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TITLE:

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

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KEYWORDS:

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

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 epithelial tissue types and for the modeling of several human diseases and conditions.

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 evolutionarily adapted by acquiring the cellular plasticity necessary to repair damaged tissue. Over the years, several efforts have been made to study epithelial plasticity using in vitro and ex vivo cell-based models. However, these efforts

have been 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. This protocol outlines the capacity of epidermal spheroid cultures to functionally model distinct stages of keratinocyte generative plasticity and demonstrates that epidermal spheroid re-plating can enrich heterogenous normal human keratinocytes (NHKc) cultures for integrinα6^{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.

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 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 for establishing 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, culture, and maintenance of epidermal spheroid cultures from normal 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:

The protocol for the collection and handling of skin specimens and isolation of human keratinocytes has been reviewed by the University of South Carolina (UofSC) IRB and classified as "research not involving human subjects", as the foreskin specimens were surgical discards

produced during routine surgical procedures (circumcision of neonate boys) and were completely devoid of identifying information. The protocol was also reviewed and approved by the UofSC Biosafety Committee on a regular basis, and all laboratory personnel underwent laboratory biosafety training. All procedures were conducted in concordance to the safety and ethics standards of UofSC.

1. Isolation and culture of human keratinocytes from neonatal foreskin tissue

1.1. Prepare wash medium by adding 0.1 M HEPES buffer to 500 mL of KSFM medium and adjust it to a pH of 7.2. Sterilize medium in a 500 mL vacuum filter (0.22 µm pore size). MCDB 153-LB basal medium can also be used as an alternative wash solution in place of KSFM.

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

1.3. 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 of wash media in a 50 mL conical tube.

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

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

1.6. 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.

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

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

1.9. Centrifuge cell suspension for 2 min at 450 *x g*, and then for 8 min at 250 *x g*.

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

1.11. Resuspend pellet in 12 mL of complete KSFM-scm medium and plate in a 10-cm dish. Incubate dish overnight (37 °C, 5% CO_2, 95% humidity).

1.12. On the following day, remove medium using an aspirating pipette and replace with 12 mL complete KSFM-scm. Repeat on days 4 and 7.

1.13. To maintain optimal growth of normal human keratinocyte (NHKc) cultures, 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 of agarose to 50 mL of 1x 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 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 resulting in pressure buildup. Release pressure buildup every 30 s. Wear appropriate protective equipment to prevent 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-warm the serological pipettes and pipette tips to 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.

NOTE: Experimentation can be stopped here and continued the next day. Polymerized agarose plates can be maintained at 37 °C up to 24 h or at 4 °C for up to 48 h when sealed with parafilm wrap. Warm plate to 37 °C in a humidified incubator for at least 1 h before use.

2.5. Passage spheroid-forming NHKc by aspirating media and washing cells in 2 mL of PBS. Aspirate PBS and add 2 mL of 0.25% Trypsin-EDTA to washed cells. Incubate for 5 min at 37 °C or until all cells completely detach. Add 2 mL of 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 of PBS. Quantify cell viability using trypan blue staining and a hemocytometer or an automated cell counter.

