Markers for an In Vitro Skin Substitute

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Abstract

The tissue engineering self-assembly approach allows the production of skin substitutes comprising both the dermis and epidermis, using methods promoting the secretion and organization of a dense extracellular matrix by skin cells. In a reconstructed epidermis, all cellular layers of the native tissue are present. An evaluation of the expression and localization of a number of specific protein markers revealed that the self-assembled, tissue-engineered skin substitute shares some common features with normal human skin, such as the expression of Ki-67, keratins 10 and 14, filaggrin, involucrin, transglutaminase, DLK, α3-integrin subunit, laminin-5, and collagens I, II, IV, and VII. At the ultrastructural level, many differentiation markers can be observed, including desmosomes, as well as an organized basement membrane presenting hemidesmosomes, lamina densa, and lamina lucida. In this chapter, protocols to generate skin substitutes by the self-assembly approach will be presented and the methods including the labeling of the principal skin differentiation markers by immunofluorescence will be examined.

Key terms

- collagens
- ibronectin
- filaggrin
- involucrin
- keratins
- laminin
- Loricrin
- markers
- skin substitute
- transglutaminase
11.1 Introduction

Multiple kinds of artificial skin substitutes are now available. They have several prospective applications including the treatment and closure of skin wounds, models of skin biology and pathology, alternatives to animals for safety testing, and drug delivery [1]. Each of these prospective applications has distinct requirements for validation of skin substitutes. These categories of applications represent, respectively, the fields of surgery, investigative dermatology, toxicology, and pharmacology [1].

The various methods used for the reconstruction of living skin substitutes give different epidermal-cell phenotypes depending on the culture conditions [2]. However, the final goal in the development of alternative in vitro models, for cytotoxicity studies as well as for extensive burn coverage, is to create an in vitro model that enables extrapolation to in vivo results. In this regard, the morphological equivalence of the tissue architecture with native tissue is not a sufficient criterion of the quality of the reconstructed tissue [3]. The expression of various specific protein markers have to be monitored and evaluated as well. An appropriate cultured skin substitute should thus exert morphological, biochemical, and functional features that approach those of the native tissue [4].

The human epidermis is a stratified squamous epithelium characterized by a high keratin (K) content and by the ability to make cornified envelopes. When keratinocytes leave the basal compartment and progress upward in the epidermis, they sequentially undergo several differentiation changes and stop dividing [5, 6]. Four layers can be distinguished morphologically in healthy epidermis: stratum basale, spinosum, granulosum, and corneum. The function of stratum basale is to proliferate and to keep the epidermis firmly anchored to the basement membrane [7]. The function of stratum spinosum is to start the differentiation process by producing new cell-cell attachments, cytoskeleton, and so forth. The function of stratum granulosum is to produce cornified envelope proteins, crosslinking enzymes and appropriate lipids. In the stratum corneum, proteins are crosslinked, lipids are extruded, and the epidermal barrier is formed [7]. The cohesion between the dermis and the epidermis is ensured by the dermo-epidermal junction, which is also required for control of epidermal growth and differentiation [8, 9].

In this chapter, protocols to generate the self-assembled, tissue-engineered skin substitute model [10, 11] will be described in detail and the quality of the skin substitutes will be examined using tissue morphology and the presence and distribution of various specific protein markers. Morphological and ultrastructural markers of epidermal differentiation include individualization of the epidermal layers such as the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum and the presence of desmosomes, hemidesmosomes, and a basement membrane at the dermo-epidermal junction. Proliferation markers such as Ki-67, as well as epidermal differentiation markers like α3-integrin subunit, keratin patterns (K14 and K10), involucrin, transglutaminase, the dual leucine zipper-bearing kinase (DLK), and filaggrin have additional specific biochemical characteristics. Other components of the basement membrane (collagens IV and VII, laminin-5) and of the dermis (collagen I) will also be examined.
11.2 Experimental Design

The concept of the self-assembly approach is to reconstruct an organ in a fashion resembling its formation in vivo, in which the use of appropriate culture and mechanical conditions induces cells to secrete a significant amount of extracellular matrix as during organogenesis [12–14]. The skin substitutes reconstructed in a 3D environment, as those produced by the self-assembly approach presented in this chapter, exhibits a well-developed epidermis that expresses differentiation markers and a well-organized basement membrane [10, 11]. Among the key developments in this procedure were the use of autologous cells and the capacity of mesenchymal cells, such as fibroblasts, to create their own extracellular matrix in vitro [15]. The model supports the hypothesis that keratinocytes and fibroblasts of individuals possess inherent properties of the native tissues as observed with skin substitutes reconstructed from psoriatic cells. The study showed that psoriatic skin reconstructed in vitro partially displays a psoriatic phenotype [16]. The immunohistochemical analyses performed to evaluate the quality of the skin model in vitro require biopsies from the native tissues as relevant controls. To insure reproducibility, at least three subjects per independent trial must be compared and the experiment performed three times.

