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Establishing a High Throughput Epidermal Spheroid Culture System to Model Keratinocyte Stem Cell Plasticity

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Abstract

Epithelial dysregulation is a node for a variety of human conditions and ailments, including chronic wounding, inflammation, and over 80% of all human cancers. As a lining tissue, the skin epithelium is often subject to injury and has evolutionary adapted by acquiring the cellular plasticity required to repair damaged tissue. Over the years, several efforts have been made to study epithelial plasticity by *in* vitro and *ex-vivo* cell-based models. However, these models are limited in their capacity to recapitulate the various phases of epithelial cell plasticity. We describe here a protocol for generating 3D epidermal spheroids and epidermal spheroid-derived cells from primary neonatal human keratinocytes (NHKc). This protocol outlines the capacity of this epidermal spheroid system to functionally model distinct stages of keratinocyte generative plasticity and demonstrates that epidermal spheroid re-plating can enrich heterogenous NHKc cultures for integrin$\alpha 6^{hi}$/EGFR^{lo} keratinocyte subpopulations with enhanced stem-like characteristics. Our report describes the development and maintenance of a high throughput system for the study of skin keratinocyte plasticity and epidermal regeneration.

SUMMARY:

Here we describe a protocol for the systematic cultivation of epidermal spheroids in 3D suspension culture. This protocol has wide-ranging applications for use in a variety of epithelia tissue types and for the modeling of several human diseases and conditions.

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Keywords

Anoikis; cell plasticity; epithelium; epidermal stem cells; epidermosphere; human keratinocytes; spheroids; 3-D culture; wound healing

INTRODUCTION:

The mammalian stratified epithelium is the most complex epithelial architecture in all living systems and is most often subject to damage and injury. As a protective tissue, stratified epithelium has evolved to generate a complex and effective cellular tissue damage response. Upon injury, these cells must activate lineage plasticity programs, which enable them to migrate to the injured site and carry out repair[1-3]. This multifaceted response occurs in several sequential steps which remain poorly understood.

A major obstacle in studying the intricate process of epithelial regeneration lies in the dearth of high throughput model systems that can capture dynamic cellular activities at defined stages of cell regeneration. While *in vivo* mouse models offer relevant insight into wound healing and most closely recapitulate the human regenerative process, their development require laborious efforts and significant cost, limiting their throughput capacity. There exists, therefore, a critical need to establish systems that enable functional investigation of human epithelial tissue regeneration at high throughput scale.

In recent years, several attempts have been made to meet the scalability challenge. This is seen through great expansion of innovative *in vitro* and *ex vivo* cell-based models that closely mimic the *in vivo* regenerative context. This include advances in organ-on-chip[4], spheroid[5], organoid[6], and organotypic cultures[7]. These 3D cell-based systems each offer unique advantages and present distinct experimental limitations. To date, spheroid culture remains the most cost-effective and widely used 3D cell culture model. And while several reports have indicated that spheroid cultures can be used to study skin stem cell characteristics, these studies have largely been conducted with animal tissue[8,9], or with dermal fibroblasts[10], with virtually no reports thoroughly characterizing the regenerative properties of human epidermal spheroid cultures. In this protocol we detail the functional development, maintenance, and characterization of epidermal spheroid cultures from neonatal human keratinocytes (NHKc). We equally describe the utility of this system to model the sequential phases of epidermal regeneration and keratinocyte stem cell plasticity *in vitro*.

PROTOCOL:

1. Isolation and Culture of Human Keratinocytes from Neonatal Foreskin Tissue

1.1. Prepare wash media by adding 0.1M HEPES buffer

1.2. to 500 mL KSFM (Thermo Fisher) basal media in order to reach a pH of 7.2. Sterilize media in a 500 mL vacuum filter (0.22-micron pore size).

1.3. In a laminar flow hood, wash neonatal foreskin with 5 mL wash media in a 50-mL conical tube. Repeat twice.

1.4. Transfer washed foreskin to a sterile Petri dish. Using a scalpel and forceps, scrape off adipose and loose connective tissues from the dermal layer. Rewash foreskin with 5 mL wash media in a 50-mL conical tube.

1.5. Place foreskin epidermis side up in a 6-well plate containing 2 mL dispase enzyme diluted in wash media (50 U/mL).

1.6. Transfer the plate to an incubator for 4 hours to overninght (37 °C, 5% CO_2, 95% humidity).

1.7. Remove foreskin from the incubator and, under sterile conditions, transfer it to a Petri dish. Using fine-tip forceps, separate the epidermis from dermis layer.

1.8. Place epidermis into a 15 mL conical tube containing 2 mL 0.25% Trypsin-EDTA (Gibco). Using a 5 mL serological pipet, mechanically crush floating epidermis. Incubate for 15 min at 37°C with periodic vortexing for 5 s every 5 min.