2.7. Aliquot 2 x 10^4 NHKc in 100 µL of 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

177 formation.
178
179 NOTE: Human epidermospheres from normal human keratinocytes can remain viable in 3D
180 suspension culture for up to 96 h, although with considerable decrease in cell viability (**Figure 2**).
181
182 **3. Epidermal spheroid re-plating assay**
183
184 3.1. 24-48 h after 3D epidermosphere formation, add 4 mL of prewarmed KSFM-scm to a 6
185 cm dish. Use a wide bore 1 mL pipette tip to transfer a single spheroid to the plate, ensuring to
186 not break it apart. Alternatively, widen a 1 mL pipette tip using a sterile razor blade to cut the tip.
187
188 3.2. Incubate the plate overnight (37 °C, 5% CO_2, 95% humidity).
189
190 3.3. Analyze seeded spheroid for attachment to the bottom of the plate and observe for
191 propagating cells using an inverted phase contrast microscope (**Figure 3**). Feed cells every 96 h
192 by gently removing 2 mL of the media inside the plate and slowly adding 2 mL fresh KSFM-scm
193 media to the plate.
194
195 3.4. Passage spheroid-derived (SD) NHKc once they reach 70-80% confluency and continue
196 with assay of choice. Monitor SD-NHKc frequently to prevent cells from reaching full confluency
197 in culture, as this results in premature differentiation and cell growth arrest (**Figure 3E-F**).
198
199 3.5. The epidermal spheroid replating assay models keratinocyte-mediated wound repair by
200 capturing each of the key sequential phases of epidermal regenerative plasticity: a) homeostatic
201 maintenance, b) differentiation halt/reversal c) stress lineage mobility, and d) tissue restoration
202 (**Figure 1B; Table 2**). This assay can also be used to produce cell populations for organotypic raft
203 cultures or to model HPV-mediated neoplasia as demonstrated in our previous work [11,12].
204
205 **4. 3D fluorescence cell tracking**
206
207 4.1. In a 6 cm dish, transfect spheroid-forming 2D monolayer NHKc cultures with 1 µg of
208 pMSCV-IRES-EGFP plasmid vector carrying the enhanced green fluorescent protein (eGFP) gene.
209 Incubate plate overnight (37 °C, 5% CO_2, 95% humidity) microscope (**Figure 4A**).
210
211 NOTE: It is important that transfection is completed in serum-free antibiotic-free conditions.
212
213 4.2. Without removing transfection mix, add 2 mL of prewarmed KSFM-scm to cells. Incubate
214 plate overnight (37 °C, 5% CO_2, 95% humidity).
215
216 4.3. Aspirate all transfection media from plate and feed cells with 3 mL of prewarmed KSFM-
217 scm. Assess for presence of EGFP-expressing cells under the FITC channel of a fluorescent
218 microscope.
219
220 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 (37 °C, 5% CO_2, 95% humidity). Visualize and monitor $EGFP^{pos}$ spheroid cell movement under the FITC channel of a fluorescence microscope (**Figure 4B-C**).

4.5. 24 h after plating, add 3 mL of 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. Incubate plate overnight (37 °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 of PBS. Remove PBS and add 2 mL of 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 of 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 of 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, created by turning off bright lights within the laminar hood.

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. 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 flow cytometer of choice containing with 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 250 *x g* for 5 min. Remove supernatant and resuspend pellet in 12 mL of 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 PBS (4 °C) 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 X-100 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 PBS (4 °C) 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 fluorescent-capable microscope with FITC and PE laser lines (**Figure 4F**).

7. Transcriptional analysis of epidermosphere cultures

7.1. Set up triplicate plates of low passage NHKc cultures to isolate RNA can be 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.

7.4. Perform reverse transcription with 1 µg of total RNA.

7.5. Using cDNA, perform real-time PCR, using GAPDH as an internal control (**Table 1**).

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

NOTE: For transcriptomic-wide profiling of epidermosphere cultures using whole-genome microarray analysis, isolate total RNA from mass cultures of a single NHKc donor and corresponding SD-NHKc from the same donor, in six replicates each.

7.7. Assess RNA quality using a bioanalyzer to achieve RNA Integrity Numbers (RIN) ranging from at least 9.0 to 9.1.

7.8. Perform microarray experiments by amplifying and biotinylating total RNA samples.

7.9. Reverse transcribe 100 ng of total RNA per sample into ds-cDNA using NNN random primers containing a T7 RNA polymerase promoter sequence.

7.10. Add T7 RNA polymerase to cDNA samples to amplify RNA, then copy RNA to ss-cDNA. Degrade excess RNA by using RNase H.

7.11. Fragment sscDNA molecules and label with biotin.

7.12. Amplify labeled samples for 16 h at 45 °C.

7.13. Hybridize samples using a hybridization oven and a wash and stain kit.

7.14. Wash and stain hybridized arrays.

7.15. Scan arrays using a system and computer workstation.

7.16. Following completion of array scans, import probe cell intensity (CEL) files into expression console software and process at the gene-level using library file and Robust Multichip Analysis (RMA) algorithm to generate CHP files.

7.17. After confirming data quality within Expression Console, import CHP files containing log2 expression signals for each probe into a transcriptome analysis software to analyze cell type specific transcriptional responses using one-way between-subject analysis of variance.

8. Assessing SD-NHKc colony-forming efficiency

8.1. Aspirate media from plates when cells reach 70-80% confluency. Wash cells three times in PBS (4 °C) for 5 min each.

8.2. Fix cells with 3 mL of 100% methanol (4 °C) for 15 min. Wash three times in PBS for 5 min each.

8.3. Stain cells in 3 mL of 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 of 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 of 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% confluent, 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 24 h 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 cultures are Integrinα6^{hi}/EGFR^{lo} cells. These cells generally make up about 25% of all SD-NHKc cultures 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; Table 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Cultivation of NHKc epidermospheres in 3D suspension. (A) Schematic representation of the epidermal spheroid re-plating assay adapted from [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.

