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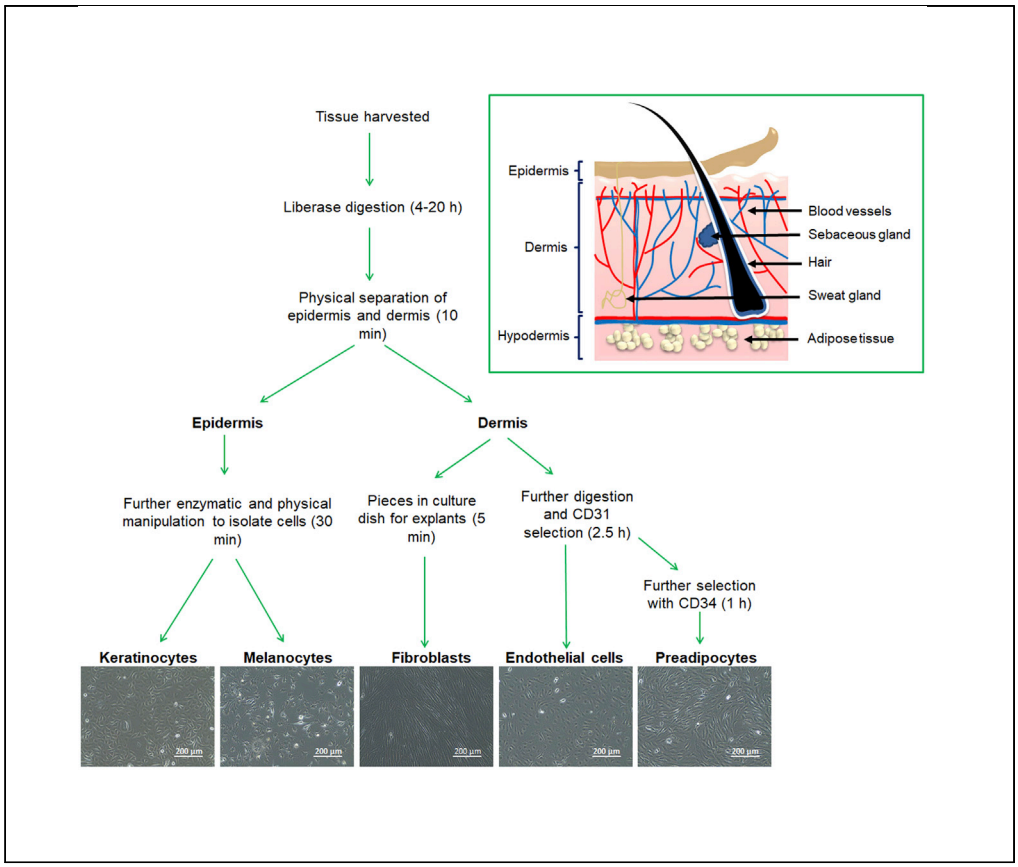
Isolation of five different primary cell types from a single sample of human skin



We have developed a technique to isolate primary keratinocytes, melanocytes, fibroblasts, preadipocytes, and microvascular endothelial cells from an individual sample of human skin. The protocol describes step-by-step instructions for processing, cells isolation, and culture of neonatal foreskin, with adaptation for more demanding adult tissues. The availability of multiple isogenic cell types derived from individual skin samples offers the ability to investigate various areas of biology, in the context of cell-type specificity without potential confounding influence of inter-individual or genetic differences.

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Highlights

Protocol to isolate five different primary cell types from human skin sample

Modification for more demanding adult tissue

Extensive characterization of each cell type

Expansion and cryopreservation for biobanking

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Isolation of five different primary cell types from a single sample of human skin

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SUMMARY

We have developed a technique to isolate primary keratinocytes, melanocytes, fibroblasts, preadipocytes, and microvascular endothelial cells from an individual sample of human skin. The protocol describes step-by-step instructions for processing, cells isolation, and culture of neonatal foreskin, with adaptation for more demanding adult tissues. The availability of multiple isogenic cell types derived from individual skin samples offers the ability to investigate various areas of biology, in the context of cell-type specificity without potential confounding influence of inter-individual or genetic differences.

For complete details on the use and execution of this protocol, please refer to Holliman et al. (2017), Horvath et al. (2019), Horvath et al. (2018), Kabacik et al. (2018), Lowe et al. (2020), Lu et al. (2019), and Lu et al. (2018).

BEFORE YOU BEGIN

Timing: 2 h reagents preparation, 1–24 h vessel coating

Over the past half century, established cell lines have been the workhorse of biological research that permitted remarkable advancements in our understanding of biological processes. Indeed, they are still used extensively for many processes from vaccine development to drugs screening (Allen et al., 2005; Folegatti et al., 2020; Pommerenke et al., 2021). Cell lines offer affordable source of large quantity of material for research as they are often grown in non-demanding conditions and are hence, easy to maintain and manage. However, being mostly of cancer origin, or ectopically immortalized or transformed, cell lines often retain little of the features that characterize normal cells that constitute their tissues of origin. Despite this, biological understanding drawn from the use of these cells has been inappropriately extrapolated and generalized to all cell types, and even to normal cells. There is a growing appreciation for the need to use primary cells in biological research and to limit conclusions to the cell types used in investigations. Although these two principles are readily understood and agreed by all, their execution is nevertheless very challenging. Access to healthy tissues is perhaps the biggest challenge, followed by the sparsity of easy and robust methods to isolate most cell types. Primary cells are also found to be more sensitive to culture conditions and require greater effort and more demanding conditions to maintain. Additionally, by their very nature, primary cells, unlike cell lines, have limited lifespan and proliferative capacity. Immortalization of primary cells with human telomerase can circumvent this issue, although some primary cells, such

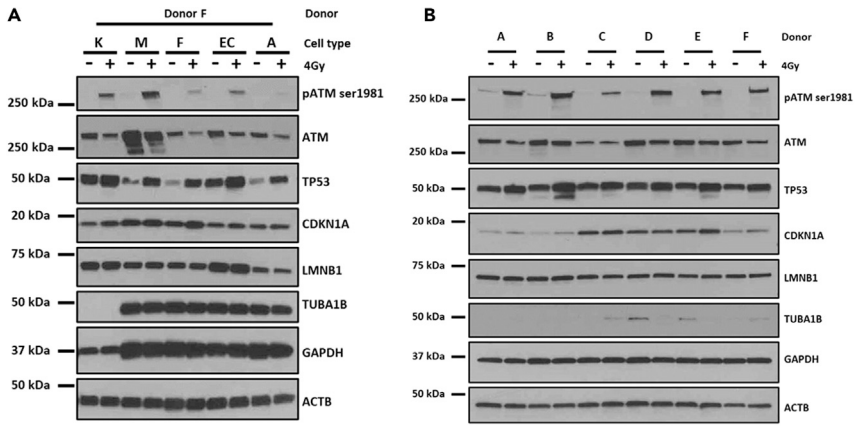

Figure 1. Intra- and inter-individual variability in protein expression in response to ionizing radiation