11.3 Materials

11.3.1 Human tissue-engineered skin substitute reconstructed by the self-assembly approach

- Confluent human fibroblasts between their second and eighth passages (for a detailed protocol concerning the extraction from human skin and the culture of fibroblasts, refer to [17])
- Human keratinocytes at 80% confluence between their third and fifth passages (cocultured with iS3T3 cells) (for a detailed protocol concerning the extraction from human skin and the culture of keratinocytes, refer to [17])
- DMEM (Invitrogen, Burlington, Ontario, Canada)
- Ham's F12 medium (Invitrogen)
- 0.22-μm low-binding disposable filter (Millipore, Billerica, Massachusetts)
- NaHCO₃ (Fisher Scientific, Ottawa, Ontario, Canada)
- Adenine (Sigma, St. Louis, Missouri)
- Fetal calf serum (HyClone, Scarborough, Ontario, Canada)
- Fetal clone II serum (HyClone)
- Penicillin G (Sigma)
- Gentamicin (Sigma)
- Insulin (Sigma)
- Hydrocortisone (Calbiochem, Gibbstown, New Jersey)
- Cholera toxin (Sigma)
- Epidermal growth factor (Sigma)
- Ascorbic acid (Sigma) (light-sensitive)
- Ingots (16-G stainless steel grade # 316, Denmar, Quebec, Canada)
Markers for an In Vitro Skin Substitute

• Merocel sponge, Medtronic (Instruments Ophtalmiques INNOVA, Laval, Quebec, Canada)
• Seeding ring (stainless steel grade # 316, Denmar); dimension: 3-cm diameter, 7/8-inch wide, 1/8-inch height, sterilized with autoclave
• Dissecting curved forceps (Fisher Scientific) sterilized with autoclave
• Tissue culture flask, 25 cm² (BD Biosciences, Mississauga, Ontario, Canada)
• Cell culture dish (BD Biosciences)
• Petri dish, size: 100 mm x 15 mm (Fisher Scientific)
• Weller Universal Dual Heat Soldering Gun (available in local hardware)
• Anchoring paper: Cut a circle with a 60-mm diameter in a Whatman sheet (Fisher Scientific); remove the concentric inside disk of 25 mm diameter after cutting; and sterilize with autoclave
• Air-liquid stand (sterile homemade stand, 1/16-inch height)
• Standard culture materials (sterile pipettes, droppers, tips, test tubes)
• Sterile laminar flow hood cabinet (class II cabinet)
• Laboratory equipment (pipetman, micropipettes, water bath, autoclave, pH meter, incubator, preparative centrifuge, magnetic stirrer, stir bar, freezer, refrigerator, vaccum)
• Laboratory glassware (graduate cylinder, beaker, pipettes, bottles)
• Immunofluorescence
• Dissecting jeweler microforceps (Fisher Scientific)
• Dissecting curved scissors (Fisher Scientific)
• OCT compound (Miles Inc., Monrovia, California)
• Small container with liquid nitrogen
• Tissue holder
• Forceps (Fisher Scientific)
• Cryostat
• Freezers (−80°C and −20°C)
• Superfrost glass slides (Fisher Scientific)
• Slide warmer (Fisher Scientific)
• Diamond pencil (Fisher Scientific)
• Distilled water (H₂O₁₂)
• NaH₂PO₄·H₂O (Fisher Scientific)
• Acetone
• NaCl (Fisher Scientific)
• KCl (Fisher Scientific)
• Na₂HPO₄ (Fisher Scientific)
• KH₂PO₄ (Fisher Scientific)
• MgCl₂·6H₂O 2.8M solution kept at −20°C (Sigma)
• CaCl₂·2H₂O (Sigma)
• Bovine serum albumin (BSA) (Sigma)
• Primary antibodies (see Table 11.1)
### Table 11.1 Antibodies

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Antigen</th>
<th>Host</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antirecombinant human Ki-67 peptide mAb (856)</td>
<td>Ki-67</td>
<td>Mouse</td>
<td>IgG1</td>
<td>BD Biosciences</td>
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<td>Anti-K14 C-terminal peptide pAb</td>
<td>Keratin 14</td>
<td>Rabbit</td>
<td></td>
<td>Dr. Normand Marceau, Centre de recherche de l'Hôtel-Dieu de Québec, Université Laval, Quebec, Canada [18]</td>
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<tr>
<td>Anticytokeratin 10 mAb (RKS60)</td>
<td>Keratin 10</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Cedarlane</td>
<td>1:100</td>
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<tr>
<td>Antifilaggrin mAb</td>
<td>Filaggrin</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Abcam, Cambridge, Massachusetts</td>
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<tr>
<td>Anti-involucrin mAb</td>
<td>Involucrin</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Sigma</td>
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<tr>
<td>Antitransglutaminase mAb</td>
<td>Transglutaminase I</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>Biomedical Technologies Inc., Stoughton, Massachusetts</td>
<td>1:400</td>
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<td>Anti-N-terminal portion of recombinant mouse DLK pAb</td>
<td>Dual leucine zipper-bearing kinase (DLK)</td>
<td>Rabbit</td>
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<td>Dr. Richard Blouin, Département de Biologie, Université de Sherbrooke, Quebec, Canada [19]</td>
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<tr>
<td>mAb antidesmosomal protein (ZK-31)</td>
<td>Epitope on desmosome</td>
<td>Mouse</td>
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<td>Sigma</td>
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<tr>
<td>Anti-a3-integrin (VM-2) mAb (HB-8530) conjugated to Alexa Fluor 488 (Invitrogen)</td>
<td>a3-integrin subunit</td>
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<td>IgG1</td>
<td>ATCC, Manassas, Virginia</td>
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<td>Anticollagen type IV pAb</td>
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<td></td>
<td>Abcam</td>
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<td>Antilaminin-5 mAb</td>
<td>Laminin 5</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Dr. Patricia Rousselle, Institut de Biologie et Chimie des Protéines, UMR 5086, CNRS, Université Lyon [20]</td>
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</tr>
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<td>Anticollagen type I pAb</td>
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<td>Isotype Control Antibody</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG1</td>
<td>Mouse</td>
<td>IgG1</td>
<td>Dako, Mississauga, Ontario, Canada</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>IgG2a</td>
<td>Mouse</td>
<td>IgG2a</td>
<td>Dako</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>IgG1a isotype control conjugated to Alexa Fluor 488</td>
<td>Mouse</td>
<td>IgG1a</td>
<td>Dako</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluorescein conjugated antimouse</td>
<td>Mouse</td>
<td>Goat</td>
<td>Millipore</td>
<td></td>
<td>1:100</td>
</tr>
<tr>
<td>FITC conjugated antirabbit IgG, H+L</td>
<td>Rabbit IgG, H+L</td>
<td>Goat</td>
<td>Millipore</td>
<td></td>
<td>1:200</td>
</tr>
</tbody>
</table>

mAb: monoclonal antibody, pAb: polyclonal antibody, #: Dilute at the same concentration than the associated primary antibody.

