg/ml trypsin and 500 µM EDTA in CMF-DPBS)
10 ml 500 µM EDTA solution in CMF-DPBS (Sigma, cat. no. E8008)
Store ≤2 weeks at 4°C

Trypsin/EDTA solution, 500 µg/ml

0.5 ml 10× trypsin-EDTA (Sigma, cat. no. T4174)
4.5 ml 500 µM EDTA solution in CMF-DPBS (Sigma, cat. no. E8008)
Store indefinitely at –20°C

Trypsin, 5 mg/ml

Dissolve trypsin powder (Sigma, cat. no. T4799) in CMF-DPBS (Gibco, cat. no. 14190-094) to 5 mg/ml. Filter-sterilize using a 0.22-µm filter. Prepare small aliquots and store indefinitely at −40°C or −80°C.

COMMENTARY

Background Information

Basic Protocol 1

Human melanocytes are a useful model for studying melanoma, cellular senescence, pigmentation, organelle biogenesis and transport, and related disease mechanisms. An advantage of studying melanocytes is that they are easy to obtain (for example, from elective surgery patients for adult cell lines, and foreskin for neonatal cell lines), and are simple to isolate and maintain in culture.

Four mitogens are used for human melanocyte growth: TPA, also known as phorbol 12-myristate 13-acetate (PMA); CT; SCF; and EDN1. The first reproducible melanocyte culture method was developed in 1982, upon the discovery that use of CT and TPA as mitogens could selectively maintain melanocyte growth in culture (Eisinger & Marko, 1982). CT acts by mimicking the effects of melanocyte stimulating hormone (MSH) by specifically activating adenylate cyclase, thereby causing production of cyclic AMP. This stimulates proliferation, or at higher concentrations cell differentiation and pigment synthesis (O'Keefe & Cuatrecasas, 1974). TPA is a phorbol ester that activates PKC signaling, leading to the phosphorylation of a number of transcription factors,

resulting in proliferation and differentiation of melanocytes in culture (reviewed by Yamasaki et al., 2009).

Keratinocytes were traditionally used as feeder cells for melanocyte culture, as a close representation of the epidermal tissue. The release of tumor necrosis factor (TNF)-α and interleukin (IL)-1α by keratinocytes has been shown to induce the production of EDN1 and SCF (Hachiya et al., 2004). It has been established that EDN1 and SCF act synergistically to stimulate the proliferation of melanoblasts and melanocytes in the presence of a cyclic AMP inducer such as CT. In a feeder-free environment, the absence of these mitogens leads to reduced melanocyte proliferation and increased apoptosis (Hirobe et al., 2010; Sviderskaya et al., 2003).

Early melanocyte culture methods also described the use of trypsin to separate the epidermis and dermis. However, dispase has the advantage of low cytotoxicity, and unlike trypsin, the thickness of the skin sample does not affect its efficiency (Kitano & Okada, 1983). Dispase is a neutral protease isolated from the bacterium *B. polymyxa*, and it acts by specifically cleaving fibronectin and type IV collagen (Stenn et al., 1989), compared to the non-specific action of the serine protease trypsin, prolonged use of which may damage cells and therefore result in lower cell yields.

Basic Protocol 2

Owing to their physiological interaction with melanocytes, keratinocytes make excellent feeder cells for melanocyte primary cultures. There is a requirement for the keratinocytes to be mitotically inactive to avoid contaminating the culture. A well-established method of producing mitotically inactive feeder cells is to treat the cells with the antibiotic mitomycin C. Mitomycin C targets guanine nucleosides in the sequence 5′CpG-3 (Tomasz, 1995), and causes inter-strand cross-links in the DNA, which means the DNA cannot replicate, and leads to cell cycle arrest. This should be an efficient process; however, it is always advisable to check that the treatment with mitomycin C has worked, as even a small percentage of dividing immortal keratinocytes will ruin the melanocyte culture.

