HHS Public Access

Author manuscript

Connect Tissue Res. Author manuscript; available in PMC 2023 September 01.

Published in final edited form as:

Connect Tissue Res. 2022 September ; 63(5): 514–529. doi:10.1080/03008207.2022.2035375.

Oral mucosa equivalents, prevascularization approaches and potential applications

Daniela S. Masson-Meyers[a], **Luiz E. Bertassoni**[b], **Lobat Tayebi**[a,*]

[a]Marquette University School of Dentistry. Milwaukee, WI 53233, USA

[b]School of Dentistry, Oregon Health and Science University. Portland, OR 97201, USA

1. Introduction

The oral cavity is covered by a mucous membrane, termed oral mucosa, which acts as a barrier to prevent underlying tissues against thermal injuries, mechanical damage, and exposure to toxic substances and pathogens [1–4]. An understanding of the native oral mucosa structure and function, its healing process, and protocols to repair oral injuries is essential to better comprehend the challenges involved with developing oral mucosa equivalents (OMEs) with potential to enhance tissue repair.

Several OMEs models have been developed over the years aiming, for instance, at reducing the need for autologous mucosa or skin grafts, as well as complications associated with these procedures. However, there is still a need for further improvements to make them more similar to the native oral mucosa, increase their potential use as *in vitro* models, and eventually translate their applications to clinical reconstruction of oral soft and hard tissues [5–7].

Another important characteristic to consider during the development and application of OMEs, is vascularization, especially when the constructs are intended to repair large-sized oral defects [5, 8]. The implantation of large tissue constructs at an ischemic injury requires fast vascularization to promote healing and prevent graft failure. Thus, approaches to develop prevascularized OMEs are on the rise. By prevascularizing the equivalents, their integration with the host tissue and faster repair will be more likely to occur. However, the attainment of effective vascularized equivalents is still a challenge.

This review starts with a brief introduction of the oral mucosa general structure, followed by an overview of healing and the reconstruction of defects in the oral cavity. Next, different oral mucosa equivalents as well as biological and tissue engineering considerations during their development and optimization are highlighted, followed by the vascularization relevance and strategies to prevascularize OMEs. Lastly, *in vitro* and clinical applications of oral mucosa equivalents are discussed.

[*]**Corresponding author: Lobat Tayebi** Associate Professor & Director of Research, Marquette University School of Dentistry, 1801 W. Wisconsin Ave., Milwaukee, WI 53233. USA. URL: tayebigroup.mu.edu, lobat.tayebi@marquette.edu, Phone: 414-288-8383.

Disclosure statement

The authors report no conflict of interest.

2. Oral mucosa structure

Human normal oral mucosa, more than any other mucosal tissue in the body, is very similar to the skin in its architecture and function. Both tissues are composed of a stratified epithelium attached to an underlying dense connective tissue by the basement membrane [2, 3, 9–11]. However, some physiological features are distinctive in the oral mucosa, such as its pink color due to the extensive blood supply, its moist surface, high permeability, and lack of appendages such as hair follicles, sebaceous, and sweat glands [1, 2].

The main cells in the epithelium are keratinocytes attached to each other by desmosomes, and arranged in distinct layers [3]. The connective tissue, i.e., lamina propria, supports and nourishes the epithelium, connects it to the underlying structures, and is composed mainly of fibroblasts, along with capillaries, inflammatory cells, an extracellular matrix (ECM), and a network of type I and III collagen and elastin fibers [2, 3]. The keratinization pattern differs between the skin and oral mucosa. The oral mucosa epithelium may be keratinized (masticatory mucosa), nonkeratinized (lining mucosa), or both (specialized mucosa on the dorsum of the tongue), depending on the region of the oral cavity and its function [1, 2, 12]. The skin epithelium is keratinized regardless of its location in the body [1, 2].

The hard palate and gingiva, where more strength is required, are covered by a keratinized squamous epithelium. In the soft palate, internal surface of the lips, ventral surface of the tongue, cheeks, and the floor of the mouth, where more elasticity is required, a nonkeratinized stratified squamous epithelium is present and characterized by the absence of a horny layer [1, 9, 10, 12]. Oral mucosa equivalents have been developed that mimic nonkeratinized or keratinized patterns depending on the region of the oral cavity where the cells are isolated from [13].

When the oral mucosa is injured, a break in the barrier function causes tissue fluid loss, increases the risk of infection, and may result in oral functional limitations. Thus, its reconstruction is required to reestablish the anatomical and physiological properties, and prevent further complications [14].

3. Wound healing and reconstruction of defects in the oral cavity

Oral injuries can be induced by a variety of conditions such as trauma, recurrent wounds, inflammation, irradiation treatment, and reconstructive surgery of congenital or pathological lesions. If the wounds are not adequately treated, they can impact the overall wellbeing of the patient due to pain and possible impairment of oral functions such as swallowing and speech [15].

The repair process in the oral cavity is still poorly understood, and the vast majority of available information on soft tissue healing comes from studies on cutaneous wound healing [16]. It is assumed that the wound healing and cellular processes during oral soft tissue repair resemble, at least in part, cutaneous healing consisting of overlapping phases of hemostasis, inflammation, proliferation, and remodeling [17, 18]. Repair of the hard palate, dental implant interfaces, periodontal, dental, and dental pulp healing also follow a similar

process, however, each of these conditions have specific healing patterns that are beyond the scope of this paper.

Oral wounds usually heal faster than cutaneous wounds, and with minimal scar formation. These aspects of oral wound healing have been attributed to the presence of excellent blood supply, differential inflammatory response, the distinct modulation of stem cells, and the antibacterial and pro-healing properties of saliva [16, 18–21]. Besides providing the oral cavity with moisture, saliva is loaded with proteins that promote cell division, enhance cell migration, and provide antimicrobial activity to support a healthy oral microflora [19]. More efficient epithelial remodeling and pro-inflammatory responses by oral keratinocytes also contribute to a faster healing process in the oral cavity, which usually only takes a few days for minor traumatic lesions [10, 16, 19].