Cells were exposed to sham dose or 4 Gy of X-ray and collected for western blot analysis 2 h after exposure.
(A) Western blot analysis of 5 different cell types isolated from the same neonatal donor; K – keratinocytes, M – melanocytes, F –Fibroblasts, EC – microvascular endothelial cells, A – preadipocytes.
(B) Western blot analysis of 6 different donors of neonatal keratinocytes; donor F is the same one as in (A). The following antibodies were used for western blotting: Ataxia Telangiectasia Mutated (ATM), Abcam (ab32420, dilution 1:5,000); pATM ser 1981, Abcam (ab81292, dilution 1:5,000); Tumor Protein P53 (TP53), Santa Cruz (sc-126, dilution 1:10,000); Cyclin Dependent Kinase Inhibitor 1A (CDKN1A), Cell Signalling (2947, dilution 1:30,000); Lamin B1 (LMNB1), Abcam (ab16048, dilution 1:5,000); Tubulin Alpha 1b (TUBA1B), Proteintech (66031-1-AP, dilution 1:100,000); Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Proteintech (60004-1-Ig, dilution 1:100,000); Actin beta (ACTB), Proteintech (20536-1-AP, dilution 1:5,000).

as human keratinocytes, cannot be immortalized in this way (Dickson et al., 2000). These challenges have curtailed the availability of these cells to the research community. As a consequence, primary cells that are available from various suppliers are often very expensive and with very limited proliferation capacity or lifespan. The protocol outlined here will contribute to the alleviation of this problem.

Skin is an excellent source of primary human cells. It is composed of many different cell types, and the two major constitutes – keratinocytes and fibroblasts, which form the epidermis and dermis respectively, originate from two different germ layers; ectoderm (Fuchs, 2007) and mesoderm (Olivera-Martinez et al., 2004), respectively, increasing its attractiveness and the potential usefulness of cells derived from it.

The protocol we present here allows robust isolation of multiple cell types from the same donor, and this presents interesting possibilities of investigating intra-individual variability in response to various factors without confounding differences between individuals (Figure 1A), a fact which is often underappreciated by researchers. Conversely, this protocol allows the easy acquisition of cells from many donors, permitting investigations into inter-individual differences in response to various factors (Figure 1B). These issues are particularly relevant in the face of reproducibility crisis in science.

As a base for protocol development, we used Normand and Karasek's method reported for isolation of keratinocytes, fibroblasts and microvascular endothelial cells (Normand and Karasek, 1995), which we refined in both the cell isolation and culturing conditions and then adapted it to suit the more demanding adult tissues. The resulting protocol allows robust isolation of keratinocytes, melanocytes, fibroblasts, preadipocytes and microvascular endothelial cells, all from a single piece of tissue. The isolated cells retain their morphology and cell-specific properties in long term culture and can be used in a variety of applications. The diversity of isolated cells can be reconstituted in 3D skin models and employed in cardiovascular and obesity research, just to name a few. The protocol described here allows harvesting of primary cells from human skin by any competent operator

who is familiar with standard cell culture techniques and has good level of dexterity. Cells are ready for passing/freezing within 7 days for neonatal tissues and up to 1 month for adult ones.

1. Ensure that ethics approval is in place and patient consent is obtained prior to tissue collection and processing.
2. All reagents and media should be prepared in biological safety cabinet and kept sterile.
3. Make sure all media, reagents and consumables are ready and of sufficient quantity.
4. On the day the tissue arrives, coat appropriate vessels for cell isolation.

Note: All centrifugations are performed at 250 g at 19 °C–21°C.

Preparation of tissue transport medium

Timing: 10 min

5. Mix 129 mL of DMEM, 15 mL of FBS (10% final concentration), 4.5 mL of Penicillin/Streptomycin (3× normal concentration) and 1.5 mL of Gentamycin/Amphotericin B (5× normal concentration) to prepare 150 mL of tissue transport medium.
6. Aliquot 15 mL per 25 mL sterile tube, label batch, date, and store at 4°C. The medium is stable for at least 1 month from the date of preparation.

Preparation of liberase solution

Timing: 10 min

7. Dissolve liberase powder in sterile water to a concentration of 5 mg/mL. Store 0.5 mL aliquots at −20 °C.

Preparation of collagenase solution

Timing: 15 min

8. Dissolve collagenase powder in HBSS with Ca^{2+} and Mg^{2+} to obtain 0.25% solution. Store 5 mL aliquots at −20 °C.

⚠ CRITICAL: HBSS with Ca^{2+} and Mg^{2+} must be used as Ca^{2+} ions are essential for collagenase activity.

Preparation of coating solutions

Timing: 5 min

9. To prepare collagen I/fibronectin coating solution, dilute both 1:100 in HBSS and store at 4 °C. This solution can be reused once as long as sterility is maintained.
10. To prepare gelatin coating solution, dilute stock gelatin 1:100 in HBSS and store at 4 °C.

Preparation of CD34 Dynabeads

Timing: 1 h

Note: Use 1 μg of CD34 antibody per 25 μL of Dynabeads, larger volumes can be prepared in advance and stored at 4 °C.

11. Resuspend the Dynabeads in the vial by vortexing for 30 s.
12. Transfer appropriate volume of Dynabeads to a 5 mL FACS tube and add the same volume of Isolation Buffer, or at least 1 mL to resuspend the beads.
13. Place the tube in a magnet for 1 min and aspirate supernatant.
14. Remove the tube from the magnet and resuspend the washed Dynabeads in the same volume of Isolation Buffer as the initial volume of Dynabeads.
15. Add desired amount of CD34 antibody and incubate for at least 30 min at 19 °C–21°C with gentle rotation.
16. Place the tube in a magnet rack for 1 min and aspirate supernatant.
17. Remove the tube from the magnet and add 2 mL Isolation Buffer.
18. Repeat steps 16 and 17 to wash off excess of antibodies.
19. Place the tube in a magnet rack for 1 min and aspirate supernatant.
20. Remove the tube from the magnet and resuspend the washed Dynabeads in the same volume of Isolation Buffer as the initial volume of Dynabeads.
21. Store the CD34-coated Dynabeads at 4 °C, they are stable for 12 months.

Preparation of cell culture media

Timing: 30 min

22. To prepare complete CnT-07 (CnT) medium, thaw supplements A, B and C provided with the medium at 19 °C–21°C and add them to the basal CnT medium, mix well and store in the dark at 4 °C for up to 6 weeks.

⚠ CRITICAL: Supplement C precipitates in CnT medium, always add supplement C to the CnT basal medium not the other way around.

23. To prepare complete Endothelial Cell Growth Medium MV (ECMV), melanocytes and pre-adipocytes media, thaw the supplements vials provided with the media at 19 °C–21°C and add to the appropriate basal medium. Mix well and store at 4 °C, use within 6 weeks.

⚠ CRITICAL: Melanocyte medium is light and temperature sensitive, make sure you warm up required volume only and protect it from light.

24. To prepare fibroblast medium supplement DMEM with 10% FBS, mix well and store at 4 °C.

Vessel coating

Timing: 1–24 h

Note: Keratinocytes are grown on collagen/fibronectin-, while melanocytes, microvascular endothelial cells, preadipocytes and adult fibroblasts are cultured on gelatin-coated vessels. Culturing of neonatal fibroblasts does not require coating. We typically grow neonatal foreskin cells in T75 flasks and adult tissue cells in smaller vessels such as 6-well plates or 6 cm dishes depending on the size of the tissue.