1.9. After incubation, add 2 mL soybean trypsin inhibitor and mix by pipetting to neutralize the trypsin.

1.10. Centrifuge cell suspension for 2 min at 450 x g and 8 min at 250 x g.

1.11. Remove floating debris with forceps, being careful not to dislodge the cell pellet. Carefully aspirate supernatant from the cell pellet.

1.12. Resuspend pellet in 12 mL complete KSFM-scm medium and plate in a 10-cm dish. Incubate dish overnight (4 °C, 5% CO_2, 95% humidity).

1.13. On the following day, remove medium and replace with 12 mL complete KSFM-scm.

1.14. Refeed cells with 12 mL medium on days 4 and 7.

1.15. To maintain optimal growth of normal human keratinocyte (NHKc) cultures, it is imperative to passage cells 1:5 on day 10 to prevent cells from achieving greater than 80% confluency.

2. Generating skin epidermosphere cultures *in vitro*

2.1. Prepare a 5% agarose mixture by adding 2.5 g agarose to 50 mL phosphate buffered saline (PBS), in a 50 mL glass bottle. Autoclave bottle under liquid cycle. Allow to cool down to room temperature

2.2. To prepare plates, place the cooled glass bottle containing agarose solution in a 1 L plastic beaker filled with 200 mL deionized water (dH_2O). Melt agarose mixture in a research-grade microwave for up to 2 minutes, mixing the agar with heat-resistant gloves every 60 s by gently tilting the bottle side to side. CAUTION: Melting agar in the microwave will cause the flask container to become extremely hot and resulting in pressure buildup. It is important to release pressure buildup every 30 s and to wear the appropriate protective equipment to prevent burn injury.

2.3. Add 3 mL of melted 5% agarose to 12 mL KSFM-scm prewarmed to 42 °C for a final concentration of 1% agarose (wt/vol). Optional: pre-warming the serological pipettes and pipette tips helps prevent premature polymerization of the soft agar.

2.4. Quickly pipette 200 µL of the 1% agar mix into individual wells of a 96-well round-bottom plate using a multichannel pipettor (Figure 1A). Leave plate in sterile environment at 25 °C for 4 h to allow agarose to fully polymerize. Warm plate to 37 °C in a humidified incubator for at least 1 h. Experimentation can be stopped here and continued the next day. However, it is not recommended to leave for more than 24 h.

2.5. Passage spheroid-forming NHKc by aspirating media and washing cells in 2 mL PBS. Aspirate PBS and add 2 mL 0.25% Trypsin-EDTA to washed cells. Incubate for 5 min at 37 °C or until all cells completely detach. Add 2 mL Soybean Trypsin Inhibitor to plate and wash off cells from the plate into a 15 mL tube. Centrifuge at 250 x g for 5 min.

2.6. Resuspend pellet in 1 mL PBS. Quantify cell viability using trypan blue staining and an automated cell counter or a hemocytometer.

2.7. Aliquot 2 x 10^4 NHKc in 100 µL KSFM-scm and seed into individual wells of previously prepared 96-well round-bottom plate. Incubate plate overnight (37 °C, 5% CO_2, 95% humidity).

2.8. Using an inverted phase contrast microscope, analyze seeded well for epidermosphere formation. Note: Human epidermospheres from normal human keratinocytes can remain viable in 3D suspension culture for up to 96 h, although with considerable decrease in cell viability (Figure 2).

3. Epidermal spheroid re-plating assay

3.1. 24-48 h after 3D epidermosphere formation, add 4 mL prewarmed KSFM-scm to a 6-cm dish. Use a wide bore 1 mL pipette tip to transfer a single spheroid to the plate, ensuring to not break it apart. Alternatively, a 1 mL pipette tip may be widened using a sterile razor blade to cut the tip.

3.2. Incubate the plate overnight (37 °C, 5% CO_2, 95% humidity).

3.3. Analyze seeded spheroid for attachment to the bottom of the plate and observe for propagating cells, using an inverted phase contrast microscope (Figure 3). Feed cells every 96 h by gently removing 2 mL of the media inside the plate and slowly adding 2 mL fresh KSFM-scm media to the plate.

3.4. Passage spheroid-derived (SD) NHKc once they reach 70-80% confluency and continue with assay of choice. NOTE: It is important to never allow SD-NHKc to reach full confluency in culture, as this results in premature differentiation and cell growth arrest (Figure 3E-F).

3.5. The epidermal spheroid replating assay models keratinocyte-mediated wound repair by capturing each of the key sequential phases of epidermal regenerative

plasticity: a) homeostatic maintenance, b) differentiation halt/reversal c) stress lineage mobility, and d) tissue restoration (Figure 1B). This assay can also be used to produce cell populations for organotypic raft cultures or to model HPV-mediated neoplasia as demonstrated by Woappi *et al.*, 2018; Woappi *et al.*, 2020 [11,12].

