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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Protocol Length

Number of Steps: 25
Number of Shots: 52

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Yvon Woappi:** This protocol presents a novel method for the cultivation and maintenance of epidermosphere cultures and a strategy to closely investigate these cultures using the spheroid re-plating assay.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

1.1. Procedures involving human skin specimens were reviewed by the University of South Carolina IRB and classified as "research not involving human subjects". The protocol was also reviewed and approved by the UofSC Biosafety Committee. All procedures were conducted in concordance to the safety and ethics standards of UofSC.

Protocol

2. Isolation and culture of human keratinocytes from neonatal foreskin tissue (Clean flowhoo

2.1. Begin by preparing wash medium as described in the text manuscript **[1]**.

2.1.1. Wash medium

2.2. In a laminar flow hood, wash neonatal foreskin twice with 5 milliliters of wash media in a 50-milliliter conical tube, then transfer the washed foreskin to a sterile Petri dish **[1]**.

2.2.1. Talent washing foreskin with wash media

2.3. Using a scalpel and forceps, scrape off adipose and loose connective tissues from the dermal layer, then rewash the foreskin with wash media **[1]**.

2.3.1. Talent scraping off the tissues

2.4. Place the foreskin epidermis side up in a 6-well plate containing 2 milliliters of dispase enzyme diluted in wash media **[1-TXT]**. Transfer the plate to an incubator for 4 hours **[2-TXT]**.

2.4.1. Talent transferring the tissue in plate **TEXT: Dispase enzyme concentration: 50 U/mL**

2.4.2. Talent transferring the plate to an incubator **TEXT: 37 °C, 5% CO2, 95% humidity**

2.5. After incubation, transfer the foreskin to a Petri dish **[1]** and use fine-tip forceps to separate the epidermis from the dermis layer **[2]**.

2.5.1. Talent transferring the foreskin to the Petri dish

2.5.2. Talent separating the epidermis from the dermis

2.6. Place the epidermis into a 15-milliliter conical tube containing 2 milliliters of 0.25% Trypsin-EDTA **[1]**. Crush the floating epidermis using a 5-milliliter serological pipet **[2]** and incubate it for 15 minutes at 37 degrees Celsius **[3]** with periodic vortexing for 5 seconds every 5 minutes **[4]**.

2.6.1. Talent transferring the epidermis in tube

2.6.2. Talent crushing the epidermis using pipet

2.6.3. Talent incubating the tube

2.6.4. Talent vortexing the tube.

2.7. After incubation, add 2 milliliters of soybean trypsin inhibitor and mix it by pipetting to neutralize the trypsin **[1]**, then centrifuge the cell suspension **[2-TXT]**.

2.7.1. Talent adding and then mixing the inhibitor with pipette

2.7.2. Talent putting the tubes inside and setting up the centrifuge **TEXT: 2 minutes at 450 *x g*, then for 8 min at 250 *x g***

2.8. Resuspend the pellet in 12 milliliters of complete KSFM-scm medium **[1]** and plate the cells in a 10-centimeter dish to incubate overnight at 37 degrees Celsius and 5 % carbon dioxide **[2]**.

2.8.1. Talent resuspending the pellet

2.8.2. Talent plates the pellet in a dish

2.9. On the next day, remove the medium using an aspirating pipette **[1]** and replace it with 12 milliliters of complete KSFM-scm. Repeat this on days 4 and 7 **[2]**.

2.9.1. Talent removing the medium

2.9.2. Talent adding medium to the plate

3. Generating skin epidermosphere cultures in vitro

3.1. Prepare a 5% agarose mixture by adding 2.5 grams of agarose to 50 milliliters of PBS in a 100-milliliter glass bottle **[1]**. Autoclave the bottle under the liquid cycle and allow it to cool to room temperature **[2]**.

3.1.1. Talent preparing agarose mixture

3.1.2. Talent putting the bottle inside and setting the autoclave and places autoclaved bottle at the room temperature

3.2. To prepare plates, place the cooled glass bottle containing agarose solution in a 1-liter beaker filled with 200 milliliters of deionized water **[1]**. Melt the agarose mixture in a research-grade microwave for up to 2 minutes **[2]**, mixing the agar every 60 seconds by gently tilting the bottle side to side **[3]**.