25. Depending on tissue size, coat a T75 flask with 2 mL, 6 cm plate with 1 mL or 6-well plate with 0.5 mL of appropriate coating solution.
26. Tilt and shake the vessels to ensure even distribution of coating solution and place it in tissue culture incubator (37 °C, 5 % CO_2) for at least 1 h up to 24 h.

Institutional permissions

Human skin samples used to establish this protocol were collected from neonatal, routine circumcision or adult facial skin following minor dermatology procedures with informed consent and ethical approval from Oxford Research Ethics Committee; reference 10/H0605/1. Researchers must obtain their own ethical approval for this protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD34 Mouse anti-Human, Unlabeled, Clone: 563, (dilution 1:12.5)	BD Biosciences	Cat#550760 RRID: AB_393870
Biological samples		
Human skin biopsies	Dermatology clinics or commercial suppliers	N/A
Chemicals, peptides, and recombinant proteins		
Y-27632 dihydrochloride	Abcam	Cat#ab120129 CAS:129830-38-2
Critical commercial assays		
CD31 Dynabeads	Life Technologies	Cat#11155D
Dynabeads™ Pan Mouse IgG	Invitrogen	Cat#11041
Other		
Dulbecco's Modified Eagle's Medium (DMEM) - high glucose	Sigma-Aldrich	Cat#D6429
Foetal Bovine Serum (FBS), qualified, heat inactivated	Gibco	Cat#10500064
CnT-07 Epithelial Proliferation Medium (CnT)	CELLnTEC	Cat#CnT-07
Endothelial Cell Growth Medium MV (ECMV)	PromoCell	Cat#C-22020
Melanocyte Growth Medium M2 (M2)	PromoCell	Cat#C-24300
Human Preadipocyte Growth Medium	Cell Applications	Cat#811-500
Trypsin-EDTA solution, 10×	Sigma-Aldrich	Cat#T4174-100mL
Soybean Trypsin Inhibitor, powder	Life Technologies	Cat#17075029
Gentamycin/Amphotericin B	Life Technologies	Cat#R01510
Fibronectin	Sigma-Aldrich	Cat#F0895
Gelatin	Sigma-Aldrich	Cat#G1393
Corning® Collagen I, Rat Tail	Corning	Cat#354236
Liberase DH	Roche	Cat#5401089001
Collagenase	Sigma-Aldrich	Cat#C9407
Bovine Serum Albumin (BSA) solution 30% sterile	Sigma-Aldrich	Cat#A9576-50mL CAS: 9048-46-8
BD Cell Separation Magnet	BD Biosciences	Cat#552311
BD Falcon Round Bottom Polystyrene Tube 5 mL With Cap	BD Biosciences	Cat#352063
Falcon 70 µm White Cell Strainer for 50 mL tube	Corning	Cat#352350
Forceps Watchmaker 7 StSteel	SLS	Cat#INS4360
Microscopy scissors, straight, sharp	VWR	Cat#233-2121
Phase-contrast light microscope	N/A	N/A
Corning™ CoolCell™ Cell Freezing Vial Containers	Corning	Cat#432000
Penicillin-Streptomycin	Sigma-Aldrich	Cat#P4333-100ML
Hanks' Balanced Salt solution (HBSS)	Sigma-Aldrich	Cat#H6648
Gibco™ HBSS, with calcium and magnesium	Fisher Scientific	Cat#11550456
Ethylenediaminetetraacetic acid disodium salt solution 0.5 M, pH 8.0 (EDTA)	Sigma-Aldrich	Cat#03690-100ML

MATERIALS AND EQUIPMENT

This protocol requires standard cell culture consumables and laboratory setup.

Tissue transport medium		
Reagent	Final concentration	Amount
FBS	10 %	15 mL
Penicillin/Streptomycin 100×	300 U / 0.3 mg/mL	4.5 mL
Gentamycin/Amphotericin B 500×	1.25 μg/mL / 0.05 mg/mL	1.5 mL
DMEM	n/a	129 mL
Total	**n/a**	**150 mL**
Store at 4 °C for up to one month.		

Isolation Buffer		
Reagent	Final concentration	Amount
BSA 30 %	0.1 %	167 μL
Filter sterilized EDTA 0.5 M	2 mM	200 μL
HBSS	n/a	49.63 mL
Total	**n/a**	**50 mL**
Store at 4 °C for up to one year.		

Soybean Trypsin Inhibitor stock solution 10×		
Reagent	Final concentration	Amount
Soybean Trypsin Inhibitor	1 mg/mL	1 g
HBSS	n/a	1 L
Total	**n/a**	**1 L**
Store at −20 °C for up to one year.		

Y-27632 stock solution		
Reagent	Final concentration	Amount
Y-27632 dihydrochloride	10 mM	50 mg
Water	n/a	15.6 mL
Total	**n/a**	**15.6 mL**
Filter sterilize and store at −20 °C for up to one year.		

Liberase solution		
Reagent	Final concentration	Amount
Liberase	5 mg/mL	100 mg
Water	n/a	20 mL
Total	**n/a**	**20 mL**
Store at −20 °C for up to one year.		

Collagenase solution		
Reagent	Final concentration	Amount
Collagenase	0.25 %	500 mg
HBSS with Ca^{2+} and Mg^{2+}	99.75 %	200 mL
Total	**n/a**	**200 mL**
Store at −20 °C for up to one year.		

⚠ CRITICAL: HBSS with Ca^{2+} and Mg^{2+} must be used as Ca^{2+} ions are essential for collagenase activity.

Collagen I/fibronectin coating solution		
Reagent	Final concentration	Amount
Collagen I	1 %	0.25 mL
Fibronectin	1 %	0.25 mL
HBSS	98 %	24.5 mL
Total	**n/a**	**25 mL**
Store at 4 °C for up to six months.		

Note: This solution can be reused once if it is kept sterile.

Gelatin coating solution		
Reagent	Final concentration	Amount
Gelatin	1 %	0.25 mL
HBSS	99 %	24.75 mL
Total	**n/a**	**25 mL**
Store at 4 °C for up to six months.		

Fibroblast medium		
Reagent	Final concentration	Amount
FBS	10 %	50 mL
DMEM	90 %	450 mL
Total	**n/a**	**500 mL**
Store at 4 °C for up to six months.		

Freezing Mix		
Reagent	Final concentration	Amount
DMSO	10 %	5 mL
FBS	90 %	45 mL
Total	**n/a**	**50 mL**
Store at 4 °C for up to a month.		

STEP-BY-STEP METHOD DETAILS

Preparation of a tissue specimen

Timing: 4–20 h

This part of the protocol describes initial tissue processing. The skin is cut into small pieces and digested in liberase.

Note: Tissue specimen should be placed in sterile cold transport medium. The medium volume can be adjusted according to the tissue size. We commonly use 15 mL of medium for neonatal foreskin samples, and less for smaller adult samples, provided the tissue is completely immersed in the liquid. The tissue can be stored for a short period at 4°C in the tissue transport medium and we have successfully isolated cells from tissues stored this way for up to 48 h.

1. Thaw 0.5 mL liberase aliquot and mix it with 4.5 mL of cold CnT and double concentration of antibiotics (100 μL of penicillin/streptomycin and 20 μL of Gentamycin/Amphotericin B) in a 15 mL tube.