4. 3D fluorescence cell tracking

4.1. In a 6-cm dish, transfect spheroid-forming 2D monolayer NHKc cultures with 1 μg pMSCV-IRES-EGFP plasmid vector carrying the enhanced green fluorescent protein (eGFP) gene using the Promega TransFast kit. Incubate plate overnight (4 °C, 5% CO_2, 95% humidity) microscope (Figure 4A). NOTE: It is important that transfection is completed in serum-free antibiotic-free conditions.

4.2. Without removing transfection mix, add 2 mL of prewarmed KSFM-scm to cells. Incubate plate overnight (4 °C, 5% CO_2, 95% humidity).

4.3. Aspirate all transfection media from plate and feed cells with 3 mL prewarmed KSFM-scm. Assess for presence of EGFP-expressing cells under the FITC channel of a fluorescent microscope.

4.4. Passage and seed 2 x 10^4 EGFP-transfected cells into individual wells of a previously prepared 96-well round-bottom plate. Incubate plate overnight (4 °C, 5% CO_2, 95% humidity). Visualize and monitor $EGFP^{pos}$ spheroid cell movement under the FITC channel of a Zeiss Axionvert 135 fluorescence microscope using Axiovision Rel. 4.5 software (Figure 4B-C).

4.5. 24 h after plating, add 3 mL prewarmed KSFM-scm to a 60-cm plate. Use a wide bore 1 mL pipette tip to transfer a single spheroid to the plate, ensuring to not break it apart. Incubate plate overnight (4 °C, 5% CO_2, 95% humidity).

4.6. Observe and analyze propagating SD-$NHKc^{EGFP}$ using a fluorescence microscope. Feed cells every 96 h by gently removing 2 mL the media inside the plate and slowly adding 2 mL fresh KSFM-scm media to the plate.

4.7. Harvest SD-$NHKC^{EGFP}$ for FACS isolation or assays of choice.

5. Characterization of spheroid-derived (SD) sub-populations by FACS

5.1. Passage SD-NHKc and corresponding autologous 2D monolayer cultures by aspirating old media and washing cells in 2 mL PBS. Remove PBS and add 2 mL 0.25% Trypsin-EDTA to washed cells. Incubate at 37 °C for 5 min or until all cells completely detach.

5.2. Add 2 mL Soybean Trypsin Inhibitor to plate and transfer cells into a 15 mL tube. Centrifuge at 250 x g for 5 min.

5.3. Resuspend pellets in 1 mL PBS. Quantify cell viability using trypan blue and an automated cell counter of hemocytometer.

5.4. Aliquot 100 μL containing 0.1-4 x 10^6 NHKc cells to 1.5 mL microcentrifuge tubes. Place tube on ice in a dark environment. NOTE: Turning off bright lights within the laminar hood is generally sufficient to create a dark working space.

5.5. Add 2 μL of FITC-conjugated anti-integrinα6 and 2 μL of PE-conjugated anti-EGFR to tubes to achieve a 1:50 dilutions. Prepare a tube with no antibodies added to serve as the unstained control. Incubate tubes on ice in dark or at 4 °C for 30 min. NOTE: The use of beads or a skin SCC line can serve as positive control, as skin SCC cells express elevated levels of integrinα6 and EGFR.

5.6. Perform flow cytometry analysis using BD FACSAria II flow cytometer or a flow cytometer of choice containing appropriate lasers.

5.7. Use the negative and positive controls to establish gates. Sort the subpopulation of integrinα6^{hi}/$EGFR^{lo}$ cells; these are the epidermal stem cell fraction. Integrinα6^{hi}/$EGFR^{hi}$ cells are the proliferative progenitor cell fraction. The integrinα6^{lo}/$EGFR^{hi}$ cells are the committed progenitor cell fraction (Figure 4D). Sorting FACS tube(s) should contain at least 1 mL of ice-cold KSFM-scm.

5.8. Test for proliferative capacity of sorted cell subpopulations by transferring the content of each respective sorting tube into a 15 mL conical tube containing 10X the volume of sorted cells in PBS. NOTE: It is important to remove all cells attached to the rim by pipetting the wall of the sorting FACS tube(s) several times, as keratinocytes can often adhere there.

5.9. Centrifuge 15-mL tubes at 1500 PRM for 5 min. Remove supernatant and resuspend pellet in 12 mL KSFM-scm. Transfer the resuspended cells into a 6-cm plate and incubate overnight (37 °C, 5% CO_2, 95% humidity).

5.10. The next day, remove media in plates and add 8 mL pre-warmed KSFM-scm. Incubate at 37 °C, 5% CO_2, 95% humidity. Re-feed cells with 12 mL medium every 3 days until 70-80% confluent (Figure 4E).