Oral wound healing usually occurs with minimal scarring, however, complex hard and soft tissue defects (e.g. surgery of congenital defects or trauma, tumor excision, reconstructive preprosthetic surgery) present major challenges [21, 22]. Large open wounds, often seen in cleft palate repair and after ablative tumor surgery, heal by secondary intention with granulation tissue development followed by wound contraction and scar formation. Mucosal tissue is often required to cover and promote healing of these types of wounds. [14, 23]. The outcomes of a successful reconstructive procedure will depend on the appropriate reconstruction protocol, defect size, as well as on the quality of soft tissue present in the defect [24]. Major progress has been made in hard tissue engineering, where growth factors, scaffolds, and cells have been clinically used for small and large craniofacial defects. However, soft tissue engineering research and clinical applications still need further investigation [22].

Procedures to promote healing and reconstruct large-sized oral defects, and generate soft tissue around teeth and dental implants, vary from autogenous/autologous grafts to three-dimensional (3D) constructs, for example, consisting of cells incorporated in biodegradable scaffolds [22]. The approaches to reconstruct oral cavity defects can be broadly grouped as: oral mucosal grafts and tissue-engineered oral mucosa equivalents (Figure 1).

Grafts to reconstruct soft tissue defects can be obtained from oral mucosa or cutaneous tissue [25]. Oral mucosa is an excellent intraoral graft material, but the quantity of tissue required for grafting can be insufficient, especially when treating large defects [2, 14, 23, 26–29]. As an alternative, skin grafts, available in higher amounts, have been used to cover and repair extensive soft tissue defects [2, 14, 15, 23]. However, skin grafts present several disadvantages when used as a mucosal replacement. These include hair growth, expression of a different keratinization pattern and potential for infection due to the wet oral environment [2, 23, 25, 28].

Oral mucosa and skin grafts require two surgical procedures: one to harvest the tissue, and another to perform the transplant, which can result in increased donor site morbidity [2, 5, 14, 23, 25, 26]. Postoperative pain, paresthesia, risk of infection, scar formation, and surgical complications are usually associated with autologous tissue grafting [27, 29]. Altogether, these disadvantages limit their use [15], and create the need for different

approaches during the reconstruction and surgical care of oral mucosa defects [2, 14, 23]. One promising alternative to replace autologous grafts and potentially shorten the time of surgical procedures and patient recovery is the use of oral mucosa equivalents, i.e., *in vitro*-engineered regenerative substitutes [2, 5, 14, 15, 22, 30].

4. Oral mucosa equivalents

The main purpose of engineering oral mucosa equivalents is to obtain models that will replicate the histological architecture and function of the native tissue for *in vitro* and *in vivo* applications [6, 14, 31].

The tissue repair process in the oral cavity and other tissues is regulated by different cell types, signaling mechanisms, cell-cell, and cell-matrix interactions. Thus, during the development of OMEs, cells, scaffolds, and signaling approaches to create functional oral tissues are needed [32].

The reconstructed oral mucosa models can be classified into two main categories: one consisting of only differentiated oral epithelium (epithelial substitutes), and the other consisting of epithelium and lamina propria [6, 33]. The models containing only epithelium are named partial, or split-thickness, oral mucosa models, while the ones with two tissue layers are named full-thickness oral mucosa models (Figure 1). The full-thickness models can be further categorized depending on the type of scaffold used, for instance: dermal substitutes (acellular or fibroblast-populated matrices) or biomaterials-based (e.g. collagen, gelatin, fibrin) [14, 33].

Epidermal cell sheets have been used to treat severe cutaneous defects such as burns and wounds, and based on this principle, small oral biopsies were used to develop autologous oral epithelial cell sheets [2, 13]. These partial-thickness OMEs, can be used for oral cavity reconstruction and for extra-oral applications (e.g. ocular surface reconstruction and to treat esophageal ulcerations) [13, 34]. They can be produced by seeding cells on temperature-responsive culture dishes, from where cells can be harvested without the need for enzymes. By adjusting the temperature, cells will attach and proliferate at 37°C and can be detached from the culture dish when the temperature decreases to 32°C [35, 36].

Oral mucosal epithelial sheets use one cell type that can be cultured in mono or multilayers, with the latter being more similar to the native oral mucosa. Keratinocytes cultured on permeable cell culture membranes at the air/liquid interface form multilayer epithelial sheets and show signs of differentiation. Monolayer oral mucosal epithelial sheets usually provide reliable data for studies such as the responses of oral mucosa to mechanical stress, addition of growth factors, and radiation damage [11].

Oral mucosal epithelial cell sheets are attained quicker in culture than skin epithelial sheets. It has been reported that epithelial sheets from small oral epithelial segments were obtained in 12 days, while it took 14 days to obtain skin epithelial sheets [2, 13]. However, oral epithelial cell sheets present some drawbacks: they are very fragile, difficult to handle, and are prone to contract [2, 3, 6, 13, 33, 37]. An alternative to overcome these issues is the

development of a more complex *in vitro* 3D full-thickness OME containing epithelium and underlying lamina propria.

Full-thickness oral mucosal equivalents are designed to mimic the native human oral mucosa structure by an association of cells (e.g. keratinocytes, fibroblasts) and scaffolds, making them more experimentally and clinically relevant than monolayer cell culture systems [31, 38]. Ideally, these equivalents should have a stratified squamous epithelium, a continuous basement membrane, and lamina propria, and the production involves the fabrication of extracellular matrix substrates (usually with fibroblasts) with oral keratinocytes seeded on top [5, 14, 33]. By incorporating different cell types in these equivalents, a high degree of cell differentiation is achieved [2, 13]. These models are easier to handle, can be processed, for example, for histological and molecular analyses, and would be clinically more beneficial to repair deep oral defects [2, 13, 14].

Optimal OMEs should be biodegradable, non-toxic, have low immunogenicity, appropriate mechanical strength, and similar density to the human oral mucosa. These equivalents should also promote cell attachment, be a suitable substrate for ECM formation, support neovascularization, and be suturable [14]. When designed to be used as *in vitro* models, OMEs should be reproducible, while a strong construct with good handling properties and optimized biodegradability is needed for *in vivo* and clinical applications [31].