Table 2. Strategies for phenotypic and molecular characterization of the epidermal spheroid

replating assay.

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 culture techniques. In this system, NHKc are cultivated as self-assembling multicellular spheroid suspensions, comprised of several 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 42 °C, can dramatically reduce premature polymerization. We observed 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 agar cushion is sufficiently firm for subsequent seeding of cells. It is important to maintain the plate) 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 outlined in this protocol, we present a strategy for propagating epidermospheres in 2D monolayer culture to generate stem-like spheroid-derived cells. The epidermal spheroid re-plating assay enriches for a stem cell-like subpopulation of integrin$\alpha6^{hi}$/EGFR^{lo} keratinocytes. These cells can be used to study epidermal reconstruction, psoriasis, or cellular neoplasia [11,12,14]. Integrin$\alpha6^{hi}$/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 unsorted autologous 2D monolayer cells as controls., skin SCC cell lines are a good alternative If these are unavailable.

In summary, this report demonstrates that human epidermal spheroid 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 muscle, 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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DISCLOSURES:

The authors do not have financial relationships to disclose.

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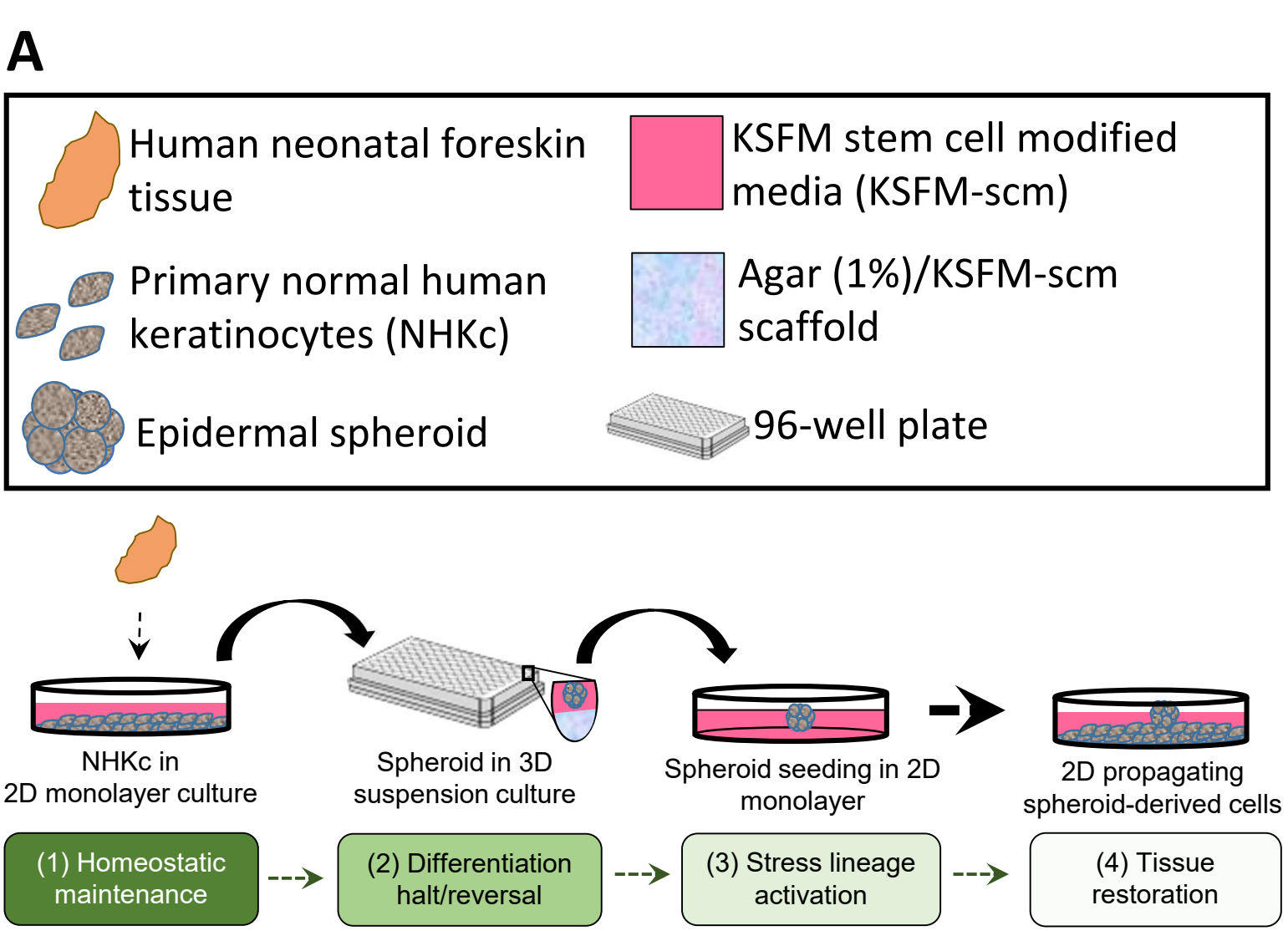
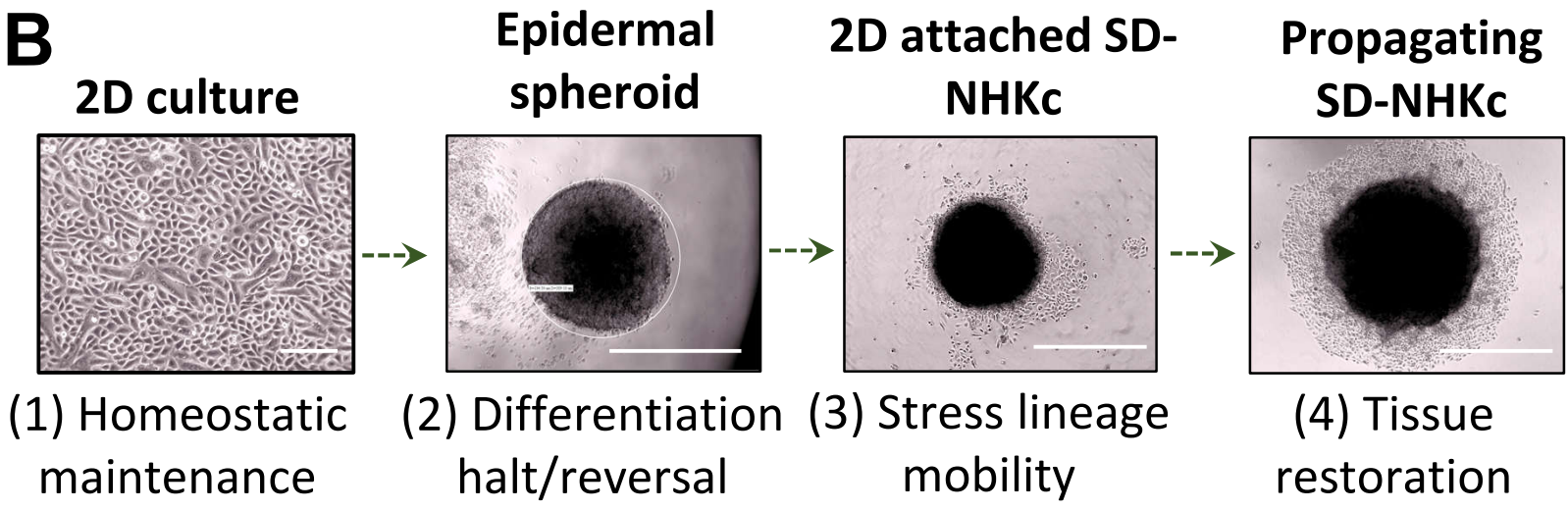