6. Immunofluorescence and staining of epidermospheres for basal stem cell markers

6.1. Transfer epidermospheres onto coverslips coated with poly-lysine. Allow SD-NHKc to propagate until 75% confluent.

6.2. Wash cells twice in ice-cold PBS for 5 min each.

6.3. Fix cells with 4% paraformaldehyde (PFA) for 20 min at room temperature.

6.4. Permeabilize cells with 0.5% Triton in 1% glycine. Block using 0.5% BSA and 5% goat serum for 30 min at room temperature.

6.5. Incubate samples with antibodies against P63 (1:200) and cytokeratin 14 (1:200) in blocking solution overnight at 4 °C.

6.6. Wash three times with ice-cold PBS containing Tween 20 (PBST), followed by incubation with FITC-and Alexa 568- conjugated secondary antibodies (1:1000 dilution).

6.7. Stain nuclei with 4', 6-diamidino-2-phenylindole (DAPI) (1:5000 dilution) before mounting cells.

6.8. Observe cells using a Nikon Eclipse E600 microscope or a Zeiss confocal laser-scanning microscope (Figure 4F).

7. Transcriptional analysis of epidermosphere cultures

7.1. Set up triplicate plates of low passage NHKc cultures so that RNA can be isolated from monolayer, spheroid, and spheroid-derived cultures from the same autologous cell line.

7.2. Pool 3-5 corresponding epidermospheres into a 1.5 mL microcentrifuge tube. Separately harvest autologous SD-NHKc and 2D monolayer cultures.

7.3. Isolate total RNA from all three groups using the All Prep DNA/RNA Mini Kit according to the manufacturer's protocol.

7.4. Perform reverse transcription with 1 μg of total RNA using the iScript cDNA Synthesis Kit.

7.5. Using cDNA, perform Real-time PCR with iQ SYBR Green Supermix following manufacturer's instructions. Use GAPDH as an internal control (Table 1).

7.6. Validate amplicon product size by agarose gel electrophoresis (2% v/v).

8. Assessing SD-NHKc colony-forming efficiency

8.1. Aspirate media from plates when cells reach 70-80% confluency. Wash cells three times in ice-cold PBS for 5 min each.

8.2. Fix cells with 3 mL ice-cold 100% methanol for 15 min. Wash three times in PBS for 5 min each.

8.3. Stain cells in 3 mL 10% Giemsa for 30 min. Wash three times in PBS for 5 min each. Allow cells to air dry overnight.

8.4. Analyze colony formation the following day and quantify the number of colonies obtained

9. Determine SD-NHKc population doublings

9.1. Passage SD-NHKc and corresponding autologous 2D monolayer cultures by aspirating old media and washing cells in 2mL PBS. Remove PBS and add 2 mL 0.25% Trypsin-EDTA to washed cells. Incubate for 5 min or until all cells completely detach (37 °C, 5% CO_2, 95% humidity).

9.2. Add 2 mL Soybean Trypsin Inhibitor to plate and transfer cells into a 15-mL tube.

9.3. Centrifuge at 250 x g for 5 min.

9.4. Remove the supernatant and resuspend pellet in 12 mL KSFM-scm.

9.5. Seed cells at low density 1-2 x 10^4 NHKc into individual 10 cm dishes and incubate overnight (37 °C, 5% CO_2, 95% humidity).

9.6. Feed plates with 8 mL KSMF-scm the following day. Feed every 4 days until at least 25% confluence, then feed every 2 days.

9.7. Serially passage cells 1:5 in 60-cm dishes until cell proliferative capacity is exhausted. Quantify cell viability by trypan blue staining. Determine population doublings at each passage using the formula: $\log(N/N_0) / \log 2$, where N represents the total cell number obtained at each passage and N_0 represents the number of cells plated at the beginning of the experiment.

REPRESENTATIVE RESULTS:

During the skin epidermosphere assay, NHKc cultures are seeded in agarose -coated wells of a 96-well plate (Figure 1A). Spheroid-forming cells should self-aggregate within 48h. Autonomous spheroid formation can be assessed as early as 24h using a standard inverted phase-contrast microscope. skin epidermosphere formation and re-plating assay model various phases of epidermal tissue regeneration (Figure 1B). Figure 2 shows high resolution images of various NHKc strains assayed for epidermal spheroid forming ability in 3D culture. It is important to examine the cells for dense sphere-shape aggregation, as this is a hallmark of spontaneous spheroid formation. We found it necessary to use more than 2 x 10^4 cells to ensure proper spontaneous aggregation. Non-spherical cell aggregation, such as seen in strains Figure 2A, is not considered adequate epidermosphere formation. Plating non-spheroid forming cell suspensions back in 2D monolayer culture seldom results in viable NHKc cell growth. However, plating of epidermospheres in 2D culture results in the proliferation of small-sized viable NHKc (Figure 3). Images of epidermospheres and SD-NHKc can be viewed and monitored using a standard inverted phase-contrast microscope. It is important to maintain these cultures below 100% confluency as this can considerably impar their growth and stem cell state in culture (Figure 3E-F). The process of epidermal spheroid formation can be functionally tracked at the single cell level by transfecting cells with a fluorescent reporter (Figure 4A-C). Under optimal conditions, keratinocyte subpopulation primarily enriched in SD-NHKc are Integrin$\alpha6^{hi}$/EGFR^{lo} cells. These cells generally make up about 25% of all SD-NHKc cells and can be readily isolated by FACS (Figure 4D). However, it is important to establish forward side scatter area (FSC-A) and side scatter area (SSC-A) gates to exclude doublets (Figure 4D). Further characterization of this stem-like keratinocyte subpopulation can be achieved by immunofluorescent staining analysis of epidermal stem cell marker expression, such as basal cytokeratin 14 (K14) and tumor protein 63 (P63) (Figure 4F).

DISCUSSION:

The use of 3D spheroid culture systems has had broad utility in assessing cell stemness. These systems have been demonstrated to enhance enrichment of tissue stem cells[13], yet their utility for the study of human epidermal stem cells has been limitedly explored. Here, we describe a strategy for enriching human keratinocyte stem cells using 3D epidermoshpere assay. In this system, NHKc are cultivated as self-assembling multicellular

spheroid suspensions, comprised of multiple keratinocyte subtypes suspended on top of agarose beds containing KSFM-scm. The setup for this protocol is time sensitive as agar polymerizes rapidly at room temperature. Preheating serological pipettes, micropipette tips, and the agar/cell mixing tube, as well as the media to up to 42 °C, can dramatically reduce premature polymerization. We've found that placing the 96-well plate in 4 °C shortly after adding agar/media mix to wells can considerably speed up polymerization and ensure that the cushion is sufficiently firm for subsequent seeding of cells. It is important to maintain plate(s) level at all times during the polymerization process, as poor polymerization of the agar/media mix will result in cells seeding or growing inside the soft agar, voiding the assay.

Also outline in this protocol, we present a strategy for propagating epidermospheres in 2D monolayer culture to generate stem-like spheroid-derived cells. The epidermosphere re-plating assay enriches for a stem cell-like subpopulation of integrin$\alpha 6^{hi}$/EGFR^{lo} keratinocytes. These cells can be used to study epidermal reconstruction, psoriasis, or cellular neoplasia[11,12,14]. integrin$\alpha 6^{hi}$/EGFR^{lo} keratinocytes can also be readily isolated by FACS and characterized by immunofluorescent staining. When conducting such experiments, we found it helpful to use the unsorted autologous 2D monolayer cells as controls. if these are not available, a good alternative can be skin SCC cell lines.

In summary, this report demonstrates that human epidermosphere culture and re-plating models keratinocyte regenerative plasticity *in vitro*, as it captures each of the four phases of regeneration: homeostatic maintenance, differentiation reversal, stress lineage mobility, and tissue restoration. However, one limitation of agar-based spheroid self-assembly is that not all NHKc stains are capable of spontaneously forming spheroids. The hanging drop method[15] is a good alternative strategy to overcome this challenge and to force induce multicellular spheroid formation. This assay can also be multiplexed with stromal or immune cells to gain further insight into the contribution of various cell populations on epidermal regeneration. it would be interesting to explore whether addition of Matrigel into the agar could enhance epidermosphere survival and potency.