3.2.1. Talent putting the glass bottle inside the beaker

3.2.2. Talent putting the beaker inside the microwave

3.2.3. Talent taking the bottle out of microwave

3.3. Add 3 milliliters of melted 5% agarose to 12 milliliters of prewarmed KSFM-scm at 42 degrees Celsius for a final concentration of 1% agarose **[1]**. Add 200 microliters of the 1% agar mix to each well of a 96-well plate using a multichannel pipettor, then leave plate in a sterile environment at 25 degrees Celsius for 4 hours **[2]**.

3.3.1. Talent adding melted agarose to prewarmed medium *Videographer: This step is important!*

3.3.2. Talent pipetting agarose in 96-well plate using multichannel pipettor *Videographer: This step is important!*

3.4. Passage spheroid-forming normal human keratinocytes by aspirating media and washing cells in 2 milliliters of PBS **[1]**. Aspirate PBS and add 2 milliliters of 0.25% Trypsin-EDTA to the washed cells, then incubate for 5 minutes at 37 degrees Celsius **[2]**.

3.4.1. Talent aspiring the media and washing the cells in PBS

3.4.2. Talent aspirating PBS and adding trypsin to the cells

3.5. After the incubation, add 2 milliliters of Soybean Trypsin Inhibitor to the plate **[1]** and wash the cells from the plate into a 15-milliliter tube **[2]**. Centrifuge at 250 times g for 5 minutes **[3]**.

3.5.1. Talent adding inhibitor to plate

3.5.2. Talent adding wash off cells to the tube

3.5.3. Talent placing the tube in the centrifuge

3.6. Resuspend the cell pellet in 1 milliliter of PBS **[1]** and quantify the cells using trypan blue staining. Aliquot 20,000 normal human keratinocytes in 100 microliters of KSFM-scm **[2]** and seed the cells in each well of the previously prepared 96-well plate **[3]**, then incubate the plate overnight **[4-TXT]**.

3.6.1. Talent resuspending the pellet in PBS

3.6.2. Talent aliquoting cells in medium

3.6.3. Talent seeding the one of the well of 96-well plate *Videographer: This step is difficult and important!*

3.6.4. Talent incubating the plate **TEXT: 37 °C, 5% CO2, 95% humidity**

3.7. Using an inverted phase contrast microscope, analyze the seeded wells for epidermosphere formation **[1]**.

3.7.1. Talent analyzing the plate under microscope

4. Epidermal spheroid re-plating assay

4.1. 24 to 48 hours after 3D epidermosphere formation, add 4 milliliters of prewarmed KSFM-scm at 37 degrees Celsius to a 6-centimeter dish **[1]**. Using a wide bore 1-milliliter pipette tip, transfer a single spheroid to the plate and incubate the plate overnight **[2]**.

4.1.1. Talent adding prewarmed medium to the dish *Videographer: This step is important!*

4.1.2. Talent transferring the spheroid to the plate. *Videographer: This step is important!*

4.2. Analyze the seeded spheroid for attaching or propagating cells using an inverted phase contrast microscope. Feed cells every 96 hours by removing the media and adding 2 milliliters fresh KSFM-scm media to the plate **[1]**.

4.2.1. SCOPE: Seeded spheroid.

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

5.1. Passage 70 to 80 percent confluent spheroid derived normal human keratinocytes and 2D monolayer cultures as previously demonstrated, then quantify cell viability using trypan blue and an automated cell counter **[1]**.

5.1.1. Talent quantifying the cells using hematocytometer/automated cell counter

5.2. Aliquot 100 microliters containing 100,000 to 4 million normal human keratinocytes into 1.5-milliliter microcentrifuge tubes **[1]**. Place the tubes on ice in a dark environment by turning off the bright lights within the laminar hood **[2].**

5.2.1. Talent aliquoting the cells to microcentrifuge tubes

5.2.2. Talent placing the tubes on ice and switching off the hood lights

5.3. Add 2 microliters of FITC *(pronounce "fit-See")*-conjugated anti-integrin-α6 *(pronounce: "alpha six")* and 2 microliters of PE-conjugated anti-EGFR to the tubes to achieve a 1 is to 50 dilutions. Prepare a tube with no antibodies added to serve as the unstained control. Incubate the tubes on ice in the dark for 30 minutes **[1]**.

5.3.1. Talent adding antibodies to the tube on ice.

5.4. Perform flow cytometry analysis with appropriate lasers. Use the negative and positive controls to establish gates **[1]**. Sort the subpopulation of epidermal stem cell fraction **[2]**, the proliferative progenitor cell fraction **[3]** and committed progenitor cell fraction **[4]**.