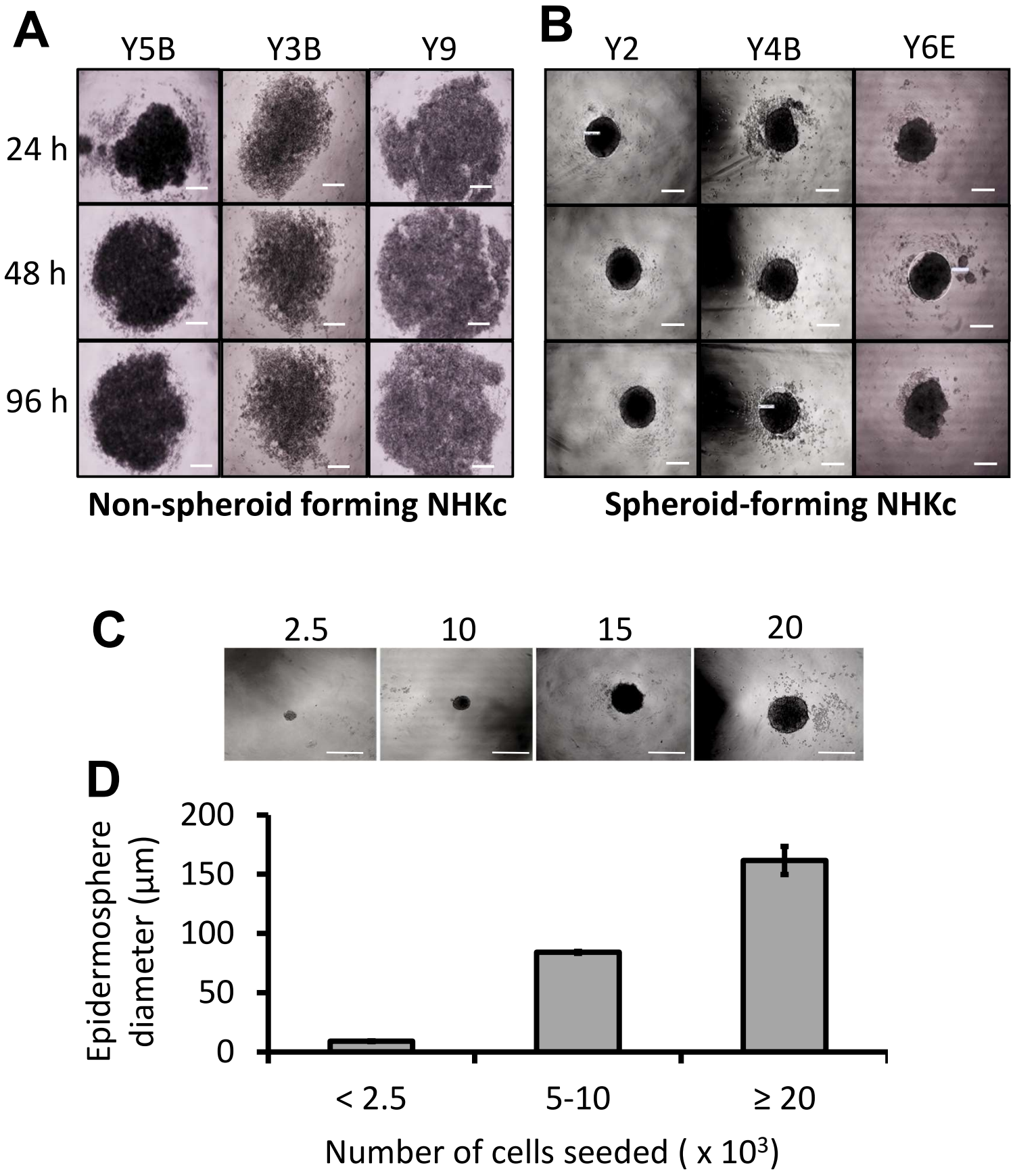
