6 Principles of Soft Tissue Engineering for Craniomaxillofacial Reconstruction

Shiuhyang Kuo, Atsuko Miyazawa, and Stephen E. Feinberg

6.1 Introduction

A tissue-engineered device is needed when the supply of autogenous native tissue is limited or scarce to repair defects due to severe wounds, tumor removal, chronic or acute degenerative changes, or congenital symptoms. Tissue engineering creates new tissue by combining cell and developmental biology with material principles. Eventually, a tissue-engineered product has to find its way into a host. The autogenous cells are used to eliminate the immune responses of the host to the tissue-engineered product. The supporting materials used in manufacturing tissue-engineered devices should cause no immunogenicity reactions and easily be integrated into a host and biodegradable and possess good mechanical properties [1].

The manufacture of tissue-engineered devices must meet current Good Manufacturing Practice (cGMP) standards which are enforced by the US Food and Drug Administration under Title 21 of the Code of Federal Regulations [2, 3]. To meet these standards, our laboratory cultured keratinocytes and constructed autogenous tissue-engineered devices in a serum-free chemically defined medium without a feeder layer. Our laboratory routinely uses a tissue-engineered device termed ex vivo produced oral mucosa equivalent (EVPOME) [4–6], which is composed of oral keratinocyte cellular layers and a supporting scaffold made from human decellularized cadaver skin, as a study model or to repair the loss of oral soft tissue in our clinical trials [7]. We cultured oral keratinocyte stem/progenitor cells to manufacture EVPOMEs. In oral mucosa tissue, oral keratinocyte stem cells reside in the basal cellular layer, and progenitor cells can residue in the basal cellular layer and the inner part of the spinous layer [8]. The percentage of oral keratinocyte stem cells is less than 10% [9, 10]. The expressions of Ki67, a proliferation marker, and integrin α6 and CD71, two frequently used oral keratinocyte stem cell markers, on native gingiva are shown in situ in Fig. 6.1. The majority of oral basal keratinocytes lack the expression of Ki67 and CD71. However, Ki67 was detected on the cellular layer on top of basal cellular layer. The expression of integrin α6 was detected along the contact area between basal membrane and basal keratinocytes and is important for cell adhesion to the underlying basement membrane. The negative expression of Ki67 suggests that the cells within the basal layer are oral keratinocytes in a quiescent stage within the native tissue. However, these oral keratinocyte stem cells will turn into highly proliferative cells when placed into an in vitro culture which simulates a

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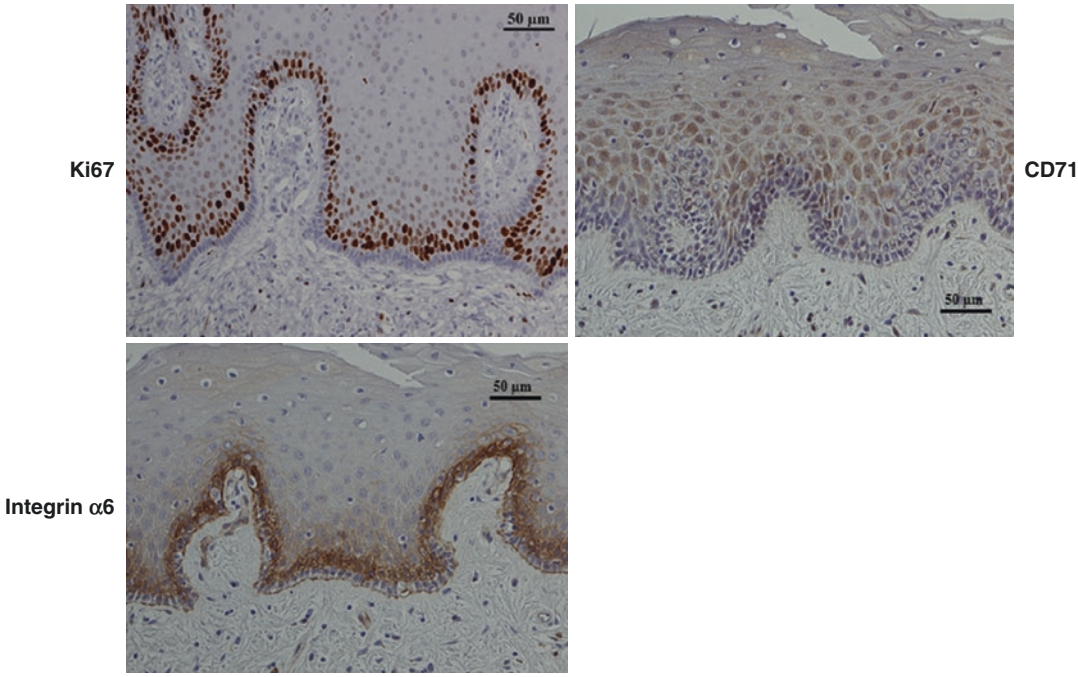

Fig. 6.1 Ki67, integrin α6, and CD71 expression on gingiva. Majority of basal keratinocytes do not express Ki67 and CD71. Integrin α6 was detected along the contact area between basal membrane and basal keratinocytes. Each scale bar represents 50 μm

wounding environment [9, 11]. It is this property that allows us to amplify and obtain a high number of oral keratinocytes possessing differentiation capacity used in manufacturing tissue-engineered devices, such as EVPOMEs.

Mammalian skin is composed of two distinct layers, epidermis and the underlying dermis. The epidermis' basal layer is composed of two keratinocyte populations, keratinocyte stem cells (KSCs) and transitory amplifying (TA) cells. Epidermal stem cells have been found to express markers which are typically expressed by stem cells in other epithelial tissues, i.e., CD34, K15, alpha6 integrin, and Lgr5 [12–14]. However, these phenotypic markers cannot be used to differentiate between the two epidermal cell populations, KSCs and TA cells, because of their lack of expression on their cell surfaces [15].

EVPOMEs manufactured by culturing a single cell type, such as oral or skin keratinocytes, can be used to repair oral mucosa or skin defects caused by diseases or trauma. There are areas within our bodies where the mucosa and skin meet and create a special structure of tissue termed mucocutaneous junctions, such as the lip, eyelid, anus, or vagina. To repair these mucocutaneous junctions, a tissue-engineered device was developed and manufactured by co-culturing oral and skin keratinocytes on either side of a cell-free zone created by a barrier [6, 16–18].

In this chapter, we present the concepts of clinical applications using tissue-engineered devices with detailed protocols that we used to culture primary human keratinocytes, skin and oral mucosa, to manufacture tissue-engineered devices such as EVPOMEs and oral and skin co-cultures that can be used to create mucocutaneous junctions. We report a unique culture system that allows cells to grow beyond contact inhibition, such that cells are produced in excess of available growth surface. The newly produced cells called "pop-up" cells float up into the culture medium and when passed into a new growth vessel reattach to the growth surface and continue to grow as adherent monolayer cells that upon confluence can begin to continuously pro-

duce additional floating "pop-up" cells [19–21]. In addition to the traditional method of using attached monolayer keratinocytes to culture keratinocytes and manufacture EVPOMEs, we also report the method of manufacturing EVPOMEs using floating "pop up" oral keratinocytes.

6.2 Proposed Clinical Applications for Reconstruction of the Lips

The lips form a dynamic complex facial structure that is difficult to reconstruct after an avulsion injury. The lips are a composite tissue consisting of the mucosa, skin, and innervated muscles. Significant loss of the lips is a functional and esthetic concern because the neuromuscular control of normal lip structures is required for eating, drinking, talking and social gesturing. Avulsion of the lips is a survivable injury; but without functional lip reconstruction, life for injured individuals is burdened by drooling, food spillage while eating, unintelligible speech, and social rejection. Functional reconstruction of the lips is compelling; when greater than 50% of the lips are avulsed, face transplantation under lifetime immunosuppression is the contemporary option.