- Secondary antibodies (see Table 11.1)
- Hydrogen peroxide 30% (v/v) (Sigma)
- Microscope slide mailer (Somagen Diagnostics Inc., Edmonton, Alberta, Canada)
- Microscope slide rack (Somagen Diagnostics Inc.)
- NaN₃ (Sigma)
- Gelatin (Fisher Scientific)
- pH paper (EMD Chemicals Inc., Gibbstown, New Jersey)
- Glycerol (MP Biomedicals, Solon, Ohio)
- Absorbent paper
11.4 Methods

11.4.1 Preparation of solutions and materials for the in vitro fabrication of human skin substitutes by the self-assembly approach

- **DMEM-Ham.** DMEM: Ham's F12 medium, 3:1, 3.07 g/L NaHCO₃ (36.54 mM), 24.3 mg/L adenine (0.18 mM), 312.5 μL/L 2N HCl. Dissolve in pyrogenic ultrapure water. Adjust pH to 7.1. Sterilize by filtration through a 0.22-μm low-binding disposable filter. Aliquot and store at 4°C.
- **Fetal calf serum and fetal clone II serum.** Thaw in cold water. Inactivate in hot water (56°C) for 30 minutes. Distribute in single-use aliquots and store at −20°C.
- **Insulin.** Dissolve 250 mg in 50-mL 0.005N HCl (125 μL 2N HCl/50 mL pyrogenic ultrapure water) to make a 1,000× stock solution (0.87 mM). Sterilize by filtration through a 0.22-μm low binding disposable filter, distribute in single-use aliquots, and store at −80°C.
- **Hydrocortisone.** Dissolve 25 mg in 5 mL of 95% ethanol (4.8 mL 99% ethanol/0.2 mL pyrogenic ultrapure water). Complete to 125 mL with DMEM-Ham to make a 500× stock solution (0.53 mM). Sterilize by filtration through a 0.22-μm low binding disposable filter, distribute in single-use aliquots, and store at −80°C.
- **Cholera toxin.** Dissolve 1 mg in 1 mL of pyrogenic ultrapure water. Complete to 118.18 mL with DMEM-Ham supplemented with 10% (v/v) fetal clone II to make a 1,000× stock solution (10⁻⁷ M). Sterilize by filtration through a 0.22-μm low binding disposable filter, distribute in single-use aliquots, and store at −80°C.
- **Epidermal growth factor.** Dissolve 500 μg in 2.5 mL of 10 mM HCl. Complete to 50 mL with DMEM-Ham supplemented with 10% (v/v) fetal clone II to make a 1,000× stock solution. Sterilize by filtration through a 0.22-μm low binding disposable filter, distribute in single-use aliquots, and store at −80°C.
- **Penicillin G and Gentamicin.** Dissolve 50,000 IU/mL of Penicillin G and 12.5 mg/mL of Gentamicin sulfate in pyrogenic ultrapure water to make a 500× stock solution. Sterilize by filtration through a 0.22-μm low-binding disposable filter, distribute in single-use aliquots, and store at −80°C.

**Preparation of culture media**

- **Human fibroblast culture medium (fDMEM).** To make 1L, refer to Table 11.2. Thaw all components at 4°C. Store at 4°C for a maximum of 10 days.
- **Complete human keratinocyte culture medium (complete hkDMEM-Ham).** To make 1L, refer to Table 11.2. Thaw all components at 4°C. Store at 4°C for a maximum of 10 days.
- **Complete air-liquid human keratinocyte culture medium (complete alhkDMEM-Ham).** To make 1L, refer to Table 11.2. Thaw all components at 4°C. Stored at 4°C for a maximum of 10 days.
Table 11.2 Medium Reconstitution

Human Fibroblast Culture Medium (fDMEM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>900 mL</td>
<td>90% (v/v)</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>100 mL</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Penicillin G-Gentamicin 500×</td>
<td>2 mL</td>
<td>Penicillin G 100 IU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamicin 25 µg/mL</td>
</tr>
</tbody>
</table>

Complete Human Keratinocyte Culture Medium (complete hkDMEM-Ham)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-Ham</td>
<td>950 mL</td>
<td>95% (v/v)</td>
</tr>
<tr>
<td>Fetal clone II</td>
<td>50 mL</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>Insulin 1,000×</td>
<td>1 mL</td>
<td>5 mg/mL</td>
</tr>
<tr>
<td>Hydrocortisone 500×</td>
<td>2 mL</td>
<td>0.4 µg/mL</td>
</tr>
<tr>
<td>Cholera toxin 1000×</td>
<td>1 mL</td>
<td>10⁻¹⁵ M</td>
</tr>
<tr>
<td>Epidermal growth factor 1,000×</td>
<td>1 mL</td>
<td>10 ng/mL</td>
</tr>
<tr>
<td>Penicillin G-Gentamicin 500×</td>
<td>2 mL</td>
<td>Penicillin G 100 IU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamicin 25 µg/mL</td>
</tr>
</tbody>
</table>