Oral tissue repair relies on bringing the appropriate cells to the wound site to secrete or stimulate the release of growth factors, enhance cellular migration, proliferation, and finally close the defect [22]. During the production of full-thickness OMEs, various scaffold materials, cell types and culture conditions have been used [31, 38].

The common cell types used to develop OMEs are primary human oral keratinocytes and fibroblasts, immortalized human keratinocytes, and immortalized mice (3T3) and human fibroblasts [7, 12, 38, 39]. Primary oral fibroblasts and keratinocytes can be isolated from different areas of the oral cavity (e.g. hard palate, gingiva) and from skin (fibroblasts), and early culture passages should be used to avoid a decrease in ECM production [33, 40].

Due to the difficulties of culturing primary cells for an extended period of time, along with the possibility of variable results due to the cells being obtained from different donors, some *in vitro* studies use immortalized cell lines such as keratinocytes (e.g. HaCaT, TR146, OKF6/TERT-2, OKG4/bmi1/TERT, KC-HPV) [7, 12, 33, 39, 40] and fibroblasts (TERT) [39]. TERT-immortalized human gingival keratinocytes and fibroblasts have been shown to produce a gingiva equivalent similar to the native healthy gingiva, or an oral mucosa tumor model (oral squamous cell carcinoma) when the equivalents were developed with HPV-immortalized keratinocytes. These equivalents could be valuable as alternatives to animal models, for example for studies involving drug targeting, biofilms, and investigation of new treatments [39].

The choice of a scaffold material is another important step in developing 3D-OMEs. It should be biocompatible, biostable, and have adequate porosity to allow for cell infiltration, proliferation, vascularization and cell-cell communication and medium perfusion. Scaffolds should be slowly biodegradable, promote tissue repair, and have mechanical and physical

properties that resemble the normal human oral mucosa. The scaffold material should not stimulate an immune response, generate a toxic reaction, or cause a severe or prolonged inflammation after implantation *in vivo* [2, 5, 32, 42, 43].

Materials used as scaffolds in oral mucosa reconstruction can be categorized in different groups, such as: 1. Naturally derived (e.g. acellular dermis and amniotic membrane); 2. Fibroblast-populated skin substitutes; 3. Collagen-based; 4. Gelatin-based; 5. Fibrin-based; 6. Synthetic (e.g. polymers); and 7. Hybrid scaffolds, i.e., a combination of natural and synthetic matrices [5, 14, 23, 28, 33, 39, 44–48]. Many tissue-engineered oral mucosa studies report the use of collagen- or fibrin-based and acellular dermal matrices as scaffold biomaterials.

The native oral mucosa lamina propria is mainly composed of collagen type I along with type III in the deeper layers, which makes collagen an excellent scaffold material for OMEs. Collagen also present low antigenicity, low inflammatory response, inherent biocompatibility, and biodegradable properties. Moreover, collagen fibers are highly tunable and can easily be shaped into hydrogels or porous scaffolds, providing support for cell proliferation [2, 49, 50]. Fibrin-based matrices also support cell adhesion, proliferation and secretion of new ECM similarly to physiological fibrin during wound healing process, where it serves as a provisional scaffold after an injury. The fibrin-based matrices are produced by a combination of fibrinogen and thrombin, and can be tunable by varying their concentration [2]. Collagen and fibrin-based matrices present many advantages and have been extensively used to fabricate OMEs and other tissue-engineered equivalents. However, their mechanical strength and biodegradability are usually limitations that require optimization (e.g., formulation, addition of cross-linkers, polymers) to enhance their stability, maintain tissue integrity and reproducibility.

Acellular dermal matrices, also often used to fabricate OMEs, are produced through a multistep process where epidermis and dermal cellular components are removed, but the basement membrane and ECM components are maintained. Their structural and biochemical properties are preserved and most importantly, they are rich in collagen and elastin fibers, which provides an architectural framework to support cell attachment, migration and proliferation *in vitro*, e.g. during fabrication of OMEs, and for clinical applications in tissue repair [51, 52]. However, their cost could be a limitation.

Along with the development of OMEs in academic research labs, there are some equivalents commercially available to be used for example as *in vitro* models to test oral products as an alternative to animal models [38]. MatTek's EpiOral and EpiGingival tissues consist of normal, human-derived oral epithelial cells cultured to form multilayered models of the human buccal (EpiOral) and gingival (EpiGingival) phenotypes, nonkeratinized and keratinized, respectively [1, 38, 53]. Episkin also has two tissue-engineered oral mucosa models: oral epithelium (SkinEthic™ HOE/Human Oral Epithelium, composed of TR146 cells derived from a squamous cell carcinoma of the buccal mucosa cultivated on an inert polycarbonate filter) and gingival epithelium (SkinEthic™ HGE/ Human Gingival Epithelium, composed of normal human gingival cells cultivated on an inert polycarbonate filter) [1, 38, 54, 55]. However, these commercial models do not have a fibroblast/collagen matrix component, which is essential in the human oral mucosa [38]. To overcome this

limitation, Organogenesis has developed GINTUIT®, which is composed of allogeneic cultured keratinocytes and fibroblasts in bovine collagen). This is the first marketed product for oral mucosa soft tissue repair, especially for periodontal therapy to regenerate keratinized gingiva [14, 56].

Despite the advances in the production of full-thickness OMEs over the traditional epithelial cell sheets, there is still a need to improve these models for clinical use and as alternatives to animal testing, for example, by introducing cells of the immune and vascular systems into the submucosal compartment [5–8]. A lack of vascularization in OMEs intended to repair large-sized oral defects is a significant limitation and still a challenge in the field, mainly because there are no standard protocols available [6].

5. Prevascularization of oral mucosa equivalents

Tissue-engineered oral mucosa equivalents are a promising approach to reconstruct defects in the oral cavity, however, their implantation, especially for large-sized constructs, has shown a relatively poor long-term viability, resulting in graft failure, mainly attributed to a lack of vascularization [1, 28].