Regeneration of oral mucosal tissue has not been previously addressed in craniomaxillofacial (CMF) soft tissue injuries. The lack of available TE/RM (tissue-engineered/regenerative medicine)-produced oral mucosa has limited surgeons to reconstruct the oral cavity and other functional units that contain an M/C junction, i.e., the lips, anal sphincter, vagina, and eyelids.

The in vitro development of a human oral mucosa is fundamental. Oral mucosa forms the inner aspect of the lip. The oral mucosa extends onto the vermillion border to unite with the skin of the face forming an M/C junction. Though skin equivalents have been developed for treatment of burns and chronic wounds, development of an *e*x *v*ivo-*p*roduced *o*ral *m*ucosal *e*quivalent (EVPOME) has lagged behind that for the skin. EVPOME for lip reconstruction will be a transforming esthetic-functional option.

The TE/RM approach faces several barriers preventing its transition into the clinical arena. These barriers are (1) inability to create composite tissue structures especially in the area of soft tissue reconstruction and (2) the difficulty in developing an in vivo perfusion system (blood vessels) to supply nutrition for large segments of tissue created in vitro. Though TE/RM in vitro culture systems have been designed to mimic the natural environment, artificial in vitro systems only allow viability of uncomplicated pieces of tissue based on oxygen perfusion of 100–200 microns. Vascularization and nutrition remain a problem limiting the size and complexity of tissue-engineered constructs that can be successfully transplanted after they are developed in vitro [22].

Most surgeons have addressed the limitations stated above by creating prefabricated flaps with their own blood supply. In this method, a vascular carrier is implanted to a new skin territory. Following a period of maturation and neovascularization, the prefabricated flap can be transferred, based on the implanted pedicle. Prefabricated flaps have been utilized in surgery for reconstruction of individual esthetic units such as the nose, ear, cheek, lip, and neck [23–26]. With flap prefabrication, a distally ligated vascular pedicle is implanted underneath the desired donor tissue, and after a period of 8 weeks of neovascularization, this donor tissue can then be transferred based on the preimplanted vascular pedicle as its axial blood supply [27]. We propose to use a parallel but distinct concept called flap prelamination. Lamination refers to the process of bonding or integration of several different tissue layers. Prelamination designates a reconstructive process where a three-dimensional structure is built at a remote site by laminating several different tissue layers together as composite grafts into a reliable existing axial vascular bed. The structure matures 2–3 weeks before transferring the unit en bloc to the defect based on its native axial blood supply. The technique of prelamination allows reconstruction to begin at a remote site. This is important as the recipient site being reconstructed may lack either the blood supply or healthy tissue substratum necessary to support construction of a sophisticated

three-dimensional construct at the defect site. Furthermore, remote reconstruction in an unscarred vascular bed offers the best chance for the composite grafts to mature [24, 25]. The technique of prelamination is often used in reconstructing structures with multiple functional layers, i.e., full-thickness reconstruction of the nose, lip, cheek, ear, maxilla, mandible, and trachea [28, 29].

Prefabricated innervated prevascularized prelaminated (PIPP) flaps increase the options available to reconstructive surgeons. Present lip reconstructions are limited to harvesting existing anatomical units. For example, the radial forearm free flap with the palmaris longus tendon is a standard option for reconstructing total lower lip defects and offers an excellent pedicle, appropriate tissue thickness, and a method to suspend the lip with the incorporated tendon rather than muscle [30]. However, existing data indicates that most injured combatants sustain multiple injuries to the extremities along with the head and neck regions [31]. The difficulty then becomes obvious if we entertain a total lip reconstruction in a battle-injured warrior by using upper extremity parts. Flap prefabrication allows the reconstructive surgeon to choose an uninjured wound bed with adequate tissue characteristics, a set of vessels, a motor nerve as well as a place to fabricate the construct.

6.2.1 Advantages of Our TE/RM Approach

The tissue-engineered human mucosa and skin keratinocytes on the same piece of dermal matrix (M/C constructs) are cultured in vitro, transferred to an existing muscle, such as the latissimus dorsi muscle (LDM), which can be harvested as an IPP flap. In a defined period of time, the M/C constructs mature, integrate, and develop a microcapillary system with the underlying muscle flap. The PIPP flap is then transferred to the area of the lips. Vascular and nerve pedicles are microvascularly anastomosed to existing pedicles in the face (Fig. 6.2). The advantages of a regenerative medicine approach to reconstruct craniofacial soft tissue injuries are:

1. There is no or minimal donor site morbidity. We use the protected torso and avoid the use of injured extremities as a tissue source thus shortening the rehabilitation phase. This results in a decrease in patient morbidity.
2. Restoration of function is accelerated; esthetic and functional outcome are better than contemporary options.
   (a) A decrease in the number of surgeries needed with a concomitant decrease in operating room time

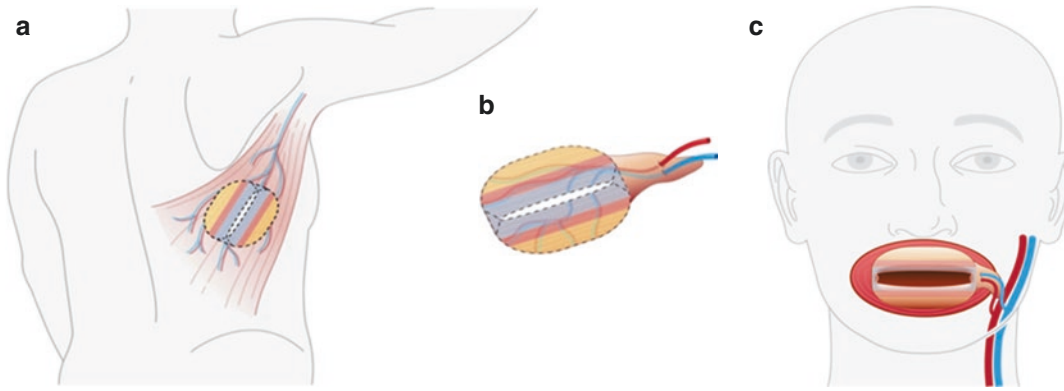

Fig. 6.2 (**a**) M/C construct integrated onto the LDM with a surgically created stoma. (**b**) The LDM with integrated M/C surgically removed to create a prevascularized prelaminated flap with thoracodorsal artery and vein. We will also harvest the thoracodorsal nerve. (**c**) The LDM prevascularized prelaminated flap inserted into the face to form lips with microanastomosis of the thoracodorsal artery and vein to the external carotid artery and external jugular vein, respectively. The thoracodorsal nerve will be anastomosed to the facial nerve for motor function (from 18 with permission)

(b) An enhancement in the quality, shape, and function of soft tissue regenerated

3. The source of the cells to develop the soft tissue constructs (lips) will come from the patient. One centimeter punch biopsies will be excised from the cheek for oral mucosa and the posterior auricular region for skin (optimal color match to face), thus making the construct autochthonous (self to self).