The XB2 cell line is an immortal line of mouse keratinocytes and was first described by Rheinwald and Green (1975). XB2 cells are differentiated in culture from teratoma cells and were originally grown on mouse feeder fibroblasts; however, Bennett et al. (1987) selected a variant line that can grow without fibroblasts. These XB2 mouse keratinocytes can be purchased from the Functional Genomics Cell Bank at St. George's, University of London (see Internet Resources).

Basic Protocol 3

Murine melanocytes are an attractive model in which to study the genetics of diseases arising from melanocyte development and pigment production; over 350 genetic loci have been established as required for normal pigmentation alone (see Internet Resources). The mouse and human genomes are of a similar length and are highly conserved. Importantly, mice have a short mating cycle, producing on average 10-15 offspring per litter, with one litter per month. This means that mice are extremely suitable for breeding congenic mutant strains.

During murine development, neural crest cells migrate dorsolaterally between the dermomyotome and epidermis before differentiating into non-pigmented progenitor melanocytes called melanoblasts (reviewed by Kunisada et al., 1996), at around embryonic day 10.5 (E10.5) (Mayer, 1973). By E12, the melanoblasts have reached the limb buds, and at E13/E14 migrate into the epidermis of the lateral trunk (Mayer, 1973), where they differentiate further to become melanocytes before and after birth (reviewed by Kunisada et al., 1996). Melanocytes are therefore present in the late stages of development, and melanoblasts will also differentiate into melanocytes in the culture system described here; however, the fragility of the skin at this stage means it is harder to separate the epidermis from the dermis, increasing the risk of fibroblast contamination. Moreover, the mother mouse has to be killed to obtain the embryos. Thus, newborn mice are more commonly used for isolation of melanocytes.

With the number of pigmentary mutant genotypes available, there is opportunity to study the effects of these mutations at multiple stages of melanocyte development, and also their effects on differentiation, including melanin production and the molecular basis of pigmentary disorders including albinism. Furthermore, murine melanocytes have proved to be extremely useful for the elucidation of the molecular pathways underlying melanoma development and metastasis.

Note the differences between human and murine melanocyte culture methods: feeder keratinocytes are used to support the murine melanocytes, and TPA and CT are the only required mitogens. As mentioned, keratinocytes

can release EDN1 and SCF, two mitogens for melanocytes; therefore, the inclusion of feeder cells replaces these extrinsic factors. It has not yet been established whether manufactured mitogens will rescue feeder-free murine melanocyte cultures, but feeder cells are valuable if attempting to immortalize mouse melanocytes, as the melanocytes can become very sparse at this stage. Similarly, we use dispase to split human skin but trypsin for mouse skin, because we have not tested the dispase method on mouse skin.

Basic Protocol 4

Small interfering RNAs (siRNAs) are short sequences of RNA, which, when transfected into cells, are able to silence corresponding genes (Dykxhoorn et al., 2003). Thus, siRNA transfection is a useful tool for studying the role of genes in important cellular processes. Indeed, the technique is very well-known and well-utilized, and many variations exist.

The transfection protocol discussed in this section has several advantages. Since the transfections are transient, the length of the experiment from start to finish is relatively short (versus stable transfections). Therefore, transfections of this type may be performed on a large scale, and data can be produced quickly. The high-throughput nature of the detailed protocol allows many siRNAs to be tested at once.

However, there are disadvantages associated with this protocol. A sophisticated plate reader is required to capture the data if 96-well plates are used; without one, it would be too laborious to process the results. Another limitation is that the ability for effects to be identified is determined by the efficiency of the transfection of any given siRNA. Therefore, if transfection efficiency is low, the effects of the knockdown may be missed and masked by the outgrowth of non-transfected cells. It is preferable to use a number of siRNAs (3-6) to target the same gene. The siRNAs could be pooled or used separately. Triplicates for each experiment are recommended. Finally, the knockdown (and possibly its effects) will be transient.

Critical Parameters

Basic Protocol 1

See the Table 1 troubleshooting guide for details.