The diffusion limit of oxygen and nutrients (100–200 μm, depending on the tissue) must be considered in any experimental or therapeutic approach to repair tissues of substantial size and prevent ischemia [57]. Insufficient vascularization of large equivalents will lead to compromised cell viability and survival, and impaired regenerative capability [58]. In *in vitro* models, nutrient supply and oxygen diffusion can be achieved by artificial perfusion. However, *in vivo*, the diffusion of oxygen and nutrients from the tissues that surround the implantation site is limited, and the ingrowth of blood vessels from the patient to vascularize the construct is slow, possibly leading to a necrotic state in the center of the construct [59, 60]. Therefore, methods to prevascularize large equivalents and assure their survival *in vivo* are greatly needed [58, 59].

5.1. Strategies to promote vascularization of oral mucosa equivalents

The strategies to promote vascularization in OMEs would fall into similar approaches used to vascularize other tissues, such as: angiogenic factor-based or growth factor delivery, scaffold-based, scaffold-free/cell sheet, *in vivo* prevascularization, and cell-based prevascularization (*in vitro* prevascularization) [57, 61–63]. According to the literature, the inclusion of endothelial cells (ECs) into scaffold materials (i.e., *in vitro* prevascularization), is the main strategy. The pre-formation of capillary-like structures into equivalents will potentially reduce the time required to anastomose with host vasculature and achieve tissue perfusion [57].

ECs are important in the blood vessel formation, the blood coagulation cascade, early inflammatory response after implantation, and during the wound healing phases that follow implantation [58]. These cells also participate in the platelet-blood vessel interaction and release several growth factors that regulate their migration, proliferation, and vascular growth such as platelet-derived growth factor (PDGF), endothelin-1 (ET-1), thrombin, basic fibroblast growth factor (bFGF or FGF-2), and interleukin-1 (IL-1) [64].

In vitro prevascularization is based on the capability of ECs co-cultured with supporting cells to form vascular networks [8]. Cells that secrete angiogenic factors, or that induce other cells to do so, have been used to promote angiogenesis within scaffolds [65]. ECM components and growth factors produced by the supporting cells also contribute to neovessel stabilization and maturation [62]. Intercellular communications and interactions among cells, growth factors, and ECM components associated with angiogenesis are essential for blood vessel formation during tissue repair [64].

Angiogenesis also relies on the activation and migration of fibroblasts, one the most common cell type co-cultured with ECs, shown to improve the formation and/or stability of capillary-like structures *in vitro*[64, 66]. Fibroblasts contribute to angiogenesis by depositing a collagen-rich ECM that enhances tubulogenesis, releasing growth factors [e.g., vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and FGF-2] [61, 67]. The cell-cell interactions (fibroblasts and ECs) in a suitable scaffold, and fibroblasts pro-angiogenic growth factors release could improve angiogenesis in the constructs without the need for exogenous biochemical stimuli [68].

Stem cells from difference sources (e.g., bone marrow, adipose tissue, induced pluripotent, embryonic, dental pulp, dental apical papilla) have also been co-cultured with ECs due to their ability to differentiate into pericytes, and hence, improve *in vitro* capillary formation [69–71]. During blood vessel formation, ECs lining the interior of the vessels are supported by pericytes covering the exterior of the vascular tube, which provide vessel stabilization, barrier function, regulation of angiogenesis and immunological response, contributing to the repair process [70, 72]. Thus, the presence of host pericytes or stem cell-derived pericytes co-cultured with ECs will likely increase the long-term patency and stability of capillaries, and the success of prevascularized constructs implantation.

The development of full-thickness prevascularized OMEs consists of preparing an underlying lamina propria layer comprised of fibroblasts and ECs embedded in a scaffold material (or not), and then culturing an epithelial layer by seeding keratinocytes on top (Figure 2). Noteworthy, the cell types and source, biomaterials and techniques to generate prevascularized OMEs will vary depending on the study.

Studies during 2010–2020 have reported vascularization of full-thickness oral mucosa equivalents [1, 6, 15, 73, 74], and others have reported vascularized oral mucosa lamina propria [29, 59]. A summary of these studies is presented in Table 1.

Heller et al. [73], generated a pre-vascularized buccal mucosa equivalent by co-culturing human gingival epithelial cells and fibroblasts, and human dermal microvascular endothelial cells isolated from human juvenile foreskin, on a collagen matrix (Bio-Gide®). Capillary-like structures were obtained inside the equivalent after 3 weeks of culture, and became functional blood vessels after implantation and anastomosis with the host vasculature (equivalents were subcutaneously implanted in the neck of mice) (Figure 3). The capillary-like structures were not homogenously dispersed throughout the collagen matrix, but rather superficially. The authors hypothesized that the EC infiltration was insufficient probably due to a lack of angiogenic factors signaling and/or the matrix structure, and suggested that

additional optimization was required to achieve a deeper infiltration of ECs. The authors used a commercial collagen matrix with porosity ranging from 5 μm to 300 nm (rough to occlusive sides). The ECs were seeded on the rough side and the decrease in pore size could have prevented cell infiltration. If the authors had designed their own collagen membrane, they could have modified its structure/porosity to achieve a deeper EC infiltration and enhance capillary formation.

Handral [1] produced OMEs using human embryonic stem cells (hESCs)-derived progenies (hESC-oral keratinocytes [Kcs], hESCs-fibroblasts [Fibs], hESC-endothelial cells [ECs], hESC-vascular smooth muscle cells [vSMCs]) cultured on a fibrin-based dermal matrix. The tissue equivalents were prepared in two stages: 1) Fabrication of vascularized dermal tissue equivalents using a tri-culture of hESC-ECs, hESC-vSMCs, and hESCs-Fibs within a fibrin-based matrix and 2) Seeding of hESC-oral Kcs on top of the prevascularized dermal equivalents cultured at the air-liquid interface. Nonkeratinized oral mucosal tissue equivalents with a network of microvasculature in the connective tissue compartment were obtained. To increase mechanical strength and decrease scaffolds contraction, fibrin was conjugated with polyethylene glycol (PEG). A protein inhibitor, Aprotinin, was also added in the scaffolds to decrease biodegradability and increase longevity, making them stable for 8 weeks (4 weeks of culture period and up to 4 weeks after the tissue formation).