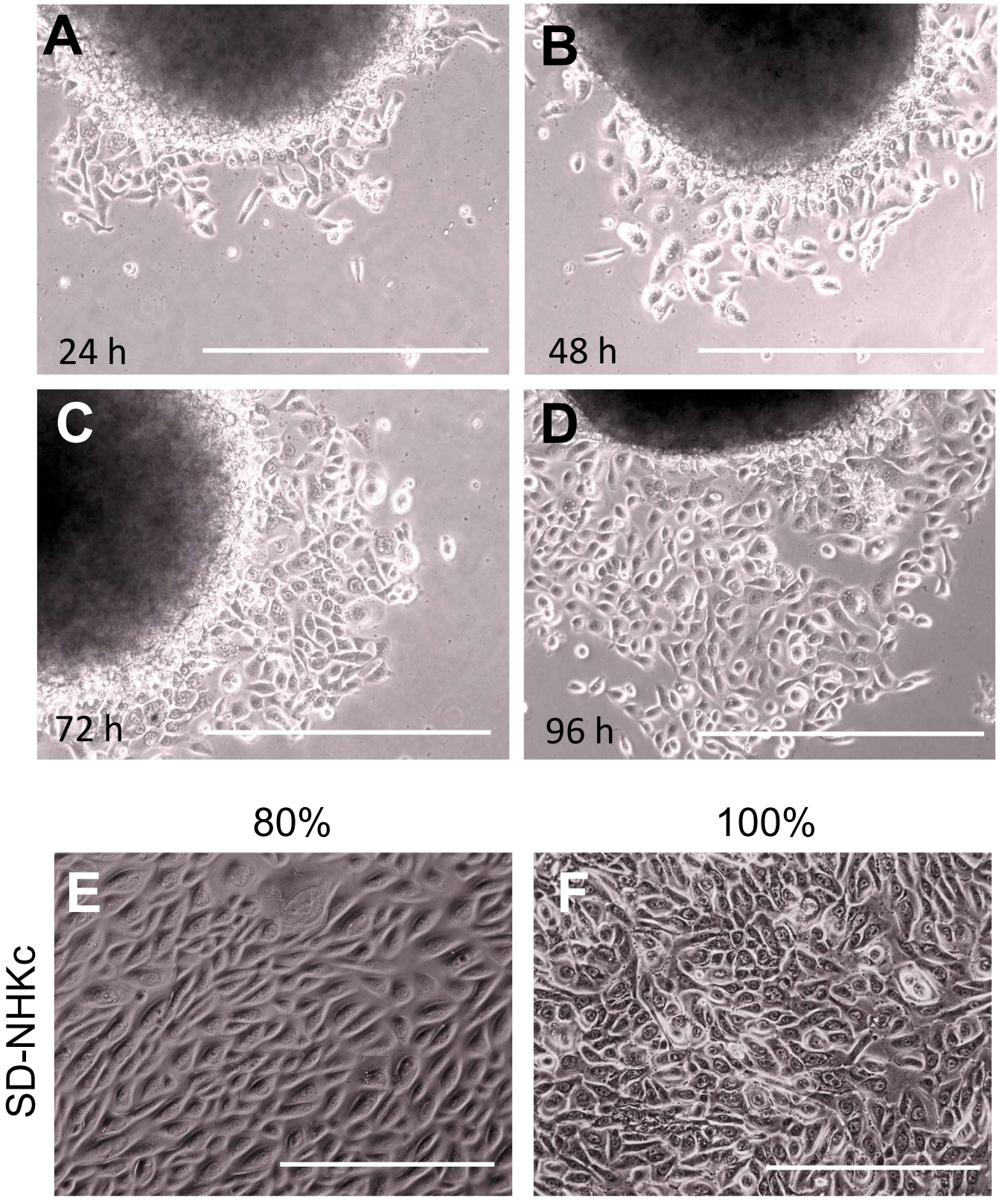