Basic Protocol 2

Owing to XB2 cells attaching very firmly, initial washes in EDTA are required. It is important to adjust the volumes of the washes according to the size of the flask/dish you are using.

Basic Protocol 3

It is critical that feeder cells be used that are mitotically inactive (see Basic Protocol 2). The animal should be hairless, and only newborn skin should be sterilized in 70% ethanol (do not sterilize embryonic skin with ethanol). When removing any muscle that may be left on the mouse skin, take care not to damage the dermis and the epidermis. Throughout the protocol, keep the skin samples wet—unless the protocol says otherwise—e.g., when incubating in trypsin we recommend 5 ml of trypsin as a guide; if you have a particularly large skin sample, make sure there is enough trypsin to cover the skin sample.

Take care when transferring the cultures to the incubator, as the dish is very full, which represents a potential source of contamination. It is recommended to remove the excess medium as soon as possible in this instance, but to leave enough time for any cells to attach.

It is important to check the cultures regularly for contamination with fibroblasts, which can take over the whole culture. (See Table 1 troubleshooting guide for details regarding fibroblast contamination).

The cells should be subcultured either when they are nearly confluent or if they are growing in large colonies. It can take an extended period of time to reach this level (3 or more weeks), but it is recommended to continue, even if you see very little growing. When trypsinizing the cells, it is important to check under the microscope that all the cells have detached, especially if the culture is sparse, in order to maximize the number of cells recovered. Feeders should be used until immortal cells take over the culture. Once this happens, CT can often be omitted (although it is best for the user to determine empirically whether the cells still require CT for routine culture). Immortal cells can be recognized because once the cells have senesced (around 4-6 weeks of culture), many cells fail to reattach at each subculture, and only a small fraction even of surviving melanocytes grows and forms colonies.

Basic Protocol 4

Treatment of cells pre-transfection

Cells should be treated well and remain healthy in the passages before transfection.

Table 1 Troubleshooting Guide for Isolation, Culture, and Transfection of Melanocytes

Protocol	Problem	Possible cause	Solution
Relevant to all protocols	Fungal or bacterial contamination	Multiple causes	This can be prevented by good aseptic technique and conditions, such as the following: a. All reagents and materials included for use in the protocol should be marked as sterile, and only opened in the laminar flow hood. All items should be sprayed with 70% ethanol before placing in the culture hood, and the culture hood should be cleaned thoroughly before use. If performing Basic Protocol 1 or 3, ensure that all forceps are sterilized in either a sterilizing oven or absolute ethanol before use. b. Wear laboratory safety clothing at all times during cell culture, ensuring gloves are pulled up under laboratory coats and no skin is exposed. c. Any individual with a cold or similar respiratory infection should at least wear a face mask or abstain from culturing during this time if possible. d. When transferring liquid from one container to another, ensure that no liquid touches the neck as this can produce a possible contamination pathway if the liquid contacts both interfaces when the container is closed. e. Only autoclaved or distilled water should be used to fill the water pan of the incubator, and this should be changed regularly. An antifungal compound, such as copper sulfate, can be added to the water if necessary. f. Regularly wipe the surface of the flask (do not spray) with 70% ethanol, or if cell growth is slow (e.g., close to senescence), trypsinize and transfer cells to a new flask around every 3 weeks. If diluting the cells less than 1 in 4 upon transfer, it is best to add soybean trypsin inhibitor to the harvested suspension (at 1 μg inhibitor per μg trypsin present) to help neutralize the trypsin. g. If fungal or yeast contamination occurs, treatment (as per the manufacturer's instructions) with an antifungal agent, such as Nystatin (e.g., Sigma, cat. no. N6261) could be tried to save the culture.
Basic Protocol 1	Unsatisfactory separation of the epidermis following dispase treatment	Skin sample too large or incubation period too short	Ensure that the skin is cut into pieces of 1 cm^2 or smaller before the initial iodine treatment, and that the skin is incubated in the dispase II/Hank's solution for ≥18 hr.