6.2.2 Proposed Study Design

We propose a multicenter, prospective, interventional non-randomized open label single arm safety and efficacy in subjects with equal to or greater than 50% loss of the lips who will be treated with a prefabricated innervated prevascularized prelaminated (PIPP) designer microvascular free flap for functional lip reconstruction. We could not ethically have a control population for randomization that was treated with contemporary surgical techniques to functionally reconstruct greater than 50% of the lips as these techniques have proven to be less than adequate. A functional lip will be reconstructed by the following steps:

1. Two 1.0 cm circular punch biopsies, the posterior auricular area for autogenous skin keratinocytes and the buccal (cheek) mucosa for autogenous oral keratinocytes, will be taken.
2. A mucocutaneous (M/C) construct will be manufactured in vitro in a cGMP facility with the autogenous skin and oral keratinocytes using an appropriate template for M/C fabrication that would address the size and geometry of the defect to be reconstructed (Fig. 6.3).
3. An ex vivo fabricated M/C construct will be grafted onto the latissimus dorsi muscle (LDM) to create a prefabricated innervated prevascularized prelaminated (PIPP) flap. The

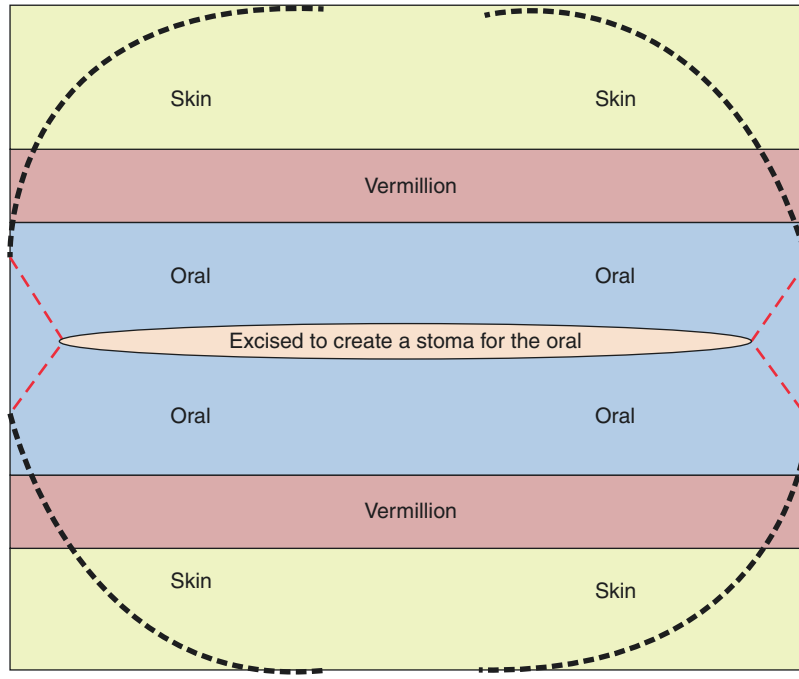

Fig. 6.3 Template for making either an upper or lower lip (50% of above template) or a complete "set" of lips. Oral = oral keratinocytes. Skin = epidermal (skin) keratinocytes. Black filled circle = excised skin tissue from the periphery of the template. Red filled circle = cuts in oral mucosa to form a commissure of the lip which will be "turned in" (from 18 with permission)

implantation of the M/C construct will be placed parallel to the muscle fibers of the LDM as well as parallel to the motor nerve and blood supply based on the thoracodorsal vessels. The parallel position of the neurovascular bundle and M/C construct will facilitate the surgical inset of the flap and the functional dilation and contraction of the muscle fibers. The location of the M/C construct will be just below the angle of the scapula in order to allow for an appropriate length of the neurovascular pedicle.

4. A stoma within the M/C construct will be created at the time of grafting onto the LDM such that the long axis of the opening is parallel to the muscle fibers to simulate the orbicularis oris muscle. The stoma or opening will be maintained with an inert obturator fabricated from a 3D printer.
5. The designer prelaminated flap at 2–3 weeks postimplantation will be harvested.
6. A microvascular free transfer to the recipient site containing the thoracodorsal artery, vein, and motor nerve to the external carotid or facial artery, external jugular or facial vein, and a branch of the facial nerve will be performed, respectively.

We can successfully restore muscle volume and function and support neovascularization and reinnervation of regenerated muscle tissue as well as structural and functional integration between the regenerated and host tissues from the transposed prefabricated innervated prevascularized prelaminated (PIPP) flap.

6.2.3 Challenges and Potential Problems

One potential problem is contraction of the devices during the manufacturing phase due to the large size of tissue-engineered mucocutaneous construct. To circumvent this untoward scenario, devices 15–20% larger is suggested to counteract the contraction based on our experience. Another challenge is that each subject will present "diverse" and "unique" types of lip defects. To address the lip diversity issue, we will group them into specific and suitable categories based on defect size, location, depth, and involvement of lip. The specific categories are:

1. Total lip reconstruction involving both commissures (Fig. 6.4a)
2. Upper lip: 50% or more of lip to be reconstructed (Fig. 6.4b)
3. Lower lip: 50% or more of lip to be reconstructed (Fig. 6.4b)
4. Involvement of 50% or more of the lower and upper lip involving the commissure (Fig. 6.4c)

Regardless of the above classification, a full lip M/C construct should be constructed, with a functional stoma, that will be fabricated based on the template in Fig. 6.3 within the prelaminated innervated prevascularized prefabricated (IPP) flap as noted in Fig. 6.2. On harvesting of the prefabricated innervated prevascularized prelaminated (PIPP) flap, it will be modified such that it is consistent with the magnitude of the defect to be reconstructed. Thus, for full lower or upper lip

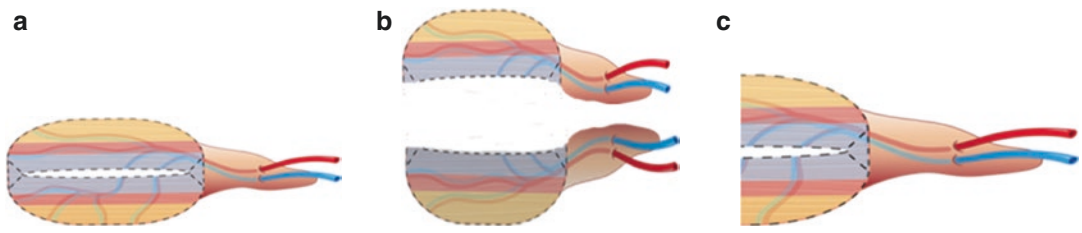

Fig. 6.4 Types of mucocutaneous constructs to be used for lip reconstruction. (**a**) M/C construct that will be used for all defects and for total upper and lower lip reconstruction, (**b**) M/C that could be used for total upper or lower lip reconstruction, and (**c**) M/C construct to be used for partial upper and lower lip reconstruction

reconstruction, only one half of the template on the LDM will be used for restoration of the lip (Fig. 6.4b), or if the area to be reconstructed involves only half of the upper and lower lip, only half of the template on the LDM, to include one commissure, will be harvested for lip reconstruction (Fig. 6.4c).

In the following sections, we will detail the protocol of cell culture and manufacturing EVPOMEs and mucocutaneous (M/C) constructs.