Figure 3



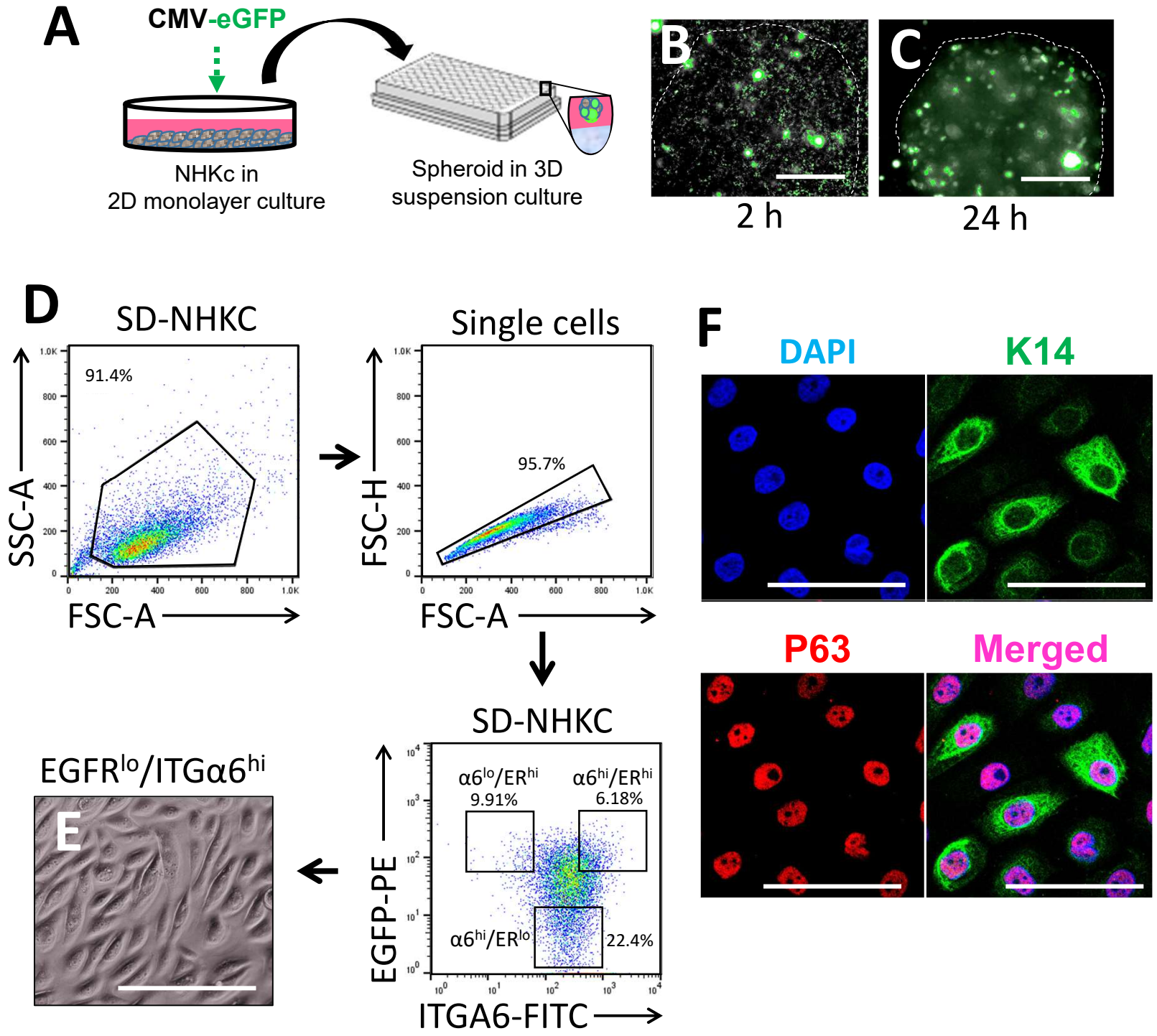


Table 1. Primers used for real-time PCR.

	Primer sequence (5'-3')
Gene name	**Forward Primer**
ALDH1	GCACGCCAGACTTACCTGTC
EGFR	AGGCACGAGTAACAAGCTCAC
GAPDH	GGAGCGAGATCCCTCCAAAAT
K14	TGAGCCGCATTCTGAACGAG
KI-67	ACGCCTGGTTACTATCAAAAGG
KLF4	CCCACATGAAGCGACTTCCC
TP63	GGACCAGCAGATTCAGAACGG
β-Actin	CATGTACGTTGCTATCCAGGC
ΔN TP63	ATGTTGTACCTGGAAAACAATGCC

Reverse Primer
CCTCCTCAGTTGCAGGATTAAAG
ATGAGGACATAACCAGCCACC
GGCTGTTGTCATACTTCTCATGG
GATGACTGCGATCCAGAGGA
CAGACCCATTTACTTGTGTTGGA
CAGGTCCAGGAGATCGTTGAA
AGGACACGTCGAAACTGTGC
CTCCTTAATGTCACGCACGAT
CAGGCATGGCACGGATAAC

Table 2

	Culture Condition	Phase Contrast Appearance	Immunostaining	FACS Analysis
Homeostatic maintenance	2D monolayer	Small sized cells < 30 µm	K14, P63	ITGα6^{med}/EGFP^{med}
Differentiation halt/reversal	3D spheroid	Compact multicellular aggregate > 50 µm	K14, P63, Ki-67	N/A
Stress lineage mobility	3D-to-2D spheroid attachment	Diffusing small-sized cells (< 20 µm) from spheroid edge	K14, P63, Ki-67	ITGα6^{hi}/EGFP^{med}
Tissue restoration	3D-to-2D spheroid monolayer	Propagating small-sized cells < 20 µm	K14, P63, IVL	ITGα6^{hi}/EGFP^{lo} and ITGα6^{hi}/EGFP^{hi}

Transcriptomic signature
Epidermal maintenance (K14, P63, IVL, K10)
De-differentiation: NANOG, SOX2, OCT4, KLF4, K14, P63, Ki-67
Proliferation of skin cells: K14, K16, K17, Ki-67
Formation of epidermis: K14, IVL, FLG