Cell sheet technology is another approach to obtain prevascularized OMEs and has not been extensively tested in oral wound healing. Lee et al. [15], investigated the potential of autologous prevascularized mucosal cell sheets comprised of oral mucosal fibroblasts, blood-derived endothelial progenitor cells (EPCs), and keratinocytes to treat deep buccal wounds in rats compared with non-vascularized cell sheets. For the prevascularized cell sheets, mucosal keratinocytes and a mixture of plasma fibrin, mucosal fibroblasts, and EPCs (isolated from peripheral blood of Sprague-Dawley rats) were used. The oral mucosal cell sheets were histologically similar to the native oral mucosa, and in the prevascularized sheets, capillary-like structures were confirmed by positive staining for CD31 (Figure 4a–c). The re-epithelialization and wound closure were faster in the cell sheet groups than in the control (silastic sheets), with positive wound healing effects more apparent in the rats treated with prevascularized cell sheets than in rats treated with non-prevascularized cell sheets (Figure 4d–g). The oral wounds treated with the prevascularized cell sheets did not present scarring or fibrosis, showing a significant similarity to the normal oral mucosa. The prevascularized oral mucosa cell sheets developed in this study showed the potential to restore the oral mucosa *in vivo* by enhancing oral wound healing. A skin graft or cell sheets with other cell types as controls to compare with the mucosal cell sheet were not included, which could be a possible limitation of the study.

In another study, Lee et al. [74], assessed the prevascularized mucosal cell sheets developed in their previous study [15] to treat third-degree cutaneous burn wounds on the back of rats, compared with skin grafts, or silastic sheets as controls. Microvessels in the fibrin-matrix layer of the oral mucosa cell sheets was observed by Hematoxylin and Eosin (H&E) staining and confirmed by the CD31-positive staining (Figure 5a–c). Prevascularization of oral mucosal cell sheets enhanced their survival after transplantation and showed tissue plasticity by enhancing healing with limited scar tissue formation (Figure 5d–o).

Nishiyama et al. [6], developed OMEs consisting of lamina propria, keratinized or nonkeratinized epithelium, and blood capillaries, using a layer-by-layer (LbL) cell coating technology. With this technique, nanofilms consisting of ECM proteins, such as fibronectin and gelatin, are formed on the cell surfaces and function as a molecular glue to promote cell-cell binding. Human oral mucosal fibroblasts were coated with ECM nanofilms to produce the lamina propria layer and human oral keratinocytes, isolated from gingiva or oral mucosa, representing either a keratinized or nonkeratinized epithelium respectively, were seeded on top. To prevascularize the OMEs, human umbilical vein endothelial cells (HUVECs) were mixed with fibronectin-gelatin coated mucosal fibroblasts. The OMEs were histologically similar to the human oral mucosa and blood capillaries were incorporated into the lamina propria, demonstrated by positive CD31 immunostaining. However, further studies should investigate structural and functional properties of constructed blood capillaries in the OMEs.

Cheung et al. [59], assessed a co-culture of HUVECs and human gingival fibroblasts (HGFs) seeded in perfused polyurethane hydrogels (degradable/polar/hydrophobic/ionic polyurethane [D-PHI]). The authors investigated how culture conditions such as perfusion, cell seeding density and ratio, and culture medium would affect *in vitro* blood vessel formation. Culturing HUVECs and HGFs under perfusion at a ratio of 1:2, respectively, using a mixture of 50% of each cell line culture medium, and having at least 80,000 cells per scaffold improved the angiogenic potential of HGFs in the scaffolds. These culture conditions also increased cell growth with the expression of TGF-β1 and FGF-2, and formation of HUVEC clusters. The culture conditions tested in the study can be relevant for optimization during the development of highly vascularized constructs intended to repair the gingival lamina propria and potentially other soft tissues.

In a previous study from Heller et al. [73], full-thickness prevascularized buccal mucosa equivalents on collagen membranes were developed, but microcapillaries formation was limited to the membranes surface. It is still not clear how angiogenic factors are involved during *in vitro* prevascularization. To better understand their role within the equivalents, the same group performed a follow-up study to investigate the impact of different concentrations of VEGF, interleukin-8 (IL-8) and bFGF in the equivalents with regards to cell viability, proliferation, migration, and tubulogenesis [29]. The oral mucosa equivalents used in this study only comprised of lamina propria (primary fibroblasts isolated from human gingiva and endothelial cells, isolated from human juvenile foreskin, seeded on a collagen membrane). Angiogenic factor levels were relevant in the microcapillaries formation and more dependent on VEGF and IL-8 levels than bFGF, suggesting that their addition to the co-cultures will likely increase capillary formation throughout the equivalents.

In summary, these studies have shown the feasibility of generating prevascularized OMEs with relevant data regarding culture conditions, scaffold fabrication and *in vivo* tissue integration and wound healing. Conditions that promote cell proliferation, pro-angiogenic factors expression, and capillary formation differed among the studies. Each study used different biomaterials (collagen, fibrin-PEG, polyurethane hydrogels), cell culture conditions, and fabrication techniques. Thus, a standardization of prevascularization methods could not be gleaned from these studies. However, some optimization parameters could be learned from these studies. For example, Heller et al. [73], tested different cell

densities seeded in the rough or occlusive side of cross-linked or native collagen membranes and found that 4×10^4 fibroblasts or HUVECs per scaffold seeded on the rough side of native collagen provided the best results in capillary-like structures formation. In a follow-up study [29], the group found that the sequential seeding of fibroblasts followed by HUVECs after 24 hours, and addition of VEGF and IL-8 improved their results. However, in the latter, they changed the cell density from 4 to 2×10^5 fibroblasts per scaffold.