Air-Liquid Human Keratinocyte Culture Medium (alkDMEM-Ham)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-Ham</td>
<td>950 mL</td>
<td>95% (v/v)</td>
</tr>
<tr>
<td>Fetal clone II</td>
<td>50 mL</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>Insulin 1,000×</td>
<td>1 mL</td>
<td>5 µg/mL</td>
</tr>
<tr>
<td>Hydrocortisone 500×</td>
<td>2 mL</td>
<td>0.4 µg/mL</td>
</tr>
<tr>
<td>Cholera toxin 1,000×</td>
<td>1 mL</td>
<td>10⁻¹⁰ M</td>
</tr>
<tr>
<td>Penicillin G-Gentamicin 500×</td>
<td>2 mL</td>
<td>Penicillin G 100 IU/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamicin 25 µg/mL</td>
</tr>
</tbody>
</table>

11.4.2 In vitro fabrication of human skin substitutes by the self-assembly approach

All further manipulations are performed under a sterile laminar flow hood cabinet.

11.4.2.1 Assembly of fibroblast sheets for dermal reconstruction

A schematic drawing of the method is presented in Figure 11.1(a).

1. Seed 2 × 10⁴ fibroblasts per 25 cm² culture flask in 5 mL of fDMEM containing 50 µg/mL ascorbic acid. Incubate in 8% CO₂, 100% atmospheric humidity at 37°C. Change culture medium three times a week.
2. After about 28–35 days, open the top of the flask with a soldering iron.
3. Using curved forceps, detach carefully one fibroblast sheet. Transfer it into a 100-mm cell culture dish.
4. Anchor peripherally the fibroblast sheet with ingots. Move the ingots one by one towards the tissue periphery to flatten the fibroblast sheet.
5. Detach a second fibroblast sheet and transfer it onto the first fibroblast sheet. Repeat step 4.
6. Place a Merocel sponge (which has been cut to fit within the ingots) on top of the superimposed fibroblast sheets. Keep the Merocel sponge in place with ingots.
Figure 11.1 Schematic representation of the in vitro production of reconstructed skin. (a) Production of stromal sheets for dermal reconstruction. Dermal fibroblasts are cultivated in fDMEM containing 50 µg/mL of ascorbic acid for 28–35 days in order to produce stromal sheets. Then two stromal sheets are detached and superimposed; ingots (small weights) are placed around the edges to keep the sheets in contact. A Merocel sponge, which has been cut to fit within the ingots, is placed on top of stromal sheets, and maintained in place with ingots (after about 2 days, Merocel sponge is removed). The resulting reconstructed dermal substitute is cultivated for one more week in fDMEM containing 50 µg/mL of ascorbic acid. (b) Assembly of reconstructed skin. Keratinocytes are seeded on top of reconstructed dermis within a seeding ring. After 1 more week under submerged conditions in hkDMEM-Ham containing 50 µg/mL of ascorbic acid, the tissue is detached carefully from the bottom of the cell culture dish and placed on an anchoring paper in order that the area with keratinocytes is on top of aperture. The reconstructed tissue together with the anchoring paper are lifted and transferred onto an air-liquid stand, and then cultured at the air-liquid interface in the maturation medium (alkDMEM-Ham containing 50 µg/mL ascorbic acid). (c) Macroscopic aspect of reconstructed skin after 21 days of maturation at the air-liquid interface.

7. Add 25 mL of the fDMEM containing 50 µg/mL ascorbic acid. Incubate in 8% CO₂, 100% atmospheric humidity at 37°C.

8. After 2 days, remove the medium and then carefully remove the ingots (those on the Merocel sponge) and the Merocel sponge.

9. Put a seeding ring in the center of the fibroblast sheets (the seeding ring can be placed between 1 and 7 days before keratinocyte seeding).
10. Add 25 mL of the fDMEM containing 50 μg/mL ascorbic acid. Incubate in 8% CO₂, 100% atmospheric humidity at 37°C. Change the culture medium three times a week.

11.4.2.2 Assembly of reconstructed skin

A schematic drawing of the method is presented in Figure 11.1(b).

11. One week after the stacking of fibroblast sheets, remove culture medium and seed 8 × 10⁶ keratinocytes within the seeding ring (from a suspension of cells in a complete hDKMEM-Ham). Incubate in 8% CO₂, 100% atmospheric humidity at 37°C.

12. About 2 to 4 hours later, add 25 mL of complete hDKMEM-Ham containing 50 μg/mL ascorbic acid. Incubate in 8% CO₂, 100% atmospheric humidity at 37°C. Change the culture medium three times a week.

13. Twelve hours later, the seeding ring can be removed.

12.4.2.3 Maturation of the tissue-engineered skin by culturing at the air-liquid interface

14. One week after the keratinocyte seeding, remove culture medium and ingots.

15. Using curved forceps, detach carefully the reconstructed skin from the bottom of the cell culture dish.

16. Place the reconstructed skin on the anchoring paper, centering it in order that the area with keratinocytes is on top of the aperture.

17. Put an air-liquid stand in a Petri dish.

18. Lift reconstructed skin together with the anchoring paper and transfer it onto the air-liquid stand.

19. Add 25 mL of alkDMEM-Ham containing 50 μg/mL ascorbic acid. Incubate in 8% CO₂, 100% atmospheric humidity at 37°C. Change the culture medium three times a week.

Note that tissue-engineered skin can be cultivated for more than 28 days at the air-liquid interface.

11.4.3 Tissue preservation and sectioning

1. Cut the tissue-engineered skin into small pieces (5 mm × 5 mm).

2. Sponge the excess tissue fluid with absorbent paper.

3. Completely coat the tissue with OCT without creating bubbles.

4. Put liquid OCT on a tissue holder and use the forceps to immerse it for 2–3 seconds in liquid nitrogen and then remove it before the OCT completely solidifies.