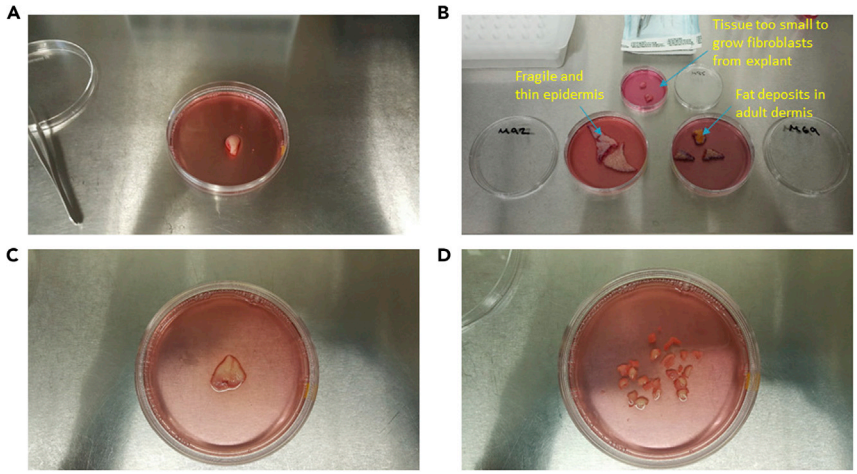

Figure 2. Initial tissue processing

(A) Neonatal foreskin tissue in 10 cm dish.

(B) Adult facial skin tissue samples from 92 and 63 years old male donors in 10 cm dishes and skin sample from 85 years old male in 6 cm dish.

(C) Neonatal foreskin cut open into a single sheet.

(D) Neonatal tissue cut into small pieces for liberase digestion.

⚠ CRITICAL: The liberase mix should be cold to prevent over-digestion of the epidermis. This is particularly important for the adult samples.

2. Empty tissue and transport media into sterile 10 cm plate (Figures 2A and 2B).

Note: Neonatal tissues are usually larger than adult ones. Adult skin samples often contain large fat deposits which should be trimmed. Adult epidermis is thin and fragile, and it should be handled with care.

3. Prepare the tissue according to neonatal foreskin protocol **a**, or adult tissue protocol **b**.
   a. Neonatal foreskin protocol.
      i. Using sterile scissors and forceps, cut down the midline of the foreskin to open to a single sheet (Figure 2C).

Note: Meticulous clean-up is not required – although this helps penetration of enzyme. Any large blood clots can be removed but removing further tissue is not advised as it reduces cell yield, particularly for microvascular endothelial cells.

   b. Adult tissue protocol.
      i. Using sterile scissors and forceps trim any fat from the dermis layer.

⚠ CRITICAL: The fat should be removed completely as it will interfere with liberase digestion.

      ii. Collect trimmed fat into a tube and add some of the tissue transport medium to keep the fat submerged and store at 4 °C.

Note: The fat is well-vascularized and its inclusion in the isolation of microvascular endothelial cells and preadipocytes significantly increases cell yield.

4. Cut the tissue into pieces of 5–6 mm^2 (Figure 2D).

⚠ CRITICAL: The pieces should never be more than 7 mm in diameter to ensure efficient penetration of liberase. The tissue can be cut into smaller pieces if faster digestion time is required.

Note: It is better to cut tissue pieces into strips rather than squares as it improves enzyme penetration. Avoid cutting tissue into very small pieces as it makes it very hard to distinguish epidermis from the dermis, especially for light colored skin samples.

5. Transfer the tissue pieces into 15 mL tube with liberase solution and invert gently.
6. Incubate the tissue for 16–20 h at 4 °C with the tube lying on its side to ensure that all the pieces are completely submerged.
   a. Neonatal foreskin protocol.
      i. Alternatively, for same-day processing, incubation can be done at 19 °C–21°C with slow rotation of the tube for 3–4 h.

Note: Same-day processing is not recommended for adult tissues as it usually leads to over-digestion of epidermis and poor keratinocytes recovery.

Pause point: There is a stopping point when tissue is digested in liberase.

Primary cell isolation

Timing: 4 h

After digestion in liberase, the epidermis is peeled off from the dermis and both are enzymatically digested and physically manipulated to produce single cells suspensions. Keratinocytes and melanocytes are isolated from epidermis, whereas dermis is the source for fibroblasts, microvascular endothelial cells and preadipocytes.

⚠ CRITICAL: Make sure all vessels are coated before starting this step.

7. Thaw an aliquot of collagenase.
8. Prepare three 6 cm dishes, add 5 mL of HBSS to one of them, 3 mL of trypsin to another and leave the third one empty.
9. Decant the digested tissue pieces into an empty 6 cm dish (Figure 3A).
10. Take one piece of tissue at a time and hold the dermis with a pair of forceps and peel the epidermis layer off with another pair of forceps; place the epidermis in the dish with trypsin and transfer the dermis to the dish with HBSS (Figure 3B).

⚠ CRITICAL: Adult epidermis is more fragile and more prone to tearing than neonatal ones; be gentle.

Note: When digestion is complete, epidermis should separate in one piece when pulled gently from the corner. If this does not happen, continue incubation of the tissue with liberase for another hour at 4 °C.

11. When all epidermis and dermis pieces are separated, use the back of the curved forceps to pull apart/rub the epidermis on the bottom on the dish to remove as many large floating pieces of epidermis as possible (Figure 3C).
12. Incubate the epidermis for 10 min at 19 °C–21°C to digest it.

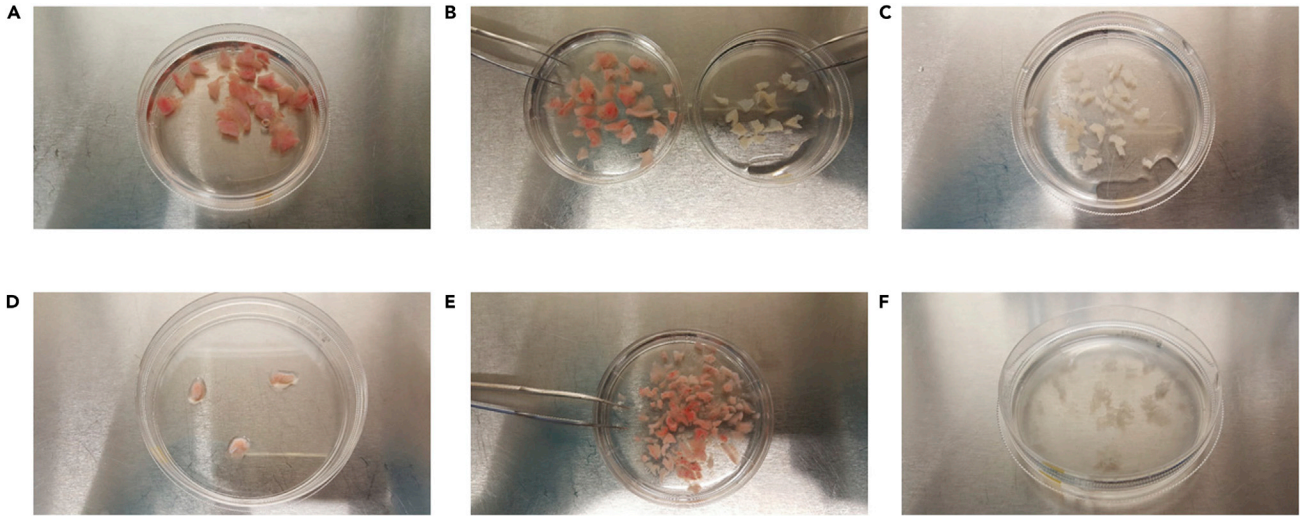

Figure 3. Tissue processing after liberase digestion

(A) Neonatal foreskin tissue in 6 cm dish after 16–20 h digestion in liberase. Please note that the tissue pieces are less compact now.