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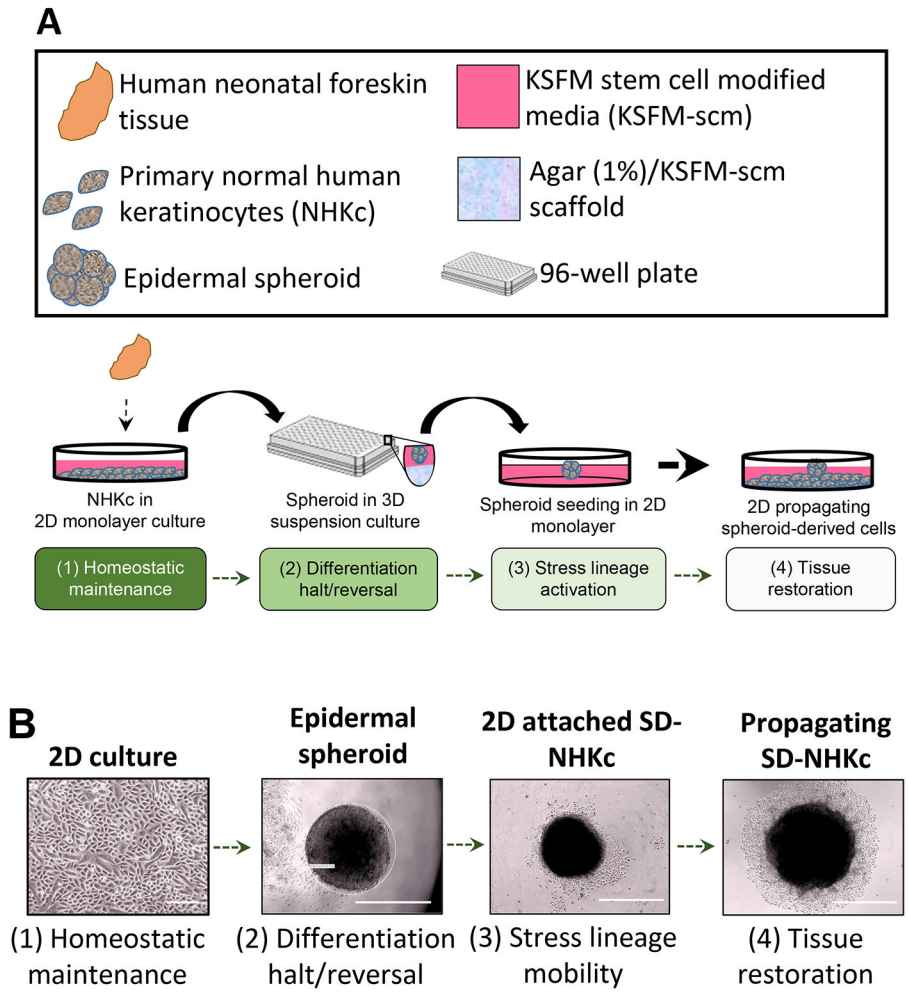

Figure 1: Cultivation of NHKc epidermospheres in 3D suspension culture.

(A) Schematic representation of the epidermal spheroid re-plating assay adapted from Woappi et al. 2018[11]. (B) Representative phase contrast images of NHKc cultures, epidermal spheroids, and SD-NHKc at each sequential step of the epidermal spheroid re-plating assay. Scale bar = 100 μM.

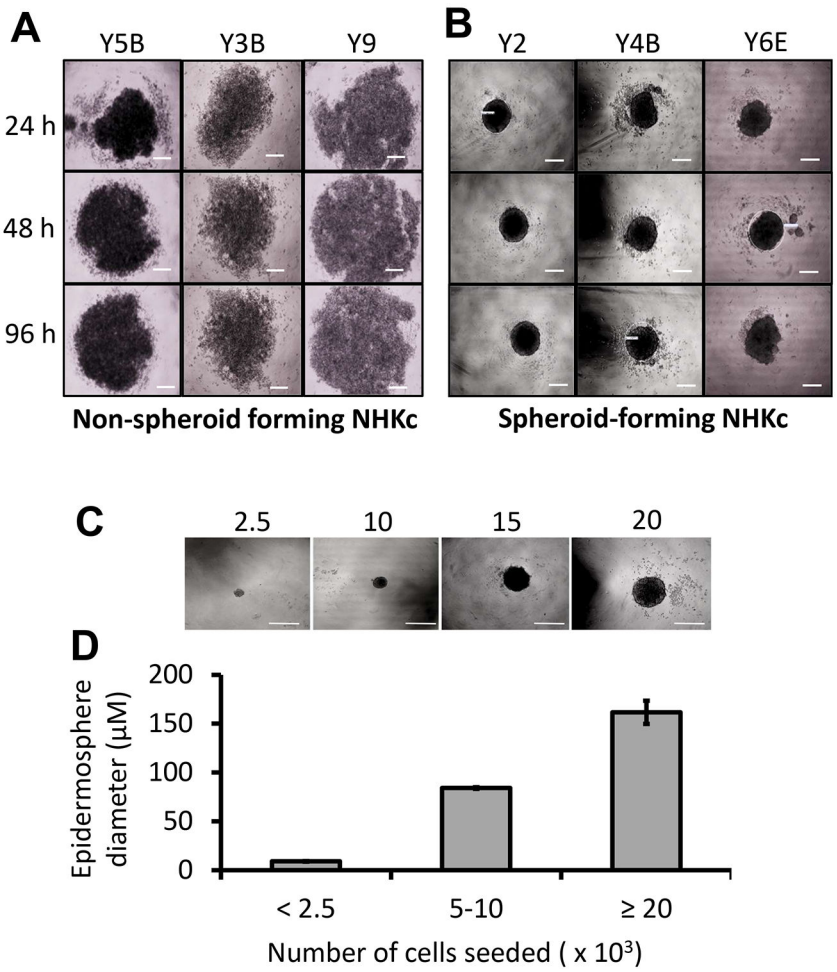

Figure 2: Assessing skin epidermosphere growth.