5. With forceps, take the OCT-coated piece of tissue and place it on top of the liquid OCT on the tissue holder.

6. Add OCT on top of the tissue sample to completely recover the tissue and prevent it from being freeze-dried.

7. Take the tissue holder with the forceps and immerse it in liquid nitrogen for approximately 15 seconds. The OCT should be white, but should not crack.
8. Store OCT blocks at -80°C until use.
9. Cut the tissue in a cryostat (4-6μm-thick sections) and place the tissue sections on the superfrost glass slides.
10. Dry the sections on a warm plate at 37°C for 30 minutes.
11. Process slides for immunohistochemistry or store the slides at -20°C (they can be kept for few weeks).

11.4.4 Preparation of solutions and materials for immunofluorescence

- **Phosphate-buffered saline-calcium (PBS-Ca).** To make 1L, dissolve 8g NaCl (137 mM), 0.2g KCl (2.7 mM), 0.92g Na₂HPO₄ (6.5 mM), 0.2g KH₂PO₄ (1.5 mM), and 0.17 mL MgCl₂·6H₂O (0.5 mM) of a 2.8M solution kept at -20°C, and 0.131g CaCl₂·2H₂O (0.9 mM) in dH₂O. Complete to 1L with dH₂O. Verify that the pH is 7.4. Note that a five-times concentrated PBS solution can be prepared and kept at 4°C, but the CaCl₂ should be omitted and added only after dilution of PBS to 1x. A ten-times concentrated solution can also be prepared and kept at 4°C.

- **PBS-Ca-BSA.** PBS-Ca containing 1% BSA. Dissolve 1g bovine serum albumin in 100 mL PBS-Ca.

- **Primary antibodies.** Dilute in PBS-Ca-BSA as specified in Table 11.2.

- **Secondary antibodies.** Dilute in PBS-Ca-BSA as specified in Table 11.2.

- **Mounting media.** To make 160 mL, dissolve 3.2g gelatin in 100-mL hot dH₂O. Add 0.896g NaCl (137 mM), 0.0224g KCl (2.7 mM), 0.1032 g Na₂HPO₄ (6.5 mM), 0.0224g KH₂PO₄ (1.5 mM), and 0.16g NaN₃ (22 mM). Adjust pH to 7.6 using pH paper. Complete to 160 mL with dH₂O. Add 48 mL glycerol.

- **Hoechst solution.** Dissolve 25 mg of Hoechst 33342 in 1 mL of dH₂O to make stock solution (25 mg/mL). Dilute 100 μL of stock 1 solution in 49.9 mL of dH₂O, and dilute 100× stock 2 solution (50 μg/mL) to make 100x stock 2 solution to 1× (0.5 μg/mL), add dH₂O.

11.4.5 Immunofluorescent labeling of human skin substitutes

1. Take the tissue sections from the freezer and leave them at room temperature for about 5 minutes to remove excess humidity. If the tissue sections are small, they can be circled by etching the slide with a diamond pencil.

2. Acetone fixation: Fix the tissue sections by immersion in cold acetone for 10 minutes at -20°C.

3. PBS-Ca washes: Put the slides in a rack and rinse three times in PBS-Ca for 5 minutes. Repeat twice.

4. First primary antibody incubation: Aspirate the liquid surrounding the tissue sections. Work rapidly to avoid drying the tissue. Cover each tissue section with 25 to 50 μL of the primary antibody. For control slides, use an unrelated antibody of the same isotype and then the monoclonal primary antibody used or PBS-Ca BSA. In the case of a polyclonal antibody, put PBS-Ca-BSA. Incubate for 45 minutes at room temperature.

5. Do three PBS-Ca washes.
6. Secondary antibody incubation: Aspirate the liquid surrounding the tissue sections. Process rapidly to avoid drying the tissue. Cover each tissue section with 25 to 50 μL of the secondary antibody. Incubate for 30 minutes at room temperature and protect from the light.

7. Do three PBS-Ca washes.

8. The tissue sections or cells can be counterstained with Hoechst (nuclei staining) as follows. Put the slides in a rack and immerse it in two successive baths of dH2O (2 minutes by wash). Cover with 25 to 50 μL of the 1× Hoechst solution. Incubate for 10 minutes in the dark. Do three dH2O washes.

9. Mount the slides as follows. Warm the solid mounting media in warm water or in the microwave to liquefy it. Place one drop of the mounting media on each tissue section. Put a cover slip gently on top while trying to avoid bubbles. Remove the excess of the mounting media by draining onto an absorbent paper.

10. Store in the dark at 4°C to avoid any bleaching of the fluorescence.

11. Examine the sections under a microscope equipped with epifluorescence.

11.4.6 Histological analysis

Cut the skin substitute into pieces of about 4 mm × 15 mm. Fix in the Histochoice MB fixative (Amresco) overnight at room temperature. Histologically process the tissue and embed in paraffin. Stain 6-μm-thick sections with Masson’s Trichrome according to standard procedures. Examine the section under a light microscope.

11.4.7 Transmission electron microscopy

Cut the skin substitute into pieces of about 1 mm³. Fix tissue with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for a minimum of 4 hours or overnight at 4°C. Wash in 0.1M sodium cacodylate buffer, post fix in 2% osmium tetroxide, and embed in Epon 812 according to standard procedures. Stain thin sections with uranyl acetate and lead citrate.