(B) Epidermis and dermis separated, please note that dermis pieces have pale, "top" side and vascularized "bottom" side.

(C) Mechanically disintegrated epidermis, epidermis pieces are no longer floating on the surface of the solution.

(D) Pieces of dermis prepared for fibroblast outgrowth, the vascularized side of dermis should be facing up.

(E) Minced dermis prepared for collagenase digestion.

(F) Digested epidermis repeatedly pipetted up and down to release cells from the tissue. Note that solution became cloudy. It is normal to see pieces of epidermis still remaining in the plate.

13. In the meantime, prepare the dermis for fibroblast isolation.
    a. Neonatal foreskin protocol.
        i. Place 2–3 pieces of the dermis with the de-epidermized (pale) side face down in the 10 cm uncoated plate (Figure 3D).
        ii. Leave the dish open in the hood for about 15 min to dry the tissue slightly to help it attach to the plate.
        iii. Add 1 mL of warm fibroblast medium supplemented with double concentration of antibiotics to the plate to almost cover the tissue pieces but not entirely.

    Note: Be careful not to dislodge attached dermis pieces, they should not be floating in the dish.

        iv. Carefully place the dish in the tissue culture incubator.
        v. At the end of the day carefully add 4 mL of warm fibroblast medium supplemented with double concentration of antibiotics to the plate.
    b. Adult tissue protocol.
        i. If the tissue is sufficiently large to yield at least 3 pieces in step 4, (Figure 2B), place a piece of de-epidermized (pale) dermis side face down in gelatin-coated well of a 6-well plate (aspirate gelatin from the plate before placing the piece of tissue).
        ii. Leave the dish open in the hood for about 15 min to dry tissue slightly to help it attach.
        iii. Add 0.25 mL of warm fibroblast medium supplemented with double concentration of antibiotics to the plate concentrating on tissue piece.

    Note: Be careful not to dislodge attached dermis pieces, they should not be floating in the dish.

        iv. Carefully place the dish in the tissue culture incubator.

v. At the end of the day, carefully add 0.25 mL more of warm fibroblast medium supplemented with double concentration of antibiotics to the plate.

Note: Growing adult fibroblasts from explants yields purer population than growing them from CD31, CD34 negative fraction (point 51b), however they grow very slowly and have lower proliferation potential.

14. Mince the remaining dermis pieces with scissors (Figure 3E) and transfer the pieces into a tube with collagenase.
    a. Adult tissue protocol.
        i. Mince with scissors any fat trimmings from the previous day and combine it with dermis pieces.
        ii. Use 2.5 mL of collagenase solution for digestion.
15. Digest dermis pieces in 37 °C water bath with frequent agitation until the tissue is completely digested (between 45 min and 1.5 h).
    a. Adult tissue protocol.
        i. After about 30 min of incubation the fat layer will appear on the top of the tube. Centrifuge the tube at 250 g for 5 min at 19 °C–21°C and aspirate the fat layer together with the supernatant.
        ii. Add the remaining 2.5 mL of collagenase solution to the tissue pieces and continue digestion until tissue dissolves.

Note: Adult tissues contain more extracellular matrix than neonatal ones and take longer to digest. Do not exceed 1.5 h of total incubation time even if some tissue pieces remain.

16. Add 3 mL of trypsin inhibitor to digested epidermis and vigorously pipet the solution up and down with a 1 mL pipette tip to further dissociate single cells from epidermis (Figure 3F).
17. Split the digested epidermis into two 15 mL tubes and centrifuge at 250 g for 5 min at 19 °C–21°C.
18. Aspirate collagen/fibronectin and gelatin solutions from the coated vessels.
19. Remove the supernatant from each 15 mL tubes and resuspend the pellets by flicking the tubes a couple of times.
20. Add 10 mL to T75, 5 mL to 6 cm plate or 2 mL to 6-well plate of warm M2 medium supplemented with double concentration of antibiotics to one of the tubes and appropriate volume of warmed CnT supplemented with double concentration of antibiotics and 10 μM Y-27632 dihydrochloride to the second tube.

Note: Y-27632 greatly improves yield of isolated keratinocytes, especially for the adult ones, it also extends their lifespan, however it can also have an impact on downstream applications.

21. Seed resuspended melanocytes and keratinocytes into appropriate vessels and place in the incubator.
22. Once pieces of dermis are digested, add 9 mL HBSS to the tube to reduce viscosity and pass the cell suspension through 70 μm cell strainer into 50 mL tube.
23. Transfer the cell suspension into 15 mL tube and centrifuge at 250 g for 5 min at 19 °C–21°C to pellet cells.
24. Prepare six 5 mL FACS tubes, each containing 1 mL of warm ECMV medium.
25. Add between 2 and 5 μL of CD31 Dynabeads (depending on the tissue size) to the first tube, mix thoroughly, place in a magnet stand for 1 min to wash.

Note: The beads settle very quickly so swirl and pipette immediately.

26. Aspirate media from washed beads when still on magnetic stand and add 1 mL of warm ECMV medium.

27. Remove supernatant from centrifuged cells, resuspend the cell pellet in 1 mL of warm ECMV medium and add cell suspension to the tube with beads.
28. Securely replace cap, invert tube several times and place it on a roller at 7 rpm.
29. Incubate for 30 min at 19 °C–21°C.

Pause point: There is a stopping point when cells are incubated with CD31 Dynabeads.

30. Briefly spin down the tube to collect all cells at the bottom of the tube.
31. Remove the cap and place the tube in magnet stand.
32. Allow beads to collect on magnet, turn the tube 180 ° to release any trapped cells and leave it for 2 min.
33. Collect the supernatant containing unbound cells into a fresh 15 mL tube while tube is sitting on a magnet and avoid disturbing the beads.
34. Take the tube out of the magnet and resuspend the beads in 1 mL of warm ECMV medium.
35. Transfer the cell supernatant into next tube and place it in the magnetic stand.
36. Repeat steps 31–35 until final tube is done, combining unbound cells from step 33 into the same 15 mL tube.
37. Aspirate gelatin coating solution from one of the coated vessels.
38. Depending on tissue size, resuspend purified CD31 positive microvascular endothelial cells in 2 mL (6-wells plate), 5 mL (6 cm plate) or 10 mL (T75 flask) of warm ECMV supplemented with double concentration of antibiotics and seed into appropriate gelatin-coated vessel.
39. Centrifuge the 15 mL tube with unbound cells for 5 min at 250 g at 19 °C–21°C.
40. Prepare six 5 mL FACS tubes, each with 1 mL of warm preadipocyte medium.
41. Add between 2 and 5 μL of CD34 Dynabeads (depending on the tissue size) to the first tube, mix thoroughly, place in a magnet stand for 1 min to wash.