Lee et al.[15, 74] showed that a completely autologous prevascularized OMEs was obtained using a density of 5×10^5 cells per scaffold (fibroblasts and ECs, at a 1:1 ratio) by cell sheet technology and the equivalents enhanced wound healing in two different conditions: deep buccal wounds and cutaneous burn wounds. Nishiyama et al.[6] obtained prevascularized OMEs using a layer-by-layer technology, and showed that an increase in HUVECs density (0.5, 1 or 2×10^5 cells/scaffold) increased tubular structure covered area, total tube length and branching points of blood capillaries. Lastly, after testing different culture conditions during the development of prevascularized OMEs with polyurethane hydrogels, Cheung et al. [59] showed that seeding more fibroblasts then HUVECs (2:1 ratio) increased HUVECs percentage survival, promoted HUVECs clustering and upregulated angiogenic factors, at a lower cell number (8×10^4 cells/scaffold) than other studies discussed in this review.

6. Potential applications

The main goal in regenerative medicine is the eventual tissue equivalent translation to clinical implantation, as an alternative to autologous grafts, to repair damaged tissues/organs [1]. However, efforts to develop, optimize, and use OMEs as *in vitro* models are also important [14].

OMEs mimic the three-dimensional human *in vivo* tissue architecture and are valuable models to study the cellular mechanisms in healthy or pathological conditions in a regulated environment, usually at a low cost and high reproducibility [1, 2]. OMEs can be suitable experimental models to assess interactions among cells, ECM, biomolecules, scaffolds, and environmental factors [25]. In addition, *in vitro* constructs provide an alternative platform to minimize the need for *in vivo* animal studies [2, 6, 14, 25].

Tissue-engineered OMEs can be fast and reproducible systems to study the dynamics of oral wound healing and fibrosis, biocompatibility of dental materials and in toxicological and pharmacological investigations of new oral care products (e.g., antiseptic mouthwashes, tooth whitening agents, anesthetic pastes) for potential irritation, inflammation, or tissue damage [1, 2, 6, 7, 14, 31, 41, 75]. OMEs can be valuable as *in vitro* oral disease modeling of oral dysplasia, early invasive oral squamous cell carcinoma, oral mucositis, oral infection models (host-pathogen interactions and efficacy of topically applied antimicrobials - antimicrobial assays) [2, 7, 31, 41], and also for the development of drug delivery systems [6, 31] (Figure 6).

Rodrigues Neves et al. [76] used an OME as a wound healing model to investigate the potential of human saliva to promote healing. They used two models to represent either an open wound or a blister. The first model consisted of 2D skin and gingiva cultures to evaluate the migration and proliferation of fibroblasts and keratinocytes. The second

model consisted of a reconstructed human gingiva or skin (oral or skin fibroblasts mixed in a collagen solution, with keratinocytes seeded on top), with full-thickness freeze blister wounds induced on their surface. Saliva induced secretion of inflammatory cytokines in blister-wounded models and improved both oral and cutaneous wound closure.

Klausner et al. [78], developed partial and full-thickness oral tissue models to test the toxicity and irritation potential of oral care products. Buccal or gingival fibroblasts and keratinocytes were obtained from normal human oral tissues from patients or cadavers and used to prepare partial or full-thickness constructs. In the partial-thickness model, keratinocytes were seeded onto a collagen-coated microporous membrane, while in the full-thickness model, buccal or gingival fibroblasts were cultured in a collagen solution to generate the lamina propria, followed by seeding of keratinocytes on top. The oral tissue constructs were reproducible models suitable for the initial toxicological evaluation of oral care products and could be beneficial during studies of oral pathologies and biology of the oral mucosa as well.

Acute effects of ionizing radiation during oral cancer treatment that can lead to severe ulceration of the oral mucosa can be studied using OMEs. Tra et al. [37], investigated the suitability of a tissue-engineered mucosa comprised of human buccal mucosa fibroblasts and keratinocytes seeded on de-epidermized dermis to evaluate DNA damage of the constructs and normal oral mucosa (biopsies) after exposure to irradiation. The authors assessed components of the epithelial layer, basement membrane, connective tissue, and expression of cytokines. The responses to irradiation were similar in the OMEs and normal oral mucosa, indicating that the model was appropriate to quantify the biological effects radiotherapy.

OMEs are not only important as *in vitro* models, but also clinically relevant to repair large defects in the oral cavity when autologous grafts would not be feasible. Potential clinical applications of these equivalents include intra-oral (e.g., cleft palate repair, periodontal surgery associated with insufficient attached gingival tissue, tissue loss caused by facial trauma and tumor excision), and extra-oral (e.g., prevention of post-endoscopic submucosal dissection esophageal stenosis, corneal epithelium restoration, and urethral reconstructions) [31, 34, 38, 41, 59, 75, 79].

Fernández-Valadés-Gámez et al. [80], reported the use of a full-thickness autologous oral mucosa equivalent (oral fibroblasts and epithelial cells seeded on an acellular fibrin–agarose scaffold) implanted in a cleft palate rabbit model. Six months after implantation, animals were euthanized, and the palatal mucosa and bone were processed for morphometric and histological analyses. The oral mucosa substitutes improved palate growth and maturation, supporting their potential clinical use to treat patients with cleft palate or other clinical cases of loss or removal of palatal tissue where grafts would be required.

Dobrowolski et al. [79], investigated an extra-oral application of oral mucosa epithelial sheets (epithelial cells isolated from buccal mucosa were cultured on a denuded amniotic membrane and placed over a fibroblast monolayer) during corneal epithelium repair in patients with aniridia. After seven days of culture, the epithelium was transplanted to the corneal surface. The oral mucosa epithelial sheets had a positive effect on restoring regular epithelial tissue on the corneal surface with a slight amelioration in visual acuity.

Applications of OMEs have increased over the years, however, clinical use of equivalents designed to reconstruct critical-sized defects is still limited mainly due to a possible lack of tissue integration and inadequate vascularization. Prevascularized full-thickness OMEs will closely resemble the native, highly vascularized normal oral mucosa, being a valuable *in vitro* model for the forementioned applications, along with studies focusing on angiogenesis, wound healing, potential treatments of oral mucosa defects, and will certainly be more clinically relevant. A properly prevascularized full-thickness OME will potentially regulate angiogenesis *in vivo*, accelerate tissue repair, and decrease the chance of implant failure by providing a pre-formed capillary network, that once integrated with the patient vasculature, will supply oxygen and nutrients to the tissue.