11.4.8 Statistical analysis

- Although variations within batches are low, there are some interbatch variations which merit that the experiment should be repeated with cells from other donors.
- Immunohistochemistry is a qualitative analysis. No statistics are usually performed except if an evaluation of the percentage of cells (for example, the proportion of Ki-67-labeled cell) or the measurement of a morphological aspect (for example, the measurement of the epidermal thickness). However, it is necessary to be sure that the noticed phenomenon is reproducible (makes at least three independent experiments).
- Since there is a set of dependent variables between experiments, a normal distribution could not be assumed because it involves an in vitro environment.
- The variation between the experiments is usually low and does not necessitate large samples. A nonparametric test is suggested to perform statistical analysis.
- Western Blot or FACS analyses could be performed to complete the study in a more quantitative manner.
11.5 Anticipated Results

As highlighted in this chapter, the development of in vitro skin substitute by tissue engineering must include the monitoring of the expression of various specific protein markers. A series of coordinated morphological and biochemical changes that result in a highly specialized and organized stratified squamous epithelium takes place during terminal differentiation and is associated with typical markers. Thus, fully developed reconstructed skin must expressed a broad range of protein products typically found in native skin [21], as observed in the tissue-engineered skin substitutes produced by the self-assembly approach (the similarities and differences are summarized in Table 11.3).

The culture at the air-liquid interface exerts a powerful effect on skin epithelial cells [22]. Macroscopically, the gross aspect of the tissue-engineered skin substitute changes in appearance from shiny to an opaque white, indicating the formation of the stratum corneum, which is induced by the contact with air [for a macroscopic picture, see Figure 11.1(c)]. Human keratinocytes cultured on an appropriated stromal substitute under submerged conditions proliferate until confluence and then form an epidermis composed of one to three cell layers [Figure 11.2(a)]. The formation of a well-organized epidermis with all histological layers present in the normal epidermis is achieved in vitro by culturing the substitute at the air-liquid interface for about 14 days [Figure 11.2(d)]. At the beginning of culture, the most differentiated layers are not present [Figure 11.2(b)].

<table>
<thead>
<tr>
<th>Table 11.3</th>
<th>Comparison Between Native Skin and the Air-Exposed Tissue-Engineered Skin Substitute</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristics</strong></td>
<td><strong>Native Skin</strong></td>
</tr>
<tr>
<td>Tissue architecture</td>
<td></td>
</tr>
<tr>
<td>Stratification</td>
<td>All epidermal strata</td>
</tr>
<tr>
<td>Desmosomes</td>
<td>SB, SS, SG, SC</td>
</tr>
<tr>
<td>Hemidesmosomes</td>
<td>Present</td>
</tr>
<tr>
<td>Basement keratinohyalin granules</td>
<td>Present</td>
</tr>
<tr>
<td>Cornified cell envelope</td>
<td>Present</td>
</tr>
<tr>
<td>Expression of epidermal differentiation-specific protein markers</td>
<td></td>
</tr>
<tr>
<td>Keratins</td>
<td>SB, SPB: K14</td>
</tr>
<tr>
<td></td>
<td>SPB: K10</td>
</tr>
<tr>
<td>Ki-67</td>
<td>SB</td>
</tr>
<tr>
<td>VM-2 (a3 integrin subunit)</td>
<td>SB</td>
</tr>
<tr>
<td>Involutrin</td>
<td>SG</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>SG</td>
</tr>
<tr>
<td>DLK</td>
<td>SG</td>
</tr>
<tr>
<td>Filaggrin</td>
<td>SG</td>
</tr>
<tr>
<td>Extracellular matrix component</td>
<td></td>
</tr>
<tr>
<td>Collagen I</td>
<td>D</td>
</tr>
<tr>
<td>Collagen III</td>
<td>D</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>BM</td>
</tr>
<tr>
<td>Collagen VII</td>
<td>BM</td>
</tr>
<tr>
<td>Laminin-5</td>
<td>BM</td>
</tr>
</tbody>
</table>

BCs, basal cells; SPBs, suprabasal cells; SBs, stratum basale; SSs, stratum spinosum; SGs, stratum granulosum; SCs, stratum corneum; Ks, keratin; BMs, basement membrane; Ds, dermis; RDs, reconstructed dermis.
The stratum granulosum appears after about 7 days [Figure 11.2(c)]. With time, the stratum corneum usually becomes thicker during the culture than normal skin because of the absence of desquamation. Thus, the tissue-engineered skin substitute is a dynamic tissue in which keratinocytes are induced to differentiate by prolonging the culture period. The distribution of keratinocyte differentiation-associated markers is going to vary according to the differentiation state of the skin substitute.

During the keratinocyte differentiation process, one of the major changes is the induction of unusually large keratins, which occurs as a cell leaves the basal layer [23]. Basal cells express mainly the keratins K5 and K14, whereas suprabasal cells express keratins K1 and K10 [7]. In a tissue-engineered skin substitute, K14 expression appears in cells of the stratum basale [Figure 11.3(a)] while K10 expression begins from the stratum spinosum [Figure 11.3(b)].

Involucrin, a marker of terminal differentiation, is among the first cornified envelope precursors to be cross-linked during keratinocyte differentiation [24]. It is expressed from the upper spinous layer of the epidermis in normal skin [25, 26]. In the mature tissue-engineered skin substitute, involucrin appears directly above the basal cells [Figure 11.3(c)], while the transglutaminase expression begins to be significant in upper spinous layers [Figure 11.3(d)]. The dual leucine zipper-bearing kinase (DLK) is a mitogen-acti-
vated protein kinase kinase kinase that has an active role in the induction of terminal differentiation [27–29]. DLK and filaggrin are not expressed in the first phases of epidermal cell differentiation [Figure 11.4(a, d)]. These proteins are induced when the stratum granulosum develops at 7 days of culture at the air-liquid interface and increase with epidermal differentiation [Figure 11.4(b, c, e, f)].