6.3 Materials and Reagents

6.3.1 Materials

1. Surgical scalpels: blade no. 15 (Fisher Scientific, catalogue no. 08-927-5C), blade no. 21 (Fisher Scientific, catalogue no. 08-927-5D). Scalpels no. 15 are used on small size of oral mucosa with the epidermal surface dimension of 5 × 5 mm or smaller.
2. Forceps: dissecting tissue forceps (Fisher Scientific, catalogue no. 13-812-41), tissue forceps with teeth (Roboz Surgical Instrument Co., catalogue no. RS8162), used for oral tissue manipulation; #5 Dumont Forceps (Roboz Surgical Instrument Co., catalogue no. RS-4955), used for dermal equivalent scaffolds manipulation.
3. Operating scissors: used for cutting dermal equivalent scaffolds, lengths, and types of scissors depending on individual preference.
4. Sterilized round petri dish: 100 × 15 mm petri dish (Fisher Scientific, catalogue no. 08-757-100D) used for oral tissue washing and dermal equivalent scaffolds rehydration; 35 × 10 mm (Fisher Scientific, catalogue no. 08-772A) used for trypsinization treatment on oral tissue.
5. Sterilized round cell culture dish: 100 × 20 mm cell culture dish (Fisher Scientific, catalogue no. 08-772-22). 150 × 25 mm cell culture dish (Fisher Scientific, catalogue no. 08-772-24).
6. Cell strainers: 100 μm pores (Fisher Scientific, catalogue no. 08-771-19).
7. Cell culture flasks: T25 (Fisher Scientific, catalogue no. 10-126-28) used for initial cell seeding when total cell number dissociated from oral tissue is equal or lower than 5M, T75 (Fisher Scientific, catalogue no. 07-202-000) used for initial cell seeding when total cell number dissociated from oral tissue is higher than 5M and for cell subculture.
8. 48-Well flat bottom cell culture plates (Fisher Scientific, catalogue no. 07-200-86): used for single cell type tissue engineering device culture.
9. Transwell multiple 6-well plate with 3 μm pore size inserts (Fisher Scientific, catalogue no. 07-200-166) and Transwell insert and dish with 75 mm diameter insert of 3 μm pore size inserts (Fisher Scientific, catalogue no. 07-200-173): used for creating air-liquid phase cell culture.
10. Sterilized rectangular 128 × 86 mm 8-well dishes (Fisher Scientific, catalogue no. 12-565-497) used for two cell types co-culture.
11. Transwell inserts and dishes: 75 mm in diameter with 3 μm pore size inserts used for two cell type tissue engineering device co-culture (Fisher Scientific, catalogue no. 07-200-173).
12. Dermal equivalent scaffolds: our laboratory uses two types of dermal equivalent scaffolds, 14-20/1000 in. thickness AlloDerm® (LifeCell Corporation, Branchburg, NJ, USA, catalogue no. 101020), and thin (0.4–0.7 mm thickness) and dehydrated Allopatch (Musculoskeletal Transplant Foundation, Edison, NJ, USA, catalogue no. 370408). Our laboratory does not use fibroblast feeder layer to culture oral keratinocytes because FDA regulations require the serum-free cell culture medium and to avoid cross contamination from feeder cellular layer.
13. 200 μm nylon mesh filter (BioDesign Inc of New York, NY, catalogue no. N200S).
14. 250 ml filter system (Fisher Scientific, catalogue no. 09-761-100).

6.3.2 Reagents

1. 1X DPBS (Dulbecco's phosphate-buffered saline) without calcium chloride and magnesium chloride (ThermoFisher Scientific, catalogue no. 14190144). 10X PBS, pH 7.4 (ThermoFisher Scientific, catalogue no. 70011069).
2. Trypsin solution for cell dissociation from oral tissue: Final concentration of 0.04% trypsin (Sigma-Aldrich, catalogue no. T-0303-10G) with 6 mM glucose (Sigma-Aldrich, catalogue no. G7021-1KG) in 1X DPBS, filter to sterilize by vacuum filter system (Fisher Scientific, catalogue no. 09-761-1), aliquot into 5 or 10 ml/tube, and stored at −20 °C.
3. Trypsin solution for cell dissociation from skin tissue: Final concentration of 0.125% trypsin in 1X DPBS and sterilize with 250 ml filter system.
4. Cell culture medium: EpiLife™ and calcium-free (ThermoFisher Scientific, catalogue no. MEPICF-500) that comes with 0.5 ml of 0.06M calcium chloride (ThermoFisher Scientific, catalogue no. 509703), stored at 4 °C.
5. Cell culture medium for skin keratinocytes: EpiLife™ with 10% FBS, and EpiLife™ with 2% FBS.
6. EpiLife™ defined growth supplement (EDGS): ThermoFisher Scientific, catalogue no. S-012-5, stored at −20 °C. EDGS will be thawed right before the addition of 5 ml of EDGS into 500 ml EpiLife™ base medium to prepare full cell culture medium.
7. Trypsin/EDTA solution for cell subculture: ThermoFisher Scientific, catalogue no. R001100. Trypsin/EDTA solutions are aliquot into 10 ml/tube and stored at −20 °C for long period of time. The solution is thawed right before the application.
8. Defined Trypsin Inhibitor (DTI, ThermoFisher Scientific, catalogue no. R007100). DTI can be stored at −20 °C for long period of time. DTI solution can be thawed and stored at 4 °C for short period of time.
9. Gentamicin/Fungizone solution for washing and sterilizing oral and skin tissue: Gentamicin (50 mg/ml, ThermoFisher Scientific, catalogue no. 15750060) and amphotericin B (250 μg/mL, ThermoFisher Scientific, catalogue no. 15290018) – Adding 12.5 ml of gentamicin into 20 ml of amphotericin B Fungizone, mix well, aliquot into 3.25 ml/vial, and store at −20 °C. Thaw and add 3.25 ml of gentamicin/Fungizone solution into 500 ml 1X DPBS to make final concentrations of 125 μg/ml gentamicin and 1 μg/ml amphotericin B.
10. Gentamicin/Fungizone solution for cell culture: Gentamicin (50 mg/ml, ThermoFisher Scientific, catalogue no. 15750060) and amphotericin B (250 μg/ml, ThermoFisher Scientific, catalogue no. 15290018) – Adding 6.7 ml of gentamicin into 20 ml of amphotericin B Fungizone, mix well, aliquot into 1 ml/vial, and store at −20 °C. Thaw and add 1 ml of gentamicin/Fungizone solution into 500 ml EpiLife™ Full Medium to make final concentrations of 25 μg/ml gentamicin and 0.375 μg/ml amphotericin B.
11. 1M calcium chloride solution (Sigma-Aldrich, catalogue no. 21115): Sterilization by filter with 0.22 μm pore size and aliquot into 1 ml/tube.
12. Human type IV collagen (Sigma-Aldrich, catalogue no. C8374): Use filter-sterilized 0.5M acetic acid to prepare 1 mg/ml human type IV collage, aliquot into 500 μl/tube, and store at −20 °C.
13. SYLGARD® 184 Silicone Elastomer Base and Curing Agent (Fisher Scientific, catalogue no. NC9644388).
14. Formaldehyde solution, 37% by weight (Fisher Scientific, catalogue no. BP531-500): Add 100 ml 10X PBS and 100 ml 37% formaldehyde into 500 ml ddH_2O, then add ddH_2O to 1 l to make 3.7% formalin solution. 3.7% formalin solution can be stored in a bottle wrapped with aluminum foil at 4 °C.
15. Antibodies: Anti-cytokeratin 10/13 antibody (Abcam, catalogue no. ab9383), anti-CD44 antibody (Cell Signaling Technology, catalogue no. 3570), anti-K2 antibody (PROGEN

Biotechnik, Heidelberg, Deutschland, catalogue no. 65191), anti-small proline-rich protein 3 antibody (Sigma-Aldrich, catalogue no. HPA044467).

6.4 Methods

6.4.1 Keratinocytes Culture

6.4.1.1 Oral Keratinocytes Culture

All materials and reagents should be sterile.