(A) Images of six individual spheroid non-forming and (B) spheroid-forming NHKc strains in floating 3D suspension. (C) Epidermospheres obtained using various amounts of NHKc. (D) Quantification of mean epidermosphere size obtained using different quantities of NHKc in floating 3D suspension culture. Bars indicate standard deviation. Scale bar = 100 µM.

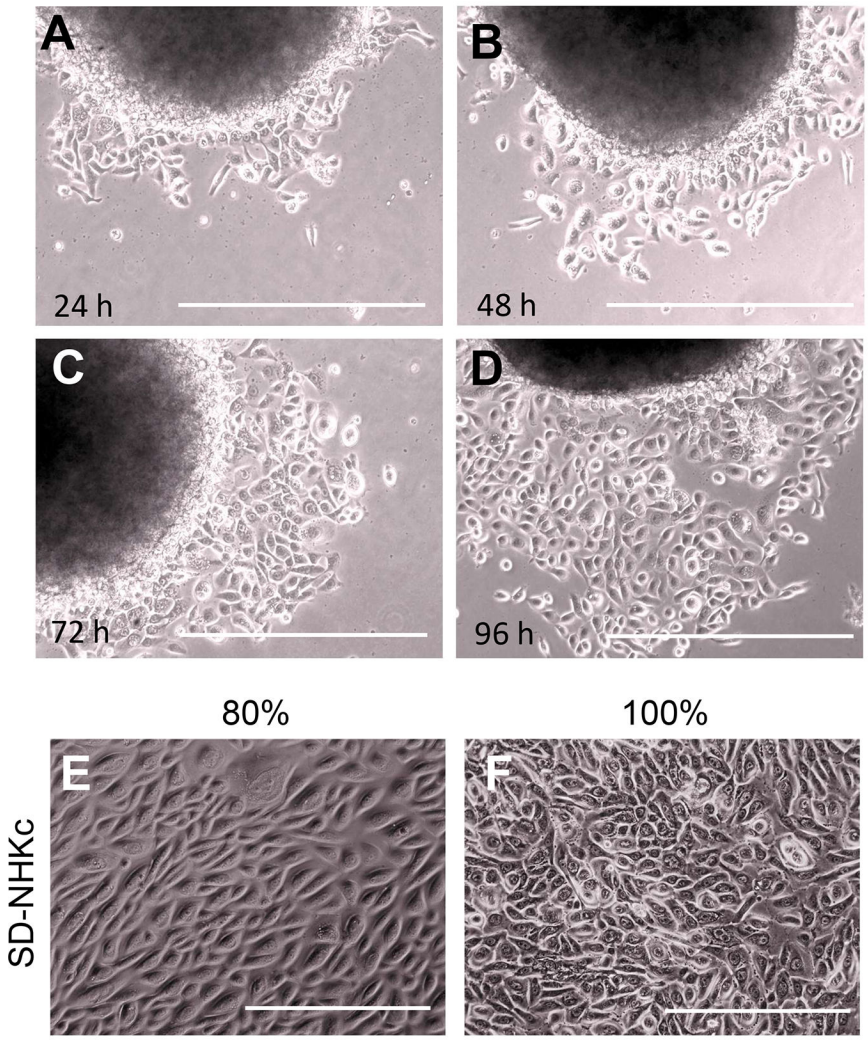

Figure 3: Growing spheroid-derived cultures.

(A) Time course phase-contrast imaging of SD-NHKc monolayer cultures propagating from an attached epidermosphere 24 h, (B) 48 h, (C) 72 h, and (D) 96 h after re-plating in 2D plastic culture. (E) SD-NHKc cultures at 80% confluency and (F) 100% confluency 15 and 20 days after replating in 2D plastic culture, respectively. Scale bar = 100 μM.