Note: The beads settle very quickly so swirl and pipette immediately.

42. Aspirate media from washed beads when still on magnetic stand and add 1 mL of warm preadipocyte medium.
43. Remove supernatant from centrifuged cells, resuspend the cell pellet in 1 mL of warm preadipocyte medium and add cell suspension to the tube with beads.
44. Securely replace cap, invert tube several times and place it on a roller at 7 rpm.
45. Incubate for 15 min at 19 °C–21°C.

Pause point: There is a stopping point when cells are incubated with CD31 Dynabeads.

46. Briefly spin down the tube to collect all cells at the bottom of the tube.
47. Remove the cap and place the tube in magnet stand.
48. Allow beads to collect on magnet, turn the tube 180 ° to release any cells trapped accidentally on the magnet and let it sit for 2 min.
    a. Neonatal foreskin protocol.
        i. Carefully aspirate the supernatant containing unbound cells while tube is sitting on a magnet avoiding disturbing beads.
        ii. Take the tube out of the magnet and resuspend the beads in 1 mL of warm preadipocyte medium.
        iii. Transfer the cell supernatant into next tube and place it in the magnetic stand.
    b. Adult tissue protocol.
        i. If the tissue is too small to yield 3 pieces in step 4, collect the supernatant containing unbound cells into 15 mL tube.
        ii. Take the tube out of the magnet and resuspend the beads in 1 mL of warm preadipocyte medium.
        iii. Transfer the cell supernatant into next tube and place it in the magnetic stand.

49. Repeat steps 47 and 48 until final tube is done, combining unbound cells from step 48b into the same 15 mL tube.
50. Aspirate gelatin coating solution from coated vessel.
51. Depending on tissue size, resuspend purified CD34 positive preadipocytes in 2 mL (6-wells plate), 5 mL (6 cm plate) or 10 mL (T75 flask) of warm preadipocyte medium supplemented with double concentration of antibiotics and seed into appropriate gelatin-coated vessel.
    a. Adult tissue protocol.
        i. Centrifuge the tube with unbound cells from step 49 at 250 g for 5 min at 19 °C–21°C.
        ii. Aspirate the supernatant and resuspend the pellet in 2 mL (6-wells plate) or 5 mL (6 cm plate) of warm fibroblasts medium supplemented with double concentration of antibiotics and seed into appropriate gelatin-coated vessel.

Note: Growing adult fibroblasts from CD31, CD34 negative population gives better yield than growing them from explants (point 13b), however these cells are not as pure and have other cell types present as well.

Culturing and expanding of isolated cells

Timing: 5–30 days

This part of the protocol guides the reader though culturing and expansion of the isolated cells.

52. Approximately 24 h after cell isolation, change media of all cell types to the ones containing single concentration of antibiotics.

⚠ CRITICAL: Antibiotics are essential in culturing the isolated cells for the first week as they prevent growth of microbes present on the skin. However, adult keratinocytes are particularly sensitive to antibiotics, therefore if changing medium is not possible the next day, it should be done by the end of the day of isolation.

Note: For the first couple of days after isolation, dermis pieces used for growing fibroblast should have only half of the normal volume of medium to prevent the pieces from detaching. It is fine if the pieces are not completely submerged in the medium, avoid aspirating dermis pieces during media changes.

Note: If cells were seeded into 6-well plate or 6 cm dish, they should be expanded before freezing.

53. Change medium every other day, use single concentration of antibiotics for 7 days, after that time, cells are grown without antibiotics.
54. When the cells become confluent and need to be passed, coat appropriate vessels with appropriate coating solution for the cell type for at least 1 h at 37 °C.

Note: We did not observe any adverse effects of fibroblasts and preadipocytes being allowed to reach confluency. Keratinocytes and microvascular endothelial cells are contact inhibited, but confluency initiates their differentiation, therefore these cells should not be kept confluent for more than a day. Melanocytes are very sensitive to confluency and should be passed when 80 % confluency is reached.

55. When the vessels are coated, wash the cells once with HBSS.
    a. All cell types except for melanocytes.

i. Add 0.5 mL (6-wells plate), 1 mL (6 cm plate) or 2 mL (T75 flask) of trypsin and incubate cells at 19 °C–21°C (fibroblasts, preadipocytes and microvascular endothelial cells) or 37 °C (keratinocytes) until cells start to round up.

⚠ CRITICAL: Keratinocytes require longer trypsinization than other cells to detach properly.

Note: Quite often colonies of keratinocytes appear in adult fibroblasts cultures. The two cell types can be separated based on the different times required for trypsinization and both cell types can be used.

Note: Microvascular endothelial cells can sometimes differentiate into another fibroblastic-looking cell type, we are unsure of the identity of these cells. When this happens, cells should be trypsinized and re-selected with CD31 Dynabeads (steps 24–38).

ii. Add equal volume of trypsin inhibitor and transfer the cell suspension into 15 mL tube.
iii. Wash the dish once with HBSS to collect any cells left behind and add it to the same 15 mL tube; cells can be counted at this point if necessary.

b. Melanocytes.
i. Add 5 mL of HBSS – and incubate the cells for 10–20 min at 37 °C.

⚠ CRITICAL: Melanocytes are extremely sensitive to trypsin and therefore should only be passed by the method described here.

ii. After melanocytes start to round up, forcefully knock the vessel to detach melanocytes from the vessel and transfer supernatant to a 15 mL tube.
iii. Wash the dish once with HBSS to collect any cells left behind and add it to the same 15 mL tube; cells can be counted at this point if necessary.

Note: Keratinocytes which were left attached can be trypsinized, expanded and cryopreserved.

iv. For subsequent passing of melanocytes, they should be incubated for 5 min at 37 °C in HBSS and then scraped from the vessel using a cell scrapper.

56. Centrifuge the tube at 250 g for 5 min at 19 °C–21°C and aspirate the supernatant.
57. Remove coating solution from the vessels and seed the cells in appropriate medium.

Freezing isolated cells

Timing: 30 min

The final part of this protocol describes how to cryopreserve isolated cells.

Note: We typically freeze cells from confluent T75 flask in 3 cryovials at concentrations 0.5–2 million cells per vial. When cells are ready for freezing follow steps 54–56.

58. Resuspend cells in 1 mL of cold freezing mix per vial frozen (e.g., if freezing 3 vials, resuspend cells in 3 mL of freezing mix).

Note: Freezing mix should be cold to minimize harmful effects of DMSO and rapid manipulation is encouraged.

59. Aliquot 1 mL of cell suspension per cryovial.

60. Place cryovials in freezing container and store it at −80 °C for 24 h.
61. The next day cells should be transferred to liquid nitrogen storage.

EXPECTED OUTCOMES

The protocol described above allows for isolation of five different primary cell types from a single sample of human skin. For neonatal tissue it takes between 5 to 7 days to obtain a confluent T75 flask of cells, while adult cells typically take between 2–4 weeks, depending on age of the donor and tissue size.