Epidermis is a self-renewing stratified epithelium. In order to localize cycling cells or to determine the proliferating index of a skin substitute, detection of Ki-67, a nuclear antigen expressed by proliferating cells in all phases of the active cell cycle (G1, G2, S, and M) but absent in resting cells (G0) can be performed [30, 31]. Typically, Ki-67 appears in the nucleus of some keratinocytes dispersed within the stratum basale of the tissue-engineered skin substitute [arrows in Figure 11.5(a)]. Also, the monoclonal anti-

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**Figure 11.4** Expression of DLK and filaggrin with the culture time. Immunofluorescence labeling of (a–c) DLK and (d–f) filaggrin in tissue-engineered skin substitutes produced by the self-assembly approach cultured (a, d) 3, (b, e) 7, and (c, f) 14 days at the air-liquid interface. Nuclei were stained with Hoechst dye (g, h, and i, respectively). Phase contrast microscopy is also provided (j, k, and l, respectively). Bars = 50 μm. (Reproduced and adapted from [28] according to the copyright policy of the publisher. © 2009 Nature Publishing Group.)
body called VM-2, another basal keratinocyte marker, which is expressed in basal cells of normal skin [32], recognizes the α3 subunit of the α3β1 integrin in the basal keratinocytes of the tissue-engineered skin substitute [Figure 11.5(b)].

Desmosomes are important structures in cell-to-cell adhesion. They serve as anchoring plates for keratin filaments [33]. In the tissue-engineered substitute, the mouse monoclonal ZK-31 antibody formed against the desmosomal protein (pan desmocollin) reveals a dot-like pattern on the keratinocyte cell membrane [Figure 11.5(d)]. The presence of desmosomes is also easily recognizable by transmission electron microscopy [Figure 11.6(a)].

A bilayered skin substitute comprising both living fibroblasts and keratinocytes presents advantages over other models. In addition to synthesizing ECM, fibroblasts secrete many components of the basement membrane such as collagen IV and VII and laminin-5 [34, 35]. In skin, the presence of a basement membrane prevents epidermal detachment from the dermis and also participates in stem cell regulation and anchoring [36, 37]. In the tissue-engineered skin substitute, collagen IV, VII as well as laminin-5 are all present at the dermo-epidermal junction [Figure 11.5(e–g)]. Hemidesmosomes, which are specialized junctions consisting of dense plaques associated to bundles of keratin filaments, are also present between the basal keratinocyte and the lamina densa in the mature tissue-engineered skin substitutes [Figure 11.6(b)]. The lamina lucida, principally composed of type IV collagen, is also apparent [Figure 11.6(b)].

The mechanical stability is an important challenge in the development of biological tissues by tissue engineering. The structure of the dermis confers considerable mechanical strength to skin. The dermis is composed mainly of collagen type I and type III fibers, which are synthesized by fibroblasts [38]. Accordingly, the reconstructed dermis of the
tissue-engineered skin substitute highly express type I [Figure 11.5(h)] and type III (not shown) collagens. During the culture, the integrity of the tissue-engineered dermis is preserved over time, thanks to the effect of ascorbic acid, which allows continuous collagen secretion and organization [39].

11.6 Discussion and Commentary

At the present time, animal models are commonly used in skin research and in the pre-clinical development of new drugs. The possibility to experimentally manipulate the mouse genome made the mouse a widely used model for advancing skin research. However, variations in histology and permeability exist between human and animal skin [11, 40]. For example, mouse dorsal skin presents two to three cell layers in the living portion of the epidermis, while human skin is rather constituted of about 5 to 15 living cell layers with less hair than mouse skin. In addition, hair follicle stem cells in mice and humans have a distinctive gene expression profile [41, 42]. Thus, the cellular and molecular characteristics of human epithelial cells could be different from those of rodents, supporting the use of human skin substitutes to take the forefront in research on human skin.

Skin substitutes provide an alternative to the animal models conventionally used [43]. As of September 11, 2004, a European Community Directive (ECD) strictly forbids the use of animals to test cosmetic products [4]. The successful application of tissue-engineered skin substitutes requires that the morphological and ultrastructural organization of the epidermis, dermis, and dermo-epidermal junction mimic the normal skin structure as closely as possible [4]. Skin substitutes produced with the self-assembly approach show an absence of exogenous material, a histologically normal and adequately differentiated tissue with the presence of a well-developed dermo-epidermal junction, and a complex collagen network [44]. This method can be used for both clinical and fundamental studies and it can be relatively adaptable to many in vitro applications: cutaneous physiology, skin disease, wound healing, stem cells, dermatopharmacology, toxicology, and angiogenesis studies.

It must be pointed out that despite the fact that the self-assembly approach has many advantages, it is a time-consuming method. It does not use any exogenous scaffold, but
instead enough time must be allowed for the cells to proliferate and to produce their own extracellular matrix. On the other hand, the absence of a scaffold can be advantageous for mechanical study of the ECM. It should be noted that beside all similarities (see Table 11.3), there are some differences in the expression and localization of various specific differentiation markers. These differences include the presence of “hyperproliferative” keratins (K6, K16, and K17) as in other models of skin substitutes produced in vitro and sometimes of the precoce appearance of involucrin, transglutaminase, and filaggrin in the lower suprabasal layers of the reconstructed epidermis. K6, K16, and K17 are considered markers of keratinocyte activation, and their expression is coordinately increased in wounded skin and in hyperproliferative skin disorders such as psoriasis [45–48]. During the in vitro culture period, most skin substitutes exhibit a hyperproliferative phenotype similar to wounded skin and Smiley et al. have confirmed that engineered skin substitutes have a gene expression profile similar to those of wounded human skin [49]. A model that lacks this hyperproliferative phenotype still needs to be developed as an in vitro mimic of normal human skin [46].