5.4.1. Talent putting the tubes in the flow cytometry instrument

5.4.2. LAB MEDIA: Figure 4D, EGFP-PE vs ITGA6-FITC plot *Video editor: Please highlight the $\alpha6^{hi}/ER^{lo}$ square with 22 % value*

5.4.3. LAB MEDIA: Figure 4D, EGFP-PE vs ITGA6-FITC plot *Video editor: Please highlight the $\alpha6^{hi}/ER^{hi}$ square with 6.18 % value*

5.4.4. LAB MEDIA: Figure 4D, EGFP-PE vs ITGA6-FITC plot *Video editor: Please highlight the $\alpha6^{lo}/ER^{hi}$ square with 9.91 % value*

5.5. Test for proliferative capacity of sorted cell subpopulations by transferring the content of each respective sorting tube into a 15-milliliter conical tube containing 10 times the volume of sorted cells in PBS **[1]**. Centrifuge the tubes at 250 *x g* for 5 minutes **[2]**.

5.5.1. Talent transferring the tube content in PBS

5.5.2. Talent centrifuging the tubes

5.6. Remove supernatant and resuspend the pellet in 4 milliliters of KSFM-scm **[1]**. Transfer the resuspended cells to a 6-centimeter plate **[2]** and incubate overnight at 37 degrees Celsius in a 5 % carbon dioxide incubator with 95% humidity **[3-TXT]**.

5.6.1. Talent removing the supernatant

5.6.2. Talent resuspending the pellet

5.6.3. Talent incubating the plate **TEXT: 37 °C, 5% CO2, 95% humidity**

5.7. On the next day, remove media from the plates and add 4 milliliters of pre-warmed KSFM-scm at 37 degrees Celsius **[1]**. Incubate the plate and re-feed the cells with 12 milliliters of medium after every 3 days until 70 to 80% confluency is reached **[2-TXT]**.

5.7.1. Talent removing and adding fresh warm media to the plate

5.7.2. Talent incubating the plate **TEXT: 37 °C, 5% CO2, 95% humidity**

Results

6. Results: Characterization of epidermal spheroid formation and spheroid-derived NHKs

6.1. Autonomous epidermal spheroid forming ability of normal human keratinocytes in 3D culture was assessed using the skin epidermosphere assay **[1]**. Non-spherical cell aggregation was not considered as adequate epidermosphere formation **[2]**.

6.1.1. LAB MEDIA: Figure 2

6.1.2. LAB MEDIA: Figure 2 *Video editor: Please highlight figure 2A*

6.2. Dense sphere-shape aggregation was considered a hallmark of spontaneous spheroid formation **[1]**. It was necessary to use more than 2 x 10^4 cells to ensure proper spontaneous aggregation **[2]**.

6.2.1. LAB MEDIA: Figure 2 *Video editor: Please highlight figure 2B*

6.2.2. LAB MEDIA: Figure 2 *Video editor: Please highlight figure 2D*

6.3. Plating of epidermospheres in 2D culture resulted in the proliferation of small-sized viable natural human keratinocytes **[1]**. It is important to maintain these cultures below 100% confluency as this can considerably impair their growth and stem cell state in culture **[2]**.

6.3.1. LAB MEDIA: Figure 3

6.3.2. LAB MEDIA: Figure 3 *Video editor: Please highlight figure 3E and figure 3F*

6.4. The process of epidermal spheroid formation was functionally tracked at the single cell level by transfecting cells with a fluorescent reporter **[1]**. Spheroid derived normal human keratinocyte sub-population are Integrinα6 *(pronounce: "integrin-alpha six")* high and EGFR low cells. These cells generally make up about 25% of cultures **[2]**.

6.4.1. LAB MEDIA: Figure 4B and 4C

6.4.2. LAB MEDIA: Figure 4D

6.5. Characterization of this stem-like keratinocyte subpopulation was achieved by immunostaining analysis of basal cytokeratin 14 and tumor protein 63 **[1]**.

6.5.1. LAB MEDIA: Figure 4F

Conclusion

7. Conclusion Interview Statements

7.1. **Yvon Woappi:** The spheroid re-plating assay is an effective strategy for epidermal stem cell enrichment from neonatal human skin. This system can be employed as a high throughput cell-based model to investigate cutaneous diseases, such as wound healing and cancer.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.