All neonatal cells except for melanocytes display enormous proliferation capacity and we can routinely grow keratinocytes for over 30 population doublings and fibroblasts, preadipocytes and endothelial cells up to 60 population doublings, before they senescence. Moreover, they retain their morphology during extensive cell culture, providing excellent source of material for research.

Adult cells have lower proliferation capacity than neonatal ones and they contain much more senescent cells in the culture, however they are still perfectly usable in a wide range of experiments.

All isolated cell types display appropriate cell morphology, function, and markers (Figure 4). Keratinocytes have cobblestone morphology, are keratin 14 (KRT14) positive and readily differentiated with high concentration (>1.2 mM) of calcium chloride (Figure 5A). Melanocytes display dendritic morphology, are tyrosinase related protein 1 (TYRP1) positive and capable of melanin production (Figure 5B). Microvascular endothelial cells are platelet and endothelial cell adhesion molecule 1 (PECAM1), CD34 molecule (CD34) and cadherin 5 (CDH5) positive and able to uptake acetylated LDL (Figure 5C). Preadipocytes display spindle morphology, are positive for CD34 and readily differentiate into mature adipocytes (Figure 5D). Finally, there are no specific markers for fibroblasts, but they demonstrate classical spindle morphology and they are positive for actin alpha 2, smooth muscle (ACTA2) and vimentin (VIM) and negative for the markers listed above.

LIMITATIONS

The protocol for primary cell isolation from human skin we present here is very robust and we have thus far isolated and banked cells from over 600 human donors, both neonatal and adult. The biggest limitation of this protocol is availability of human skin samples which is not readily accessible to many laboratories. We hope that by highlighting the importance of primary cell use in research and providing an easy-to-follow protocol, clinicians will be more willing to collaborate to provide such samples that can be used for high quality and essential research.

Neonatal foreskin samples provide good size tissues that allows isolation of large quantity and very high-quality cells with high proliferating potential. Obviously, due to the nature of these samples, cells isolated from them come from male donors and have limited use for any sex-effect studies.

Alternatively, adult skin samples can be easily obtained from both sexes and at different ages, however, they provide a different challenge. In general, adult tissue biopsies are smaller and hence the number of cells harvested are lower and therefore it takes longer to establish initial culture. This is compounded by adult cells having lower proliferation capacity due to both older age and larger number of divisions required at the beginning to establish the culture. Moreover, adult tissues contain more extracellular matrix per tissue volume than neonatal ones, meaning that there are fewer cells available for isolation. Additionally, the fat present in adult tissue impairs enzymatic

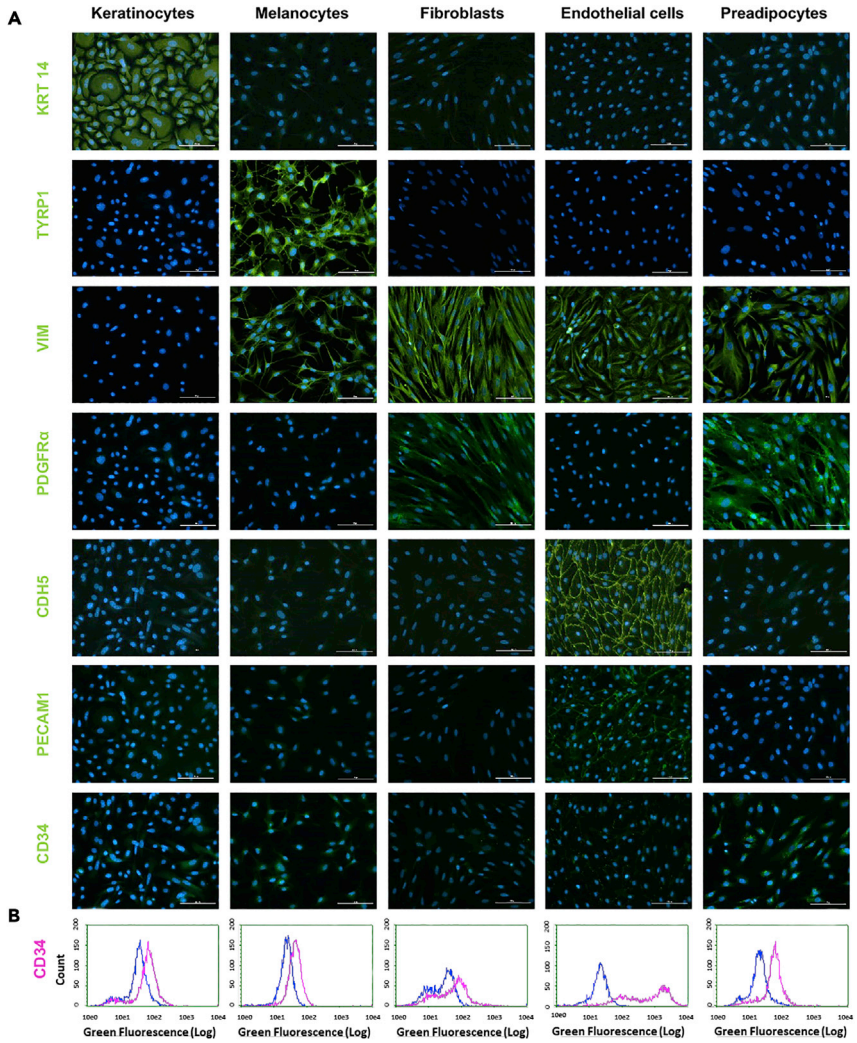

Figure 4. Expression of cell-type-specific markers in cells isolated from skin samples

(A) Immunofluorescence detection of cell type specific markers. Cells were seeded on coverslips, fixed, permeabilized and stained with appropriate antibodies. Each coverslip was incubated with two different antibodies raised in different hosts and then the signal was pseudocolored green. The scale bar represents 100 μm. The following antibodies were used for immunofluorescence: Keratin 14 (KRT14), Abcam (ab7800, dilution 1:100); Tyrosinase Related Protein 1 (TYRP1), Covance (SIG-38150, dilution 1:500); Vimentin (VIM), Santa Cruz (sc-6260, dilution 1:100); Platelet Derived Growth Factor Receptor Alpha (PDGFRα), Abcam (ab203491, dilution 1:500); Cadherin 5 (CDH5), Santa Cruz (sc-9989, dilution 1:100); Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1), Millipore (04-1074, dilution 1:100); CD34 Molecule (CD34), Abcam (ab81289, dilution 1:100).

(B) FACS analysis of CD34 expression (CD34 antibody, BD Biosciences, cat. no. 550760, dilution 1:50). Cells were trypsinized and fixed without permeabilization to detect membrane bound CD34 protein. Blue color represents control with only secondary antibody, pink shows CD34 expression.

digestion of the tissue and therefore additional mitigation steps must be incorporated into the protocol. Finally, adult cells in contrast to neonatal samples, would have been exposed to various environmental factors during the lifetime, such as pollution or UV, which should be considered when using these cells for downstream applications.

TROUBLESHOOTING

Problem 1

Epidermis does not peel easily from dermis (step 10).