1. All procurement of human tissues needs to be approved by experimenters' institutes.
2. Freshly dissected oral mucosa tissues can be transported in washing solution containing 125 μg/ml gentamicin/1 μg/ml amphotericin B (Fungizone) and stored at 4 °C for short period of time before processing tissues. Oral tissues can also be stored in full medium before tissue process procedures begun to maintain the viability of cells inside tissue. However, it can also increase the chance of contamination.
3. All procedures should be performed inside the hood of a biological safety cabinet from this point on.
4. Using forceps to transfer oral tissues into a 100 × 15 mm petri dish containing 15 ml washing solution. Blood on tissues and unwanted parts of tissues can be removed by a scalpel during this step.
5. Transfer oral tissues into a new 100 × 15 mm petri dish containing 15 ml washing solution. The tissues are held by forceps, scraped gently by a scalpel, submerged in washing solution, and incubated for 20–30 min. The purpose of this step is to sterilize the tissues.
6. Transfer tissues into a new petri dish and repeated step 5.
7. Repeat step 6.
8. Transfer tissues into a 35 × 10 mm dish containing 5 ml of 0.04% Trypsin with 6 mM glucose, and incubate for 16–17 h at room temperature inside the hood of the biological safety cabinet. Depending on the size of tissue, the size of dish and the volume of trypsin/glucose solution should be adjusted.
9. Transfer tissues into a 100 × 15 mm petri dish containing 5 ml (equal volume) of DTI solution.
10. A loose epidermal part of tissue should be observed at this point. Using forceps to hold down the tissue and a scalpel to scrape epidermal part of tissue, where the keratinocyte basal cells reside, away from the rest of tissue, which is discarded.
11. Pipette the DTI solution containing cells dissociated from oral tissue, and filter them through a 100 μm cell strainer placed on top of a 50 ml tube. Apply 2–3 ml of fresh DTI solution or full cell culture medium in the dish to wash the remaining cells and filter them through the same cell strainer.
12. Count viable oral keratinocytes' number.
13. Seed oral keratinocytes onto up to 5M cells/T25 cell culture flask. If cell number is higher than 5M, cells can be divided and seeded into multiple T25s or up to 15M cells/T75 cell culture flask.
14. Incubated cells inside a 5% CO_2 incubator at 37 °C for 48 h without disturbing cells.
15. Change medium every 2 days and subculture oral keratinocytes when cells reach appropriate confluence.

6.4.1.2 Skin Keratinocyte Culture

1. For preparation of isolation of primary adult human skin keratinocytes, in laminar flow hood, clean blood and fat off of tissues with washing solution. If the specimen is not going to be used immediately, it can be put on ice or alternatively left at 4 °C and submersed in DPBS.
2. Hold the sample with the forceps with teeth, and scape the sample with scalpel to remove excess fat and blood residue with washing solution in the petri dish.
3. Remove spent washing solution and rinse sample with fresh washing solution then transfer to new petri dish.
4. Repeat three times this process for each 30 min. If rinsing solution comes out too red,

repeat washing in order to eliminate blood cells.
5. After the last washing, slice full-thickness epithelium into 5-mm-wide strips fringe with scalpel. The thinner the strips, the better the trypsinization will be.
6. Add 0.125% trypsin solution in petri dish to be exposed in maximum area to trypsin.
7. Leave in laminar hood for 16 h for trypsinization. At this time, epidermal side is up and dermis side is down.
8. After 16 h, aspirate off most of the trypsin solution and add the same volume of EpiLife™ with 10% FBS. The serum can help to stop the action of trypsin.
9. Gently scrape off the epidermal layer down to the basal layer by a #21 scalpel with holding one end of the tissue with sterile forceps. Discard dermis afterward.
10. Filter the cell suspension through 200 μm nylon mesh (see Note 1) into 50 ml tubes using a sterile serological 10 ml pipette.
11. Rinse petri dish with fresh EpiLife™ with 10% FBS and filter into cell suspension 50 ml centrifuge tubes.
12. Count cells and centrifuge cells for 5 min.
13. Aspirate supernatant and resuspend cell pellet in warmed EpiLife™ with 2% FBS.
14. Plate 2–3 × 10^6 cells/ml.
15. Incubate at 37 °C, 5% CO_2 for 24 h for cells to attach.
16. Change medium to regular EpiLife™ medium without serum.
17. Feed regular EpiLife™ every other day and subculture cells when 70–80% confluence.

6.4.2 3D Organotypic Culture Using EVPOMEs (Ex Vivo Produced Oral Mucosa Equivalents) to Manufacture Soft Tissue Engineering Devices

There is a need to restore damaged craniomaxillofacial soft tissue either intraorally and/or extraorally. The tissue-engineered device manufactured by autogenous cells became a solution to meet the requirements with minimal morbidity to the patient. The tissue-engineered device has to integrate into host tissue easily and in as short a period of time as possible without causing any immunogenic reaction within the recipient. The tissue-engineered device has to facilitate neovascularization such that the major vascular supply to the construct is based on the major vessels of the implant site, i.e., muscle. EVPOMEs were proven to have such properties [5, 7, 32]. The manufacture of EVPOMEs is basically composed of two phases, liquid phase and an air-liquid phase. The liquid phase establishes the seeding and proliferation of basal cellular layer on top of scaffold. The air-liquid phase promotes the stratification of keratinocytes and differentiation, i.e., keratinization.

6.4.2.1 EVPOME Using Single Cell Type, Oral Keratinocytes, Culture

1. Decellularized cadaver skin used as a scaffold is cut into 1 cm in diameter by scissors or by an 8 mm in diameter punch. The size of cut scaffolds is determined according to the experimental designs. In our laboratory, we use AlloDerm® or Allopatch as scaffolds. The ideal thickness of scaffolds should be around 0.5 mm. A thick scaffold will impede cell growth and development on scaffolds; however, it will be difficult to physically manipulate a too thin scaffold.
2. Rehydrate scaffolds in 100 mm dish containing 15 ml 1X DPBS for 30 min. Use forceps to gently tap the scaffolds to make sure air is pressed out of the scaffolds.
3. Aspirate old DPBS and replace with fresh DPBS in the same dish. Tap the scaffolds, and let the scaffolds submerge in DPBS for 30 min.
4. Repeat step 3.
5. Transfer scaffolds into wells of 48-well plate with epidermal side up. Epidermal side is the cell seeding side. Epidermal side is the side DPBS won't stay on the surface which appear rougher than the dermal side. Dermal side is the side where DPBS will stay on the surface and look shinier than the epidermal side. There is a notch on one corner of AlloPatch scaffolds to assist the identification of epidermal side.

6. Add 100 µl 1X DPBS and 5 µl of 1 mg/ml human type IV collagen onto each scaffold. Rock 48-well plate back and forth a few times, and incubate the plate at 4 °C overnight. The volumes of 1X DPBS and 1 mg/ml collagen should be proportionally adjusted according to the culture surface area of the well.
7. On the next day, place the 48-well plate inside the hood of the biological safety cabinet to allow the temperature warm up to the room temperature.
8. Add 1 ml full medium into each well with scaffold to test pH value. The color of the medium should be pink. If color of the medium is yellowish orange or yellow, then aspirate out the solution, and replenish with fresh 1 ml full medium.
9. Add trypsin/EDTA solution to just cover cell surface to dissociate cells from culture flasks, and incubate at 37 °C inside 5% CO_2 incubator for approximately 5 min.
10. Add equal volume of DTI solution into culture flask to stop trypsin activity. Rock culture flask gently a few times to allow DTI and trypsin/EDTA to mix homogenously.
11. Collect cell suspension into a 50 ml tube.
12. Count viable cells.
13. Centrifuge cells at 100 g for 5 min.
14. From this step on, full medium with 1.2 mM calcium (0.6 ml 1M calcium chloride in 500 ml full medium) should be used to culture cells.
15. Aspirate trypsin/EDTA/DTI solution completely, and resuspend cell pellet in full medium to make cell density 100 k cells/100 µl.
16. Seed 200 k cells onto each scaffold, add additional full medium drop by drop by up to the top of each well, and incubate cells at 37 °C inside a 5% CO_2 incubator.
17. Change medium every day for 4 days.
18. Transfer each scaffold onto an insert in 6-well plate to create an air-liquid phase to promote cellular stratification. Add 1.5 ml full medium in each well where the insert sits or the appropriate volume of full medium to allow medium to meet the bottom of the insert if different setup is used.
19. Change medium every 2 days for 7 days.
20. Evaluate EVPOMEs by histology and immunohistochemistry at the end of 11 culture days (Fig. 6.5) (See note 2).