(*Continued*)

Table 1 Troubleshooting Guide for Isolation, Culture, and Transfection of Melanocytes, *continued*

Protocol	Problem	Possible cause	Solution
Basic Protocol 1	Keratinocyte contamination	Carry over during isolation protocol	Following isolation, diploid keratinocytes may be observed growing in culture as isolated colonies. However, these cells tend to die and detach from the flask before subculturing is required, and any remaining adhered cells will not proliferate after subsequent passaging.
Basic Protocol 1	Cells die during protocol	Low density of primary melanocytes following isolation	Initially, the plating density of primary melanocytes is low in comparison to the number of cells plated: ~30% of the 1.5×10^5 cells plated will adhere to the flask within 2-3 days. To prevent cell death, several points can be addressed: a. Ensure that any large pieces of peeled epidermis are torn or cut into smaller pieces before trypsin incubation. b. While carrying out step 4, transfer any removed piece of epidermis to the diluted trypsin quickly, to avoid drying of the skin. c. The epidermis should be incubated in trypsin for 15 min only; any longer than this increases the risk of cell death. d. Care should be taken not to shear the cells when creating the cell suspension in step 6; the suspension should be mixed carefully using a slow and steady speed. e. Following step 8, cells should be incubated on ice. Melanocytes are easily able to attach to plastic (even tubes), which is inhibited by low temperatures. The cells can be kept on ice for ~1 hr. f. During subculture, cells should be kept on ice as much as possible following step 14, to avoid loss and to ensure that the cell counts accurately represent the number of cells in culture. This is especially important should a growth curve graph be required.

(*Continued*)

Table 1 Troubleshooting Guide for Isolation, Culture, and Transfection of Melanocytes, *continued*

Protocol	Problem	Possible cause	Solution
Basic Protocol 1	Fibroblast contamination	Carry over during isolation protocol or excessive force during separation of dermis from epidermis	During isolation: two pairs of blunt forceps should be used to separate the dermis and the epidermis; one to grip each layer. The relevant forceps should then be used exclusively for that layer of skin to prevent fibroblasts being transferred to any other solutions or any skin samples (if processing more than one sample). If the epidermis remains 'stuck' to the dermis, do not forcibly pull the layers apart, as dermal fibroblasts may adhere to the epidermis. Only a small piece of epidermis is required; one or two pieces of skin 1 cm^2 in size are sufficient for a 25-cm^2 culture flask. Post-isolation: Fibroblasts grow rapidly and can take over a culture once present but can be removed by the addition of 75-150 μM geneticin (G418) to the culture medium for 3-4 days. Geneticin is not toxic to melanocytes under these conditions, and if the contamination has not subsided after a few more days, the addition can be repeated. If fibroblasts appear whilst culturing human melanocytes, EDN1 can be initially excluded as it can aid fibroblast growth.
Basic Protocol 2	XB2 cells retain ability to divide	Ineffective treatment with mitomycin C	Ensure that cells are not confluent and are still growing prior to treatment with mitomycin C. It is recommended to test 10^6 cells from each batch of feeder cells, as described. If any growing colonies are seen that batch should be discarded and replaced with fresh stocks.
Basic Protocol 3	Dermis does not easily separate from epidermis	Inadequate time in trypsin	When trying to split the dermis from the epidermis it is important not to force their separation, as fibroblast contamination may result. If skin does not split easily, place it back in the trypsin at 37°C and leave for a further 10-15 min. Repeat until the two layers split easily.
	Fibroblast contamination		See above advice for Basic Protocol 1
Basic Protocol 4	Unreliable data	Initial plating density not determined	Cell density can affect the quality and reliability of the results. This is particularly true when investigating parameters that affect cell growth or death. Select a range of seeding densities, perform the transfections, and then assess the results. For example, in a screen of siRNAs involving growth for 5 further days, the optimized seeding density for melanoma cells was 2000-3000 per well. This ensured that a reasonable density of cells remained in both positive and negative control wells.

This will increase the quality and reliability of the results.