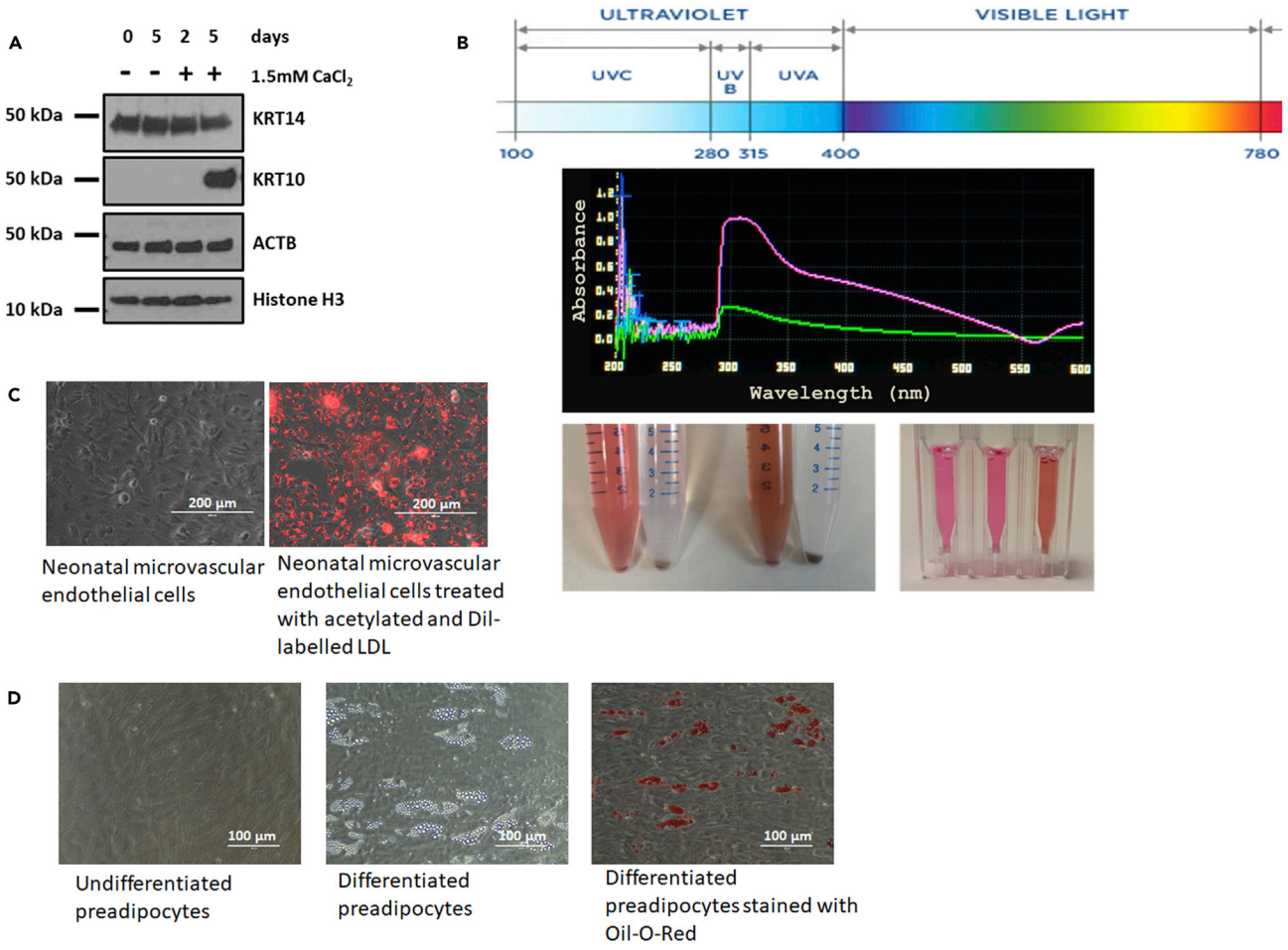

Figure 5. Functional analysis of different cell types

(A) Western blot analysis of keratinocytes differentiation by treatment with 1.5 mM $CaCl_2$. Neonatal primary keratinocytes were sham-treated or treated with 1.5 mM $CaCl_2$ for 5 days. Samples for western blot analysis were collected before treatment, on day 2 and 5. The following antibodies were used for western blotting: Keratin 14 (KRT14), Abcam (ab7800, dilution 1:20,000); Keratin 10 (KRT10), Abcam (ab76318, dilution 1:5,000); Actin beta (ACTB), Proteintech (20536-1-AP, dilution 1:5,000); Histone H3, Novus Biologicals (NB500-171, dilution 1:100,000).

(B) Spectroscopic analysis of melanin release into media by melanocytes after 48 h treatment with 10 µM PMA (pink line with PMA and green line without PMA, showing peak absorption differences in the UVB spectrum), picture below shows collected media and pelleted melanocytes without PMA and with PMA displaying dark melanin in both cells and released into the media; right shows media in cuvettes compared to fresh melanocyte media.

(C) Acetylated LDL uptake by endothelial cells. Microvascular endothelial cells were seeded on collagen fibronectin coated coverslip, next day Dil-Ac-LDL was added to the media at concentration 10 µg/mL and incubated for 4 h at 37°C. After incubation cells were washed in HBSS, fixed in formalin and imaged. The scale bar represents 200 µm.

(D) Differentiation of preadipocytes into mature adipocytes. Preadipocytes were seeded into gelatin coated coverslips and differentiated into matured adipocytes by using StemPro™ Adipogenesis Differentiation Kit according to manufacturer's protocol. After 14 days of differentiation, cells were fixed, stained with Oil Red O and imaged. The scale bar represents 100 µm.

Potential solution

Usually, it means the tissue pieces were too large for the enzyme to digest the tissue sufficiently. It is better to cut the tissue into strips rather than chunks. The tissue can be cut to smaller pieces and incubated for additional hour. Sometimes, especially for the adult skin, if the fat was not removed completely it will interfere with the digestion. Fat should be meticulously trimmed before liberase digestion.

Problem 2

Epidermis does not peel in one sheet but tears (step 10).

Potential solution

Tissue was over digested in liberase, either incubated too long or the digestion solution was not cold. In general, adult epidermis is more fragile and it tears more easily than the neonatal, gentle manipulation is encouraged.

Problem 3

Fibroblast-like cells appear in culture of microvascular endothelial cells (step 55ai).

Potential solution

Microvascular endothelial cells can sometimes differentiate into another fibroblastic-looking cell type, we are unsure of the identity of these cells. When this happens, cells should be trypsinized and re-selected with CD31 Dynabeads (steps 24–38).

Problem 4

Melanocytes acquire fibroblast-like morphology (step 55bi).

Potential solution

Most often this happens when melanocytes are allowed to reach confluency or are exposed to trypsin. Melanocytes must be passed only by scraping at maximum 80 % confluency.

Problem 5

Bacterial or yeast contamination during cell culture (step 53).

Potential solution

The antibiotics were removed too early from cell culture or were not stored properly, and they have degraded. Adhere to manufactures' instructions for antibiotics storage and keep them in culture for a minimum of 7 days.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ken Raj (kraj@altoslabs.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

S.K. developed and optimized the protocol, adapted it to the adult tissues, and wrote the manuscript; D.L. developed and optimized the protocol and edited the manuscript; H.C., S.F., and J.S. provided skin tissue samples; K.R. initiated protocol development, acquired funding, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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