The various methods used for the reconstruction of living skin give different epidermal-cell phenotypes depending on the culture conditions [2]. However, immunohistochemistry is not a quantitative technique. In this regard, Western Blot and FACS analysis allow more subtle comparisons between the expression level of a given protein and can be run in parallel on cells harvested from the reconstructed tissue. Also, it should be noted that for some markers, the stratum corneum can exhibit an important background [see Figure 11.5(c)] corresponding to the nonspecific binding of the primary or secondary antibody. This is true for tissue-engineered skin substitutes as well as for native skin.

Progress in tissue engineering and cell culture has led to the development of various organized tissues offering the possibility of creating a model for research applications. As examples, human tissue-engineered skin substitutes have been produced and applied to percutaneous absorption studies and to the characterization of epithelial stem cells [10, 11, 21, 50]. Wound-healing models composed of human epithelial and mesenchymal cells also permit a better understanding of the mechanisms of reepithelialization of the skin and cornea [51, 52]. The similarities observed between these models and the in vivo wound healing process support their use for investigating basic mechanisms. Using living cells, the reconstruction of organs such as the bladder, blood vessels, cornea, and adipose tissue have been developed and represent alternative tools to the use of animal in research [12, 15, 52, 53].

11.7 Application Notes

- When cultured in flasks immersed in the medium, keratinocytes do not reach the late stage of terminal differentiation. To lead keratinocyte differentiation study, the utilization of skin substitute cultured at the air-liquid interface is indicated.
- The expression and localization of differentiation markers in various skin models provide good parameters for the evaluation of the quality of skin substitutes.
- Epidermal differentiation is characterized by a series of coordinated morphological and biochemical changes over time, which allow for a step-by-step evaluation of the differentiation process.
### Troubleshooting Table

<table>
<thead>
<tr>
<th>Problem</th>
<th>Explanation</th>
<th>Potential Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue-engineered skin substitute</td>
<td>Irregular distribution of keratinocytes</td>
<td>Be careful to seed keratinocytes slowly, drop by drop, on the entire surface defined by the seeding ring.</td>
</tr>
<tr>
<td>The surface of the reconstructed tissue appears macroscopically nonhomogenous</td>
<td>Poor attachment between stromal sheets</td>
<td>During the superimposition of the stromal sheets, be sure to eliminate bubbles.</td>
</tr>
<tr>
<td>Detachment or blistering between dermal sheets</td>
<td>The primary and the secondary antibodies must exhibit a good match</td>
<td>Use antibodies made from different species; the secondary antibody must be raised against the immunoglobulin type corresponding to that of the primary antibody.</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>Photobleaching</td>
<td>Protect immunolabeled slides from light.</td>
</tr>
<tr>
<td>No labeling</td>
<td>Microscope equipped with the wrong filter</td>
<td>Microscope must be equipped with the filter corresponding to the dye used.</td>
</tr>
<tr>
<td>No or unexpected labeling</td>
<td>The choice of the antibody is crucial since there are many nonspecific ones on the market</td>
<td>Always run in parallel an appropriate positive control.</td>
</tr>
<tr>
<td>High background level</td>
<td>Sources and characterization performed such as Western blot analyses and purification must be carefully checked before use</td>
<td>Prepare cell extract and use Western blotting to verify that the targeted protein is present.</td>
</tr>
<tr>
<td>Basal keratinocyte marker expressed in first suprabasal cell layers [for example, see VM-2 labeling in Figure 11.5(c)]</td>
<td>Primary antibody is too concentrated</td>
<td>Run a titration curve to determine the optimal dilution.</td>
</tr>
<tr>
<td>Epidermal cyst within the dermal compartment</td>
<td>Secondary antibody too concentrated</td>
<td>Run a titration curve to determine the optimal dilution.</td>
</tr>
<tr>
<td></td>
<td>Slope angle in the cross-section</td>
<td>Reorient the tissue block in order to cut in a vertical plane perpendicular to the skin surface.</td>
</tr>
<tr>
<td></td>
<td>Slope angle in the cross-section</td>
<td>Orient the tissue block in order to cut in a vertical plane perpendicular to the skin surface.</td>
</tr>
</tbody>
</table>

- According to the proliferation-differentiation ratio (+/- proliferation or +/- differentiation), the appearance of the tissues will change: number of layers and thickness of tissues or delay/advance in the expression of epidermal differentiation markers.
- The number of proliferating cells could be modulated by the culture conditions.
- In vitro tissue-engineered skin models with genetically modified cells can be used as a tool to understanding mechanisms regulating keratinocyte growth and differentiation (as an example, see [54]).

### 11.8 Summary Points

1. The in vitro system described here combines the three critical skin components: epithelia, basement membrane, and stroma.
2. The tissue-engineered skin substitute produced by the self-assembly approach represents a promising tool both for basic and clinical applications or pharmacological research.
3. Different skin markers are available and represent useful tools to monitor the quality of a skin substitute or its differentiation status when the appropriate culture conditions are used.

4. The tissue-engineered skin substitute produced by the self-assembly approach is an interesting alternative to the utilization of animal models for skin research.

5. The self-assembly approach of tissue engineering can be adapted for the in vitro reconstruction of various organs.

6. Future studies will tell whether modifications of the culture conditions allow for the reconstruction of a skin substitute that fully mimics human skin.

Acknowledgments

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References