6.4.2.2 EVPOME Using Floating Keratinocytes Culture

Huang et al. [33] showed that trypsin can damage cellular membranes and cellular proteome structures and upregulate p53 and p21 Waf1/Cip1 involving cellular senescence process. It becomes obvious that an important advantage of using floating cells is to eliminate the insult of trypsinization, which is a required step to dissociate attached monolayer cells.

1. The processes of scaffolds are the same as in steps 1–7 under Sect. 6.4.2.1.
2. Use 1 ml full medium to test the pH value of collagen-coated scaffold, and then aspirate the solution right before cell seeding.
3. Collect floating cells in spend medium and count viable cells. Centrifuge cells to concentrate viable floating cells if cell density is lower than 150 k cells/ml (See note 3).
4. Add full medium containing 0.06 mM into the spent medium to make the floating cell density equal to 150 k cells/ml.
5. Seed floating drop by drop onto the scaffolds.
6. Add full medium containing 0.06 mM calcium drop by drop into the well until medium reaches the top of the well.
7. Incubate inside a CO_2 incubator at 37 °C for 24 h.
8. Change medium with fresh full medium containing 1.2 mM calcium. Follow steps 17–20 under Sect. 6.4.2.1.
9. Evaluate EVPOMEs by histology and immunohistochemistry at the end of 12 culture days (Fig. 6.6).

6.4.2.3 EVPOME Using Two Cell Types Co-cultures

The mucocutaneous junction, an area where mucosa and skin tissue meet, is a complex structure. The restoration of such structure by a tissue-engineered device requires a co-culture of oral and skin keratinocytes separated by cell-free

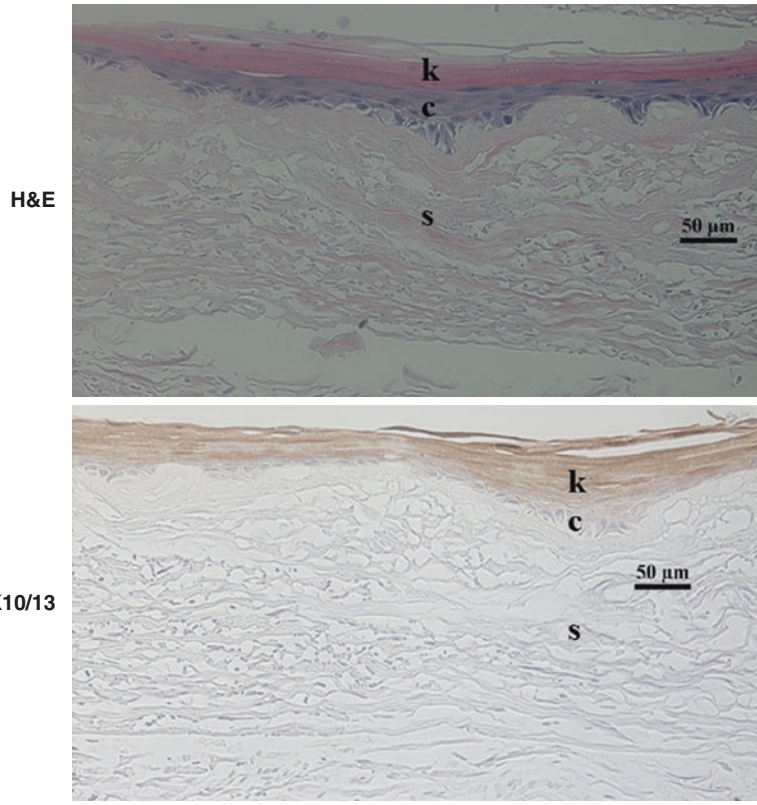

Fig. 6.5 Histology of oral keratinocyte EVPOME. H&E histology was shown on the top and immunohistochemistry of CK10/13 with dilution of 1:300 on the bottom. K represents keratin layer, c cellular layers, and S scaffold. Each scar bar represents 50 µm

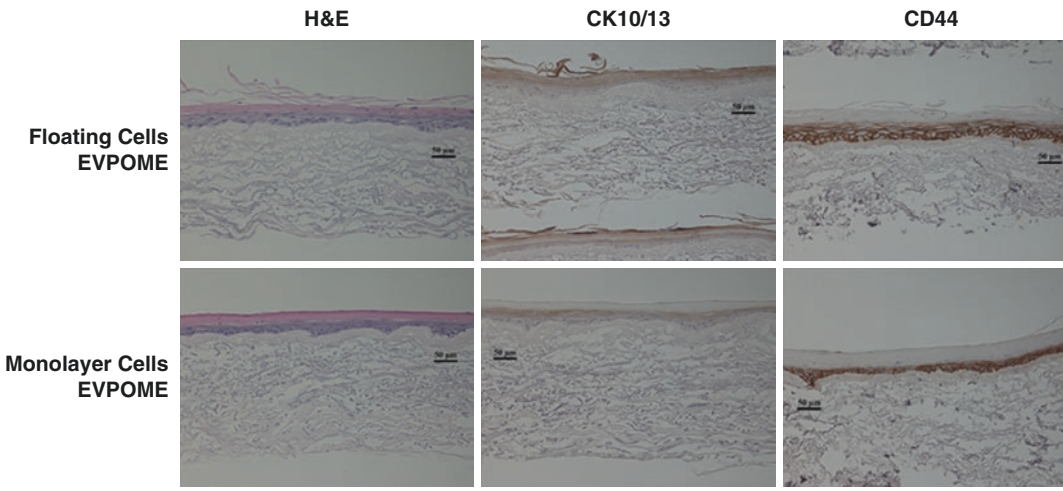

Fig. 6.6 Histology of floating and monolayer oral keratinocyte EVPOMEs. Floating and monolayer oral keratinocytes were collected from the same cell culture flask. H&E, CK10/13 with dilution of 1:300, and CD44 with dilution of 1:50. Each scar bar represents 50 µm

zone that allows both types of cells to gradually merge and migrate toward one another to create the mucocutaneous junction. In the case of lip restoration, the tissue-engineered mucocutaneous junction construct will be implanted into the latissimus dorsi muscle, with the stoma or opening in the device paralleling the muscle fibers. Once the device has been integrated into the mus-

cle bed, it will be harvested along with the thoracodorsal neurovascular bundle to create a prefabricated prelaminated microvascular free flap mimicking lip muscular structure [18]. The following are protocols for manufacturing an in vitro tissue-engineered mucocutaneous junction construct.

Making of Barrier, Corral, and Waffle

1. Corral, waffle, and 2.8 mm wide barrier frames were made out of ABS*plus* plastic by a Dimension Elite 3D Printer (Stratasys, Eden Prairie, MN, USA). The designs of corral and barrier based on dimensions of the well of 128 × 86 mm 8-well dishes and waffle based on 150 × 25 mm dish were shown in Fig. 6.7. The designs of barrier, corral, and waffle should be based on the culture dishes used in experiments.
2. Place each barrier inside a well of rectangular 128 × 86 mm 8-well dishes, and make sure the barrier sits tightly inside the well.
3. Mix SYLGARD® 184 with a ratio of base to curing agent ranging from 10 to 11 by weight.