Seeding density

It is vital that the seeding density for each cell line be optimized. Ideally, this should be performed before any large-scale transfections are performed.

siRNA master plate

Use an siRNA master plate to ease the transfer of siRNAs to the experimental plates. This will reduce errors and shorten the time it takes to transfect each plate.

siRNAs

It is ideal to use a number of siRNAs (as a pool or separately) to target the same gene. This increases the efficiency of transfection (and the cost of the experiment).

Control siRNAs

At least three control siRNAs are recommended:

Positive control: Choose an siRNA that will induce the required effect (growth arrest, for example).

Negative control: Choose a scrambled siRNA.

Transfection positive control: Use an siRNA that is toxic for the cell and therefore is a good test of transfection efficiency. A good example of this is AllStars Hs Cell Death Control siRNA from Qiagen.

Pipetting

The use of fresh pipette tips is important to reduce cross-contamination of siRNAs. Thorough mixing of solutions is paramount to the success of the transfections.

Troubleshooting

Table 1 summarizes commonly encountered technical issues along with suggested solutions.

Anticipated Results

Basic Protocol 1

The inclusion of only one to two pieces of adult skin 1 cm^2 in size is sufficient to yield 1.5 $\times$ 10^5 cells. The culture will become confluent after 3 weeks. Subsequent passages will take between 10 and 14 days to become confluent; later passages take up to around a month. Fibroblasts and especially keratinocytes are commonly found within this first passage, but growth of these is easily remedied as described in the troubleshooting section. An example of growing human melanocytes can be seen in Figure 2A. Figure 2B shows senescent melanocytes, which are characterized by their large, flat appearance.

Basic Protocol 2

If using XB2 cells as feeder cells for primary cultures, it is advisable to make a large stock of mitomycin C-treated cells. Once tested to make sure the cells are no longer dividing, stocks can be stored in liquid nitrogen and used when required. From a 175-cm^2 flask (35 ml culture), the expected yield of mitomycin C-treated cells is $\sim 1 \times 10^7$ cells, and we store these in liquid nitrogen in aliquots of either 0.5×10^6 or 1×10^6 per vial, giving 10-20 vials of mitomycin C-treated cells. Figure 2C shows a growing primary culture from mouse skin containing melanocytes, melanoblasts, and feeder cells.

Basic Protocol 3

Melanocyte colonies should form after roughly 2-3 weeks; however, this may take longer, and so it is recommended to persevere. The diploid cells will grow for only a few passages under the conditions described. However, ~ 1 in 10^6 normal mouse melanocytes spontaneously becomes immortal, and these form growing colonies of small, less-pigmented cells which should all survive and proliferate after subculture. If any melanocytes are proliferating by 3 months after primary culture, they are almost certainly immortal. It may take up to 6 months to obtain enough of the immortal cells to freeze stocks. Note that this time can be greatly shortened by crossing the required genotype of mouse with *Cdkn2a* (*Ink4a-Arf*) null mice, as *Cdkn2a* null melanocytes do not senesce at all (Sviderskaya et al., 2002). Figure 2D shows a culture of mouse melanocytes, with the appearance of senescent melanocytes, which are larger and highly pigmented.

Basic Protocol 4

Post-transfection, cells can be permeabilized and fixed for subsequent cell and nuclear staining, using established staining protocols (not described here; Soo et al., 2011). If using siRNAs that are anticipated to inhibit growth or induce cell death, for example, a simple pan-nuclear stain such as DAPI could be used. In this case, the following results would be expected:

Positive siRNA control (e.g., inhibits growth): cell number will decrease.

Negative siRNA control (e.g., growth unaffected): cell number will increase.