7. Conclusion

The aim of this review was to present an overview of human oral mucosa structure, function and reconstruction, development of oral mucosa equivalents, approaches for their vascularization and potential *in vitro* and clinical applications.

Many advances have been made to construct oral mucosa equivalents, however, there is still a need to make these equivalents more physiologically and structurally similar to the native human oral mucosa. For instance, the incorporation of ECs in the submucosal compartment and biomaterials optimization to enhance cell proliferation and promote capillary formation, will make the equivalents more clinically relevant in oral mucosa repair, particularly to treat large-sized oral defects, and more effective as *in vitro* models.

In vitro OMEs prevascularization is still challenging, yet a feasible approach, and have shown promising results with enhanced scaffold integration and healing in animal models, however, different techniques, biomaterials and culture conditions have been reported for their fabrication. The studies reviewed in this article reported that prevascularized OMEs were generated on different scaffolds (e.g. collagen membranes, fibrin-based matrices, polyurethane hydrogels) with several sources of endothelial cells (e.g. HUVECs, dermal microvascular endothelial cells, human embryonic stem cells derived-endothelial cells, endothelial progenitor cells), cultured at different densities. While reasonable that different approaches have been investigated, it is likely that a combination of multiple methodologies will be required for the complex process of blood vessel formation. Once *in vitro* prevascularization is achieved, it is critical that the microvascular networks be highly organized, stable *in vitro* and remain functional after implantation, which are among the greatest challenges still to be overcome.

Different approaches from scaffold modifications to cell culture conditions to obtain a more conducive environment for capillary formation are needed. Among promising techniques to optimize vascular network formation and distribution, are spatially controlled micropatterns using bioprinting and templating techniques to form flow channels for endothelialization. These techniques can provide more controlled distribution of ECs in the scaffolds than incorporating them within hydrogels or seeding them on the scaffolds' surface, which can potentially lead to a randomly distributed blood vessel formation. Other modifications in the biomaterials to make them a better platform for EC's migration and self-assembly into

capillary-like structures include fabrication of composite scaffolds (e.g. collagen-hyaluronic acid, collagen-glycosaminoglycans-chitosan) and changes in structural properties (e.g. creation of interconnected pores by freeze-drying). Another approach that is promising to optimize capillary formation and stabilization is the use of co-cultures of ECs not only with often used fibroblasts, but with pericytes and vascular smooth muscle cells.

An optimal protocol to fabricate OMEs that closely resemble the native oral mucosa and the attainment of a well-organized and stable vascular network in these equivalents will likely combine different strategies. The knowledge gained from research in optimizing these equivalents in the past few years, although broad, can certainly provide a valuable foundation for future studies.

Funding

This work was supported by the National Institute of Dental & Craniofacial Research/National Institutes of Health (NIDCR/NIH) under Grants [numbers R15DE027533, 3R15DE027533–01A1].

References

1. Handral HK. In vitro establishment of vascularised skin and oral mucosa from human embryonic stem cells for pre-clinical studies and industrial applications [dissertation]. 2017.
2. Kinikoglu B, Damour O, Hasirci V. Tissue engineering of oral mucosa: A shared concept with skin. J Artif Organs 2015:18(1): 8–19. [PubMed: 25326194]
3. Liu J, Bian Z, Kuijpers-Jagtman A, Von den Hoff J. Skin and oral mucosa equivalents: Construction and performance. Orthodontics & craniofacial research 2010:13(1): 11–20. [PubMed: 20078790]
4. Mohd Nor NH, Berahim Z, Ahmad A, Kannan TP. Properties of cell sources in tissue-engineered three-dimensional oral mucosa model: A review. Curr Stem Cell Res Ther 2017:12(1): 52–60. [PubMed: 27538403]
5. Kriegebaum U, Mildenberger M, Mueller-Richter UD, Klammert U, Kuebler AC, Reuther T. Tissue engineering of human oral mucosa on different scaffolds: In vitro experiments as a basis for clinical applications. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology 2012:114(5): S190–S198. [PubMed: 23063397]
6. Nishiyama K, Akagi T, Iwai S, Akashi M. Construction of vascularized oral mucosa equivalents using a layer-by-layer cell coating technology. Tissue Engineering Part C: Methods 2019:25(5): 262–275. [PubMed: 30838934]
7. Dongari-Bagtzoglou A, Kashleva H. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nat Protoc 2006:1(4): 2012–2018. [PubMed: 17487190]
8. Tomasina C, Bodet T, Mota C, Moroni L, Camarero-Espinosa S. Bioprinting vasculature: Materials, cells and emergent techniques. Materials 2019:12(17): 2701.
9. Mescher A Junqueira's basic histology. New york city. In. Junqueira's basic histology. New york city: Mc Graw Hill; 2013.
10. Skin and oral mucosa. date.
11. Moharamzadeh K, Brook I, Van Noort R, Scutt A, Thornhill M. Tissue-engineered oral mucosa: A review of the scientific literature. Journal of dental research 2007:86(2): 115–124. [PubMed: 17251509]
12. Bierbaumer L, Schwarze UY, Gruber R, Neuhaus W. Cell culture models of oral mucosal barriers: A review with a focus on applications, culture conditions and barrier properties. Tissue Barriers 2018:6(3): 1479568. [PubMed: 30252599]
13. Kinikoglu B Tissue engineering of full-thickness human oral mucosa. 2010.
14. Izumi K, Kato H, Feinberg SE. Tissue engineered oral mucosa. In. Tissue engineered oral mucosa. Stem cell biology and tissue engineering in dental sciences; 2015.