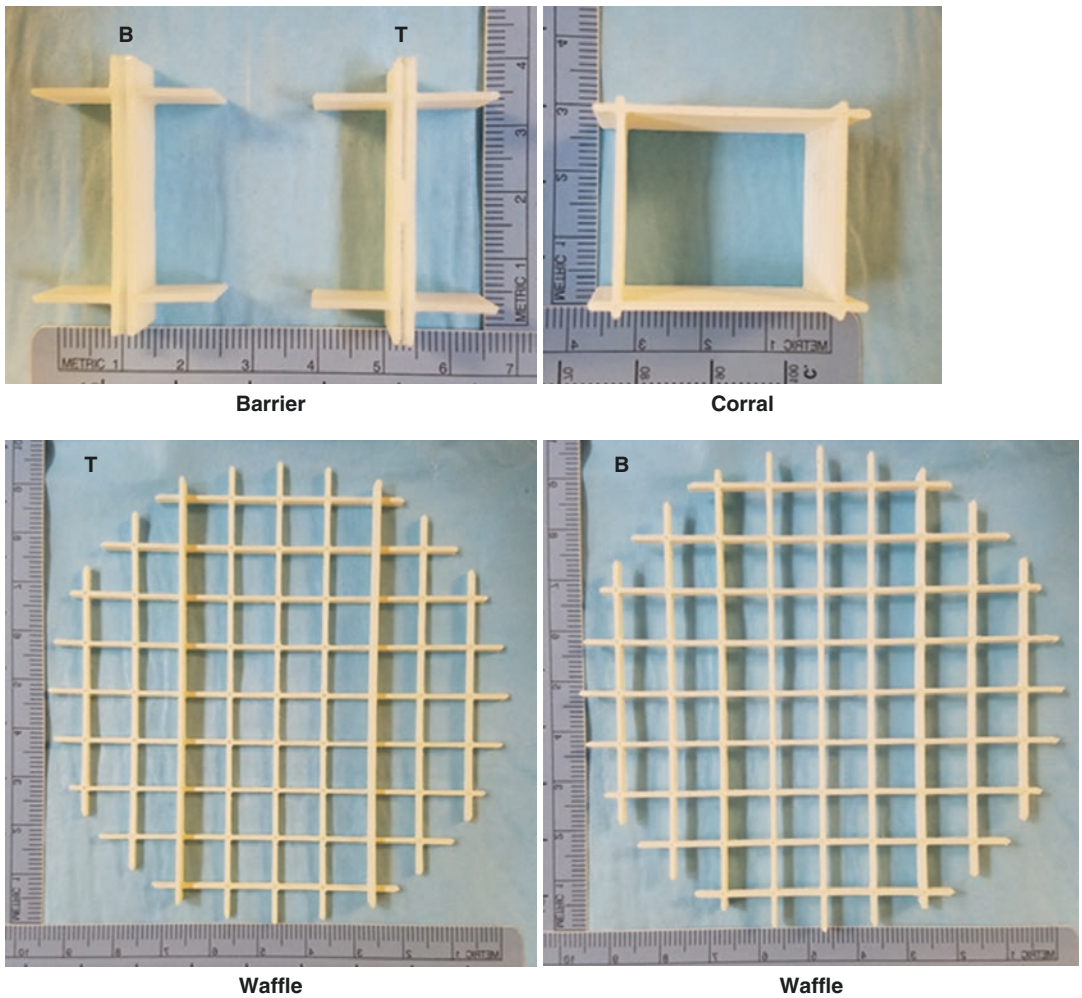

Fig. 6.7 Barrier, corral, and waffle used for oral and skin keratinocytes co-cultured EVPOMEs. Barrier is used to create a cell-free zone for oral and skin keratinocytes co-culture, corral is used during liquid phase culture, and waffle is used during air-liquid phase culture. T is the top side facing up. B is the bottom side facing up. Barrier bottom side is the side contacting the scaffold. Waffle top side is the side where the insert sits

4. Fill the central space of the frame with thoroughly mixed SYLGARD® 184 [PDMS, poly(dimethylsiloxane)] and cured for 24 h at room temperature followed by an additional 4 h at 65 °C to complete curing.
5. Remove PDMS-filled barrier from the well and trim extra PDMS on the surface of barrier.
6. Place the barrier in a new well to examine whether there is a leak by adding 1 ml of dyed solution in one side of barrier and wait for a few minutes. The dyed solution will flow into the other side of barrier if the barrier cannot make a tight seal.
7. Sterilize corrals and barriers with ethanol and/or UV.

Manufacturing EVPOME Using Oral and Skin Keratinocytes Co-cultures

1. Cut scaffolds into 3 × 3 cm squares. The size of scaffolds depends on the choice of culture dishes and experimental designs. Cut two notches on two corners.
2. Rehydrate the scaffolds in 15 ml 1X DPBS inside a 100 mm dish. Tap and press air out of the scaffolds and soak in DPBS for 20–30 min.
3. Aspirate DPBS and replenish with fresh DPBS and repeat step 2 two more times.
4. Let the scaffolds soak in fresh DPBS overnight at room temperature inside the hood of the biological safety cabinet.
5. On the next day, prepare 2 ml 0.05 mg/ml human type IV collagen by adding 100 µl 1 mg/ml collagen into 1900 µl 1X DPBS.
6. Transfer DPBS-soaked scaffolds from dish into the well of rectangular 128 × 86 mm 8-well dish with epidermal side up and two notched corners on the same side, either right or left.
7. Add 2 ml 0.05 mg/ml human type IV collagen into the well and incubate at 4 °C overnight.
8. Aspirate collagen solution completely from the well.
9. Insert the sterilized barrier into well with forceps, and place it firmly on top of scaffold along the central line of scaffold to make a tight seal and create a cell-free zone between the two cellular areas (Fig. 6.8).
10. Add 0.5 ml full medium containing 0.06 mM calcium into one side of barrier to make sure there is no leaking. If no leaking is observed, then add 0.5 ml full medium containing

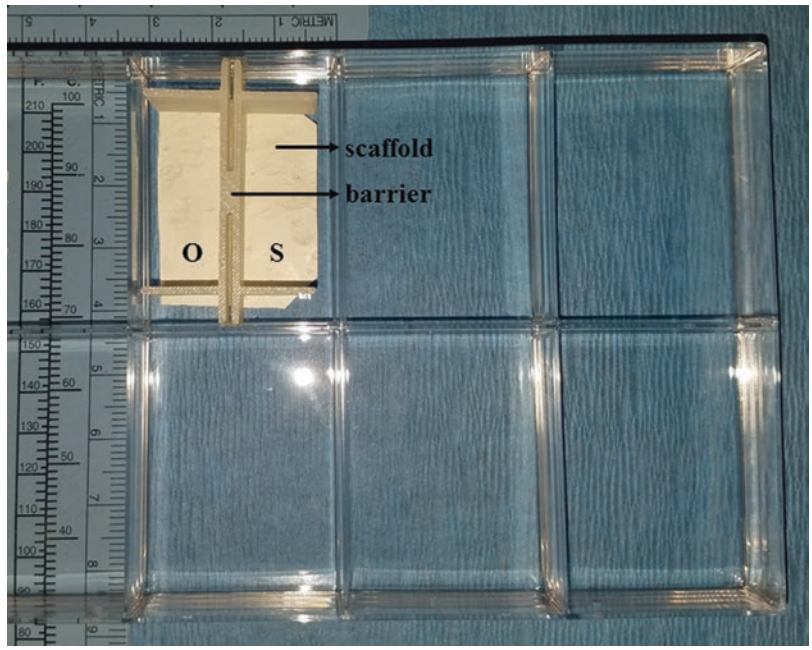

Fig. 6.8 Barrier setup for oral and skin keratinocytes co-culture cell seeding. Barrier is firmly pressed on top of the scaffold whose two notched corners were placed on the same either right or left side of the barrier. Oral (O) and skin (S) keratinocytes will be seeded on either side of the barrier, respectively

0.06 mM calcium into the other side of barrier.