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Affymetrix platform	Affymetrix		For microarray experiments
Affymetrix's HuGene-2_0-st library file	Affymetrix		Process
Agilent 2100 Bioanalyzer	Agilent		For microarray experiments
All Prep DNA/RNA Mini Kit	Qiagen	80204	Used for RNA isolation
Analysis Console Software version 3.0.0.466			analyze cell type specific transcriptional responses using one-way between-subject analysis of variance
BD FACSAria II flow cytometer	Beckman		For flow cytometry
Console Software version 3.0.0.466/Expression console Software	Affymetrix/Thermo Fisher Scientific		For confirming data quality
Cytokeratin 14	Santa Cruz Biotechnology	sc-53253	1:200 dilution
Dispase	Sigma-Aldrich	D4818	For cell media
FITC-conjugated anti-integrinα6	Abcam	ab30496	For FACS analysis
GeneChip Command Console 4.0 software	Affymetrix/Thermo Fisher Scientific		For confirming data quality
GeneChip Fluidics Stations 450 (Affymetrix/Thermo Fisher Scientific)	Affymetrix/Thermo Fisher Scientific		For washing and staining of hybridized arrays

GeneChip HuGene 2.0 ST Arrays	Affymetrix/Thermo Fisher Scientific		For hybridization and amplifycation of total RNA
GeneChip Hybridization Oven 640	Thermo Fisher Scientific		For hybridization and amplifycation of total RNA | Amplify labeled samples
GeneChip Hybridization Wash, and Stain Kit (Affymetrix/Thermo Fisher Scientific).	Affymetrix/Thermo Fisher Scientific		For washing and staining of hybridized arrays
GeneChip Scanner 3000 7G system	Affymetrix/Thermo Fisher Scientific		Scanning hybridized arrays
GeneChip WT PLUS Reagent Kit	Affymetrix/Thermo Fisher Scientific		For amplifycation of biotinylating total RNA
Human Basic Fibroblast Growth Factor (hFGF basic/FGF2)	Cell Signaling Technology	8910	For cell media
Human Epidermal Growth Factor (hEGF)	Cell Signaling Technology	8916	For cell media
Human Insulin	Millipore Sigma	9011-M	For cell media
iQ SYBR Green Supermix (Bio-Rad)	Bio-Rad	1708880	Used for RT-qPCR
iScript cDNA Synthesis Kit	Bio-Rad	1708890	Used for RT-qPCR

KSFM	ThermoFisher Scientific	17005041	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
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
MCDB 153-LB basal medium	Sigma-Aldrich	M7403	MCDB 153-LB basal media w/ HEPES buffer
NEST Scientific 1-Well Cell Culture Chamber Slide, BLACK Walls on Glass Slide, 6/PK, 12/CS	Stellar Scientific	NST230111	
P63	Thermo Scientific	703809	For immunostaining 1:200 dilution
PE-conjugated anti-EGFR (San Jose, CA; catalog number)	BD Pharmingen	555997	For FACS analysis
pMSCV-IRES-EGFP plasmid vector	Addgene	20672	For transfection
Promega TransFast kit	Promega	E2431	For transfection
Qiagen RNeasy Plus Micro Kit	Qiagen		For microarray experiments
Thermo Scientific™ Sterile Single Use Vacuum Filter Units	Thermo Scientific	09-740-63D	For cell media

Zeiss Axionvert 135 fluorescence microscope	Zeiss	Use with Axiovision Rel. 4.5 software

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

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Rebuttal to the Reviewer's Edits: We would like to thank the editor for their comments and suggestions to improve this manuscript. All comments have been addressed in the revised application and are summarized bellow.

Editorial comments:
Changes to be made by the Author(s):

1. **Please include an ethics statement before the numbered protocol steps.**
    a. **Authors' response:** we thank the editor for these excellent suggestions. The revised manuscript has been edited to include an ethics consideration statement.

2. **In Figure 2D: Please specify micrometer instead of micromolar in the y axis.**
    a. The revised manuscript has been edited to specify micrometer instead of micromolar on the y-axis.

3. **Figure 4B: Please include a space between 2h so it says "2 h".**
    a. We have included a space between 2 and h to designate time.

4. **Where is Table 1? Please upload Table 2 as an xls/xlsx file instead of docx file.**
    a. We thank the editor for pointing this out. We have included Table 1 in the revised document. We also uploaded Table 2 as an xls file..

5. **Additional comments are in the attached manuscript).**
    a. All comments made in the manuscript have been answered or modified accordingly.