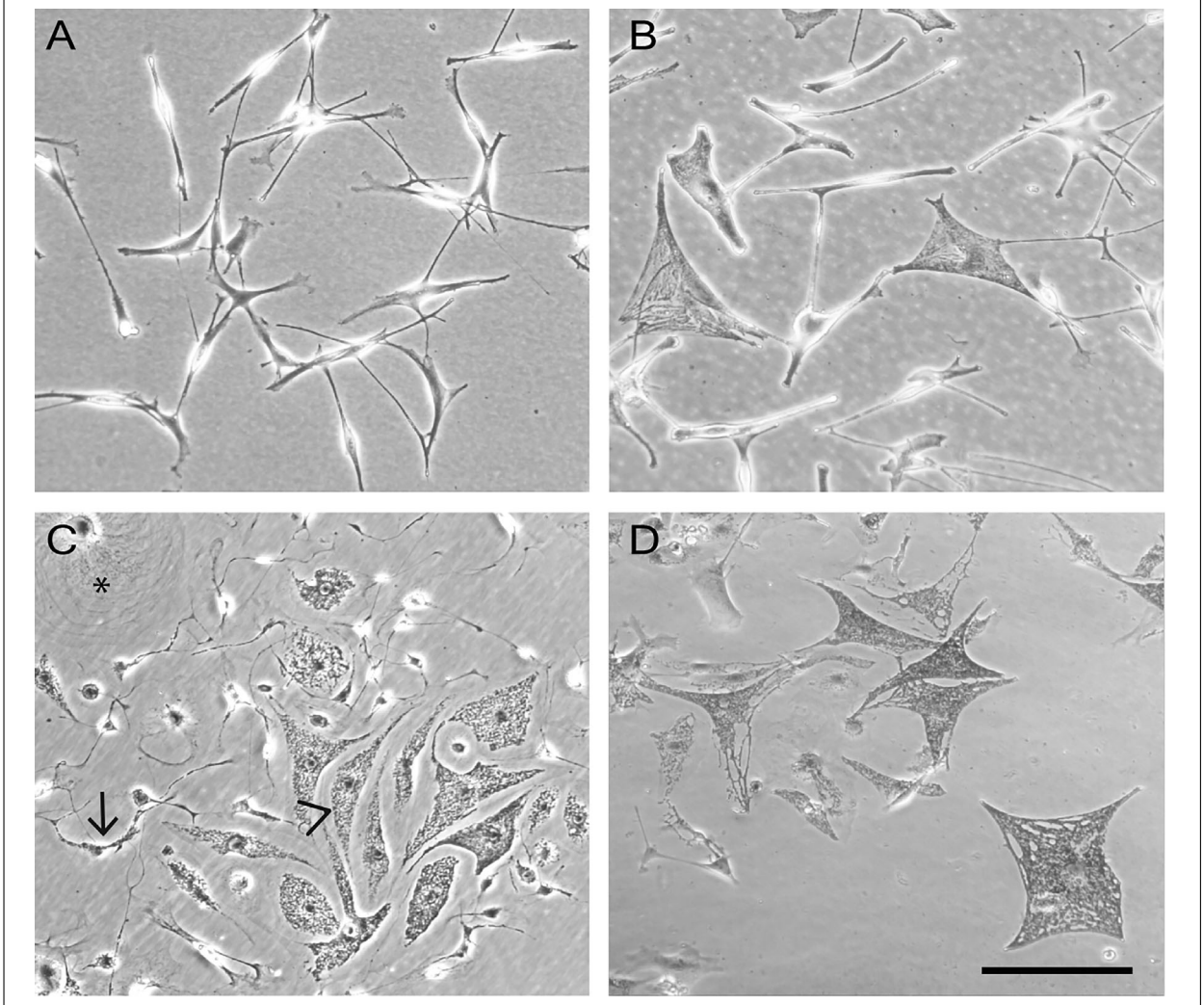

Figure 2 (**A**) Growing human melanocytes showing elongated, bi- and tri-polar morphology. (**B**) Human melanocytes showing appearance of larger, flatter senescent cells. (**C**) Growing primary culture from mouse skin showing melanocytes (ˆ)(elongated and bi-polar), melanoblasts (↑), and feeder cells (*). (**D**) Mouse melanocytes showing the appearance of senescent mouse melanocytes which are larger and show increased pigmentation. Scale bar = 200 µm.