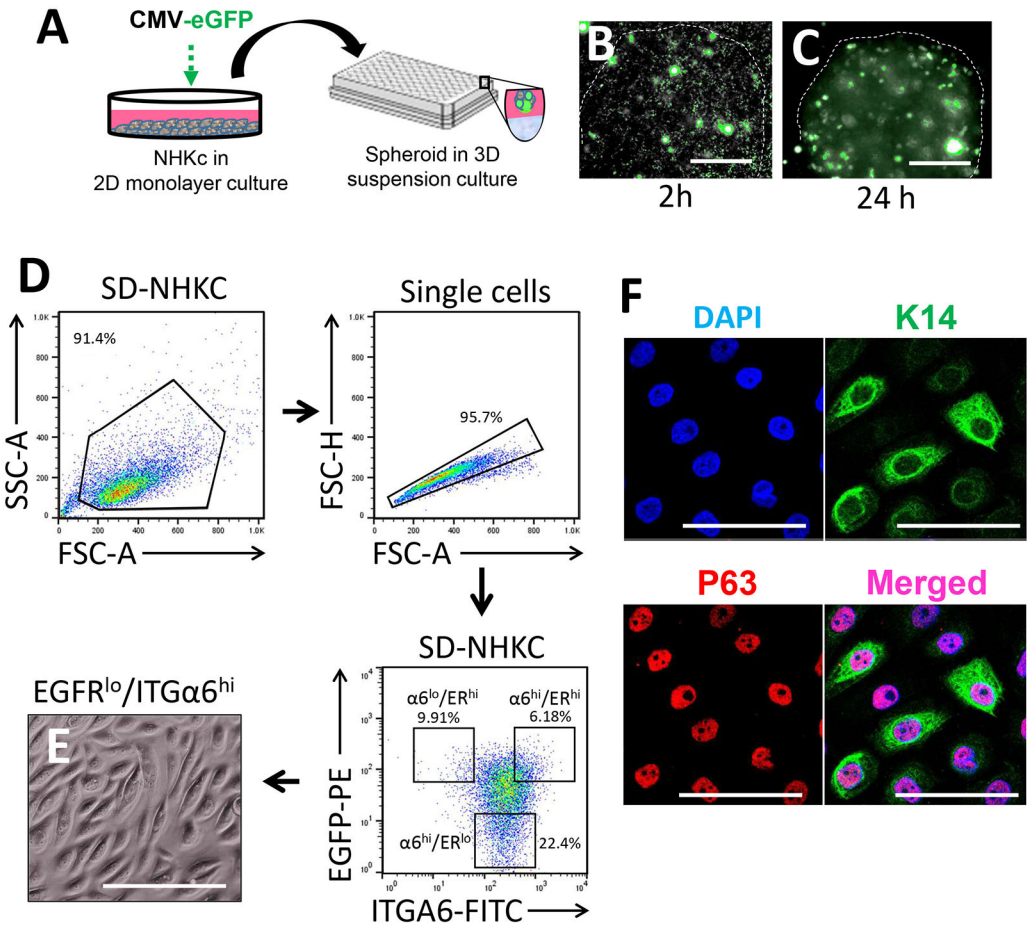

Figure 4: Characterization of spheroid-derived (SD) sub-populations.

(A) Schematic representation of 3D cell tracking assay of epidermospheres *in vitro* as described by Woappi *et al.* 2020[12]. (B) EGFP-expressing epidermospheres 2 h and (C) 24 h after seeding in 3D culture. (D) Fluorescence activated cell sorting (FACS) of SD-NHKc subpopulations. Approximately 1/4th of all cells should be integrinα6^{hi}/EGFR^{lo}. (E) Integrinα6^{hi}/EGFR^{lo} subpopulations produce keratinocyte holoclones. (F) Immunofluorescent staining analysis of basal epithelial stem cell marker expression in Integrinα6^{hi}/EGFR^{lo} cells. Scale bar = 50 μM.

Table 1.

Outlines PCR primer sequences used for the detection of select genes involved in neonatal keratinocyte plasticity.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
MCDB 153-LB basal media	Sigma-Aldrich	M7403	MCDB 153-LB basal media w/ HEPES buffer
KSFM-scm	ThermoFisher Scientific	17005042	Supplemented with 1% Penicillin/Streptomycin, 20 ng/ml EGF, 10 ng/ml basic fibroblast growth factor, 0.4% bovine serum albumin (BSA), and 4 μg/ml insulin
Human Basic Fibroblast Growth Factor (hFGF basic/FGF2)	Cell Signaling Technology	8910	
Human Epidermal Growth Factor (hEGF)	Cell Signaling Technology	8916	
Human Insulin	Millipore Sigma	9011-M	
Thermo Scientific™ Sterile Single Use Vacuum Filter Units	Thermo Scientific	09-740-63D	
FITC-conjugated anti-integrinα6	Abcam	ab30496	
PE-conjugated anti-EGFR (San Jose, CA; catalog number)	BD Pharmingen	555997	
Dispase	Sigma-Aldrich	D4818	
NEST Scientific 1-Well Cell Culture Chamber Slide, BLACK Walls on Glass Slide, 6/PK, 12/CS	Stellar Scientific	NST230111	
P63	Thermo Scientific	703809	1:200 dilution
Cytokeratin 14	Santa Cruz Biotechnology	sc-53253	1:200 dilution
All Prep DNA/RNA Mini Kit	Qiagen	80204	
iScript cDNA Synthesis Kit	Bio-Rad	1708890	
iQ SYBR Green Supermix (Bio-Rad)	Bio-Rad	1708880	
Promega TransFast kit	Promega	E2431	
pMSCV-IRES-EGFP plasmid vector	Addgene	20672	