11. Prepare oral and skin keratinocytes for cell seeding. Seed 500 k cells/cm^2 in 0.25 ml medium containing 0.06 mM calcium of oral and skin keratinocytes onto each side of the scaffold, respectively (See note 4). Make sure cells are evenly disturbed over the scaffold surface, and allow cells to sit for 30 min without disturbance.
12. Add 2.75 ml medium containing 0.06 mM calcium into each side of the barrier. The total volume of the medium of each side will be 3.5 ml.
13. Incubate cells inside a 5% CO_2 incubator at 37 °C for 24 h (See note 5).
14. Aspirate medium completely first, remove the barrier, transfer scaffold to a 100 × 20 mm dish, place a corral on top of the scaffold to pin down the scaffold and prevent its movement, and add 30 ml medium containing 1.2 mM calcium to continue liquid phase culture (Fig. 6.9).
15. Change medium containing 1.2 mM calcium every 2 days for a total of 4 days.
16. Transfer scaffold onto a 75 mm diameter insert sitting on top of a waffle inside a 150 × 25 mm cell culture dish, and add 80 ml medium containing 1.2 mM calcium to create an air-liquid phase (Fig. 6.10). The volume of the medium applied should depend on the height of the waffle and the size of culture dish.
17. Change medium every 2 days for 10 days (See note 6).
18. Evaluate by histology and immunohistochemistry at the end of 15 culture days (Fig. 6.11).

There is a continued need for reconstruction of complex soft tissue defects of the head and neck tissue. There have been advances in reconstruction through regenerative medicine, tissue engineering, and advanced surgical reconstruction. However, each of these endeavors has its positive and negative aspects. Throughout our past and present studies, the issue of immunosuppression and graft rejection has been addressed by harvesting the patient's own cells. We have also addressed the junctions involved in mucocutaneous transition, by creating a tissue-engineered mucocutaneous junction. We have addressed vascularity of engineered tissue by the prefabrication and prelamination and transfer via free tissue

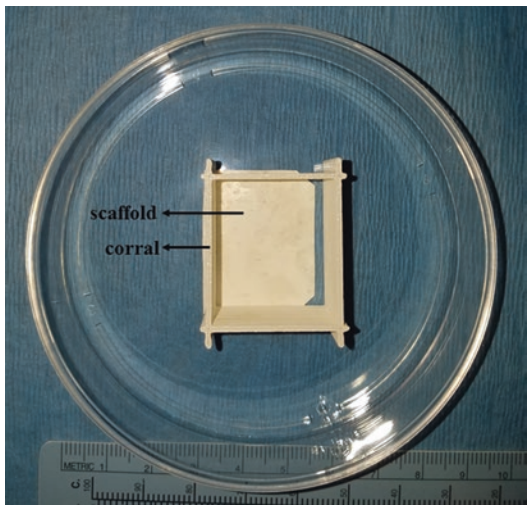

Fig. 6.9 Liquid phase setup for oral and skin keratinocytes co-cultured EVPOMEs. The corral is placed on top of scaffold to prevent floating around of scaffold and ensure the stability of cell growth

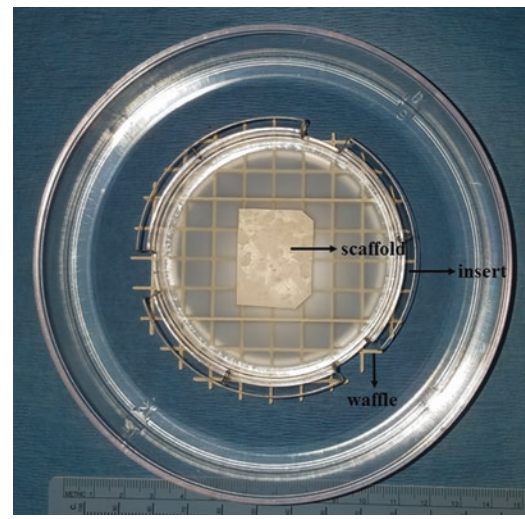

Fig. 6.10 Air-liquid phase setup for oral and skin keratinocytes co-cultured EVPOMEs. Insert sits on top of the waffle, which functions as a raise in order to create an air-liquid phase culture. Scaffold is placed on the insert

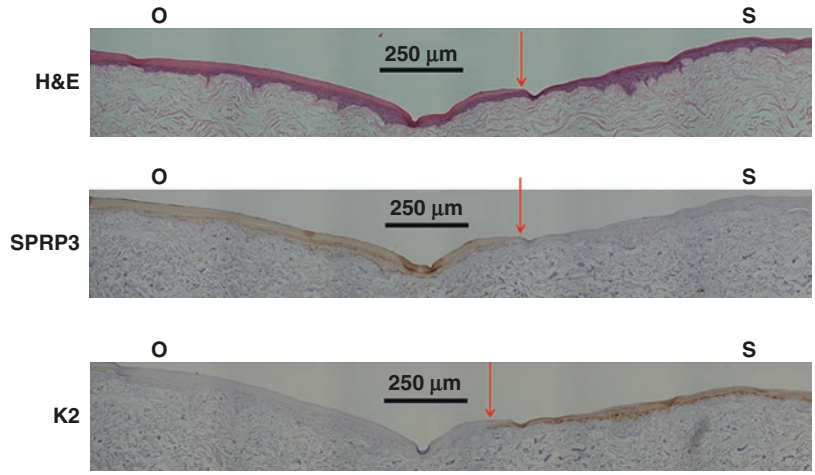

Fig. 6.11 Histology of oral and skin keratinocyte co-culture EVPOME. Cells were culture in medium with 0.06 mM Ca^{+2} for 1 day, at liquid phase in medium with 1.2 mM Ca^{+2} for 4 days, and at air-liquid phase in medium with 1.2 mM Ca^{+2} for 10 days. O is oral keratinocytes culture side and S skin keratinocytes culture side. Red arrows represent where oral and skin keratinocytes met and merged. Anti-K2 antibody at a dilution of 1:250 and anti-small proline-rich protein 3 antibody at a dilution of 1:2000 were used to distinguish skin and oral keratinocytes, respectively

transfer. Further studies are needed to address function and sensation as well as multicenter clinical trials to evaluate the viability and effectiveness of development of this type of specialized therapy. In addition, long-term studies are needed to assess color, function, and durability of patients with prelaminated/prefabricated rotational and free flaps.

6.5 Notes

1. Prepare a funnel-shaped nylon net filter by folding the round-shaped filter twice and then stapling at the edge of the overlapping portion. Soak several filters in 95% ethanol overnight for sterilization.
2. Evaluation of EVPOMEs by histology and immunohistochemistry:

   At day 11 post-seeding, EVPOME samples were collected and fixed in 5 ml of 3.7% phosphate-buffered formalin in wells of 6-well plate at 4 °C overnight. The fixing time can be evaluated empirically to get the best signals of specific biomarkers examined. EVPOMEs are washed in 1X PBS solution, cut into 1 mm wide strips, and then stored in 70% ethanol for paraffin embedding.
3. Centrifuge floating cells at 10 g for 2.54 cm radius, 4 °C for 5 min. Aspirate spent medium and resuspend cell pellets in appropriate volume of full medium containing 1.2 mM calcium.
4. The larger the cell seeding surface, the higher the cell seeding number/cm^2 is required to obtain a well-developed EVPOME. However, the relationship between cell seeding number/cm^2 and cell growth area is not lineally proportional. The optimal cell seeding number/cm^2 needs to be determined empirically based on the surface area of dish where the scaffold sits not the surface area of scaffold itself.
5. The number of cell culture days in medium containing 0.06 mM calcium can be changed based on the experimental designs.
6. The number of air-liquid phase culture days should be determined empirically based on the width of cell-free zone.

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