Test siRNA: Cell number will increase or decrease depending on the targeted gene.

Time Considerations

Basic Protocol 1

The protocol for the preparation of the skin sample and isolation of melanocytes is described in steps 1-11 and performed over 2 days. The preparatory stages of cleaning and submerging the skin in dispase solution should take ∼30 min, and the second day protocol consisting of the removal of the epidermis and isolation of melanocytes will take 1-2 hr, depending on the number of samples received.

The RPMI growth medium should be changed every 3-4 days, taking no longer than 5 min per flask, and subculturing of cells at subsequent passages as described in steps 13-17 will take ∼30 min. If freezing the cells, allow a further 15-30 min, depending on the number of cryotubes required.

As mentioned previously, the primary melanocyte cell culture at passage 0 will take ∼3 weeks to become confluent, with subsequent passages taking 10-14 days. The time required to reach confluency will increase with age of the cell culture; if the cells are near senescence, this may take as long as 6 weeks. In general, it will take primary melanocyte cultures about 3-12 months to become fully senescent, varying with donor age, among other parameters.

Basic Protocol 2

The subculture of XB2 keratinocytes can be achieved in ∼20-25 min, depending on the time taken for the cells to detach from the

flask. When preparing mitomycin C-treated XB2 keratinocytes, the total incubation time is between 3 and 3.5 hr, with subsequent incubation in DMEM growth medium for 10 min to elute any remaining mitomycin C. This is then followed by harvesting and freezing of the treated cells. Batch testing takes ∼2 weeks.

Basic Protocol 3

The plating out of mitomycin C-treated XB2 keratinocytes can be achieved in ∼15-25 min and should be carried out 1-3 days before the rest of the protocol. The number of skin samples determines the time for the explantation protocol. If preparing more than one skin sample, it is recommended to space them out throughout the day, for example, half the samples in the morning and the rest in the afternoon. For one sample, the initial period (steps 2-6) should take 15-20 min. This is then followed by a 1 hr incubation. If the skin is able to be split after this period, then the remaining steps (8-19) should take 15-20 min (if not able to be split, place back into the trypsin and check every 10-15 min to see if skin can be split). After practice and increased familiarity with the technique, ∼8 skin samples can be processed in a day. Subculture should take between 15 and 20 min (depending on how easily the cells detach). The cells start to senesce around 4-6 weeks after explantation. Time for immortalization is discussed above.

Basic Protocol 4

Pre-transfection

It is vital that cells are healthy and growing well before starting the protocol. Do not allow the cells to become 100% confluent in the passages prior to transfection, as this can change the results.

Day 1

Cells are transfected and seeded on the same day. While learning, one 96-well plate per cell line would be advised to ensure efficient transfection. However, with practice, five to ten plates could be transfected in one day.

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Author Contributions

Philip S. Goff: Writing - original draft, writing - review and editing; **Joanna T. Castle:** Writing - original draft; **Jaskaren S. Kohli:** Writing - original draft; **Elena V. Sviderskaya:** Methodology, supervision, writing - review and editing; **Dorothy C. Bennett:** Methodology, supervision, writing - review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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Eisinger and Marko (1982). See above.

A description of the first long-term culture of human melanocytes.

Sviderskaya et al. (1997). See above.

Includes the current mouse melanocyte culture method.

Internet Resources

https://www.sgul.ac.uk/about/our-institutes/molecular-and-clinical-sciences/research-sections/cell-biology-research-section/genomics-cell-bank/materials-and-methods

Our materials and methods page with further and related details for melanocyte culture methods.

https://www.sgul.ac.uk/about/our-institutes/molecular-and-clinical-sciences/research-sections/cell-biology-research-section/genomics-cell-bank/cell-bank-holdings

Web site of the Functional Genomics Cell Bank at St. George's, University of London with access to, among others, mouse melanocyte and melanoblast lines carrying a variety of pigmentary mutations.

http://www.ifpcs.org/colorgenes/

Information on the mapped color genes in mice, zebrafish, and human models.