15. Lee J, Kim EH, Shin D, Roh J-L. Accelerated oral wound healing using a pre-vascularized mucosal cell sheet. Scientific reports 2017:7(1): 1–10. [PubMed: 28127051]
16. Iglesias-Bartolome R, Uchiyama A, Molinolo AA, Abusleme L, Brooks SR, Callejas-Valera JL, Edwards D, Doci C, Asselin-Labat M-L, Onaitis MW. Transcriptional signature primes human oral mucosa for rapid wound healing. Science translational medicine 2018:10(451).
17. Hämmerle CH, Giannobile WV, Abrahamsson I, Blanco J, Bosshardt D, Dahlin C, Donos N, Ellingsen JE, Fickl S, Gruber R. Biology of soft tissue wound healing and regeneration–consensus report of group 1 of the 10th european workshop on periodontology. Journal of clinical periodontology 2014:41(S1-S5.
18. Sculean A, Gruber R, Bosshardt DD. Soft tissue wound healing around teeth and dental implants. Journal of clinical periodontology 2014:41(S6–22.
19. Gibbs S, Roffel S, Meyer M, Gasser A. Biology of soft tissue repair: Gingival epithelium in wound healing and attachment to the tooth and abutment surface. Eur. Cells Mater 2019:14(63–78.
20. Khan I, Arany P. Biophysical approaches for oral wound healing: Emphasis on photobiomodulation. Advances in wound care 2015:4(12): 724–737. [PubMed: 26634185]
21. Politis C, Schoenaers J, Jacobs R, Agbaje JO. Wound healing problems in the mouth. Frontiers in physiology 2016:7(507. [PubMed: 26903873]
22. Aghaloo TL, Hadaya D. Basic principles of bioengineering and regeneration. Oral and Maxillofacial Surgery Clinics 2017:29(1): 1–7.
23. Kato H, Marcelo CL, Washington JB, Bingham EL, Feinberg SE. Fabrication of large size ex vivo-produced oral mucosal equivalents for clinical application. Tissue Eng Part C Methods 2015:21(9): 872–880. [PubMed: 25760802]
24. Engstrand T, Kihlström L, Neovius E, Skogh A-CD, Lundgren TK, Jacobsson H, Bohlin J, Åberg J, Engqvist H. Development of a bioactive implant for repair and potential healing of cranial defects. Journal of neurosurgery 2014:120(1): 273–277. [PubMed: 23909245]
25. Almela T, Brook IM, Moharamzadeh K. Development of three-dimensional tissue engineered bone-oral mucosal composite models. J Mater Sci Mater Med 2016:27(4): 65. [PubMed: 26883949]
26. Khmaladze A, Ganguly A, Kuo S, Raghavan M, Kainkaryam R, Cole JH, Izumi K, Marcelo CL, Feinberg SE, Morris MD. Tissue-engineered constructs of human oral mucosa examined by raman spectroscopy. Tissue Eng Part C Methods 2013:19(4): 299–306. [PubMed: 22992065]
27. Llames S, Recuero I, Romance A, García E, Peña I, Del Valle ÁF, Meana A, Larcher F, Del Río M. Tissue-engineered oral mucosa for mucosal reconstruction in a pediatric patient with hemifacial microsomia and ankyloglossia. The Cleft Palate-Craniofacial Journal 2014:51(2): 246–251. [PubMed: 23879858]
28. Perez-Amodio S, Tra WM, Rakhorst HA, Hovius SE, van Neck JW. Hypoxia preconditioning of tissue-engineered mucosa enhances its angiogenic capacity in vitro. Tissue Eng Part A 2011:17(11–12): 1583–1593. [PubMed: 21303226]
29. Heller M, Bauer HK, Schwab R, Blatt S, Peters K, Nezi-Cahn S, Unger RE, Hasenburg A, Brenner W. The impact of intercellular communication for the generation of complex multicellular prevascularized tissue equivalents. Journal of Biomedical Materials Research Part A 2020:108(3): 734–748. [PubMed: 31788941]
30. Tevlin R, McArdle A, Atashroo D, Walmsley G, Senarath-Yapa K, Zielins E, Paik K, Longaker M, Wan D. Biomaterials for craniofacial bone engineering. Journal of dental research 2014:93(12): 1187–1195. [PubMed: 25139365]
31. Moharamzadeh K, Colley H, Murdoch C, Hearnden V, Chai W, Brook I, Thornhill M, Macneil S. Tissue-engineered oral mucosa. Journal of dental research 2012:91(7): 642–650. [PubMed: 22266525]
32. Larsson L, Decker A, Nibali L, Pilipchuk S, Berglundh T, Giannobile W. Regenerative medicine for periodontal and peri-implant diseases. Journal of dental research 2016:95(3): 255–266. [PubMed: 26608580]
33. Moharamzadeh K. 14 - oral mucosa tissue engineering. In: Tayebi L and Moharamzadeh K editors. 14 - oral mucosa tissue engineering. Biomaterials for oral and dental tissue engineering; 2017/01/01/: Woodhead Publishing; 2017.

34. Yamaguchi N, Isomoto H, Kobayashi S, Kanai N, Kanetaka K, Sakai Y, Kasai Y, Takagi R, Ohki T, Fukuda H. Oral epithelial cell sheets engraftment for esophageal strictures after endoscopic submucosal dissection of squamous cell carcinoma and airplane transportation. Scientific reports 2017:7(1): 1–12. [PubMed: 28127051]
35. Moschouris K, Firoozi N, Kang Y. The application of cell sheet engineering in the vascularization of tissue regeneration. Regen Med 2016:11(6): 559–570. [PubMed: 27527673]
36. Matsuura K, Utoh R, Nagase K, Okano T. Cell sheet approach for tissue engineering and regenerative medicine. J Control Release 2014:190(228–239.
37. Tra W, Tuk B, Van Neck J, Hovius S, Perez-Amodio S. Tissue-engineered mucosa is a suitable model to quantify the acute biological effects of ionizing radiation. International journal of oral and maxillofacial surgery 2013:42(8): 939–948. [PubMed: 23583647]
38. Llopis-Grimalt M, Munar-Bestard M, Ramis J, Monjo M. Tissue-engineered oral mucosa constructs for in vitro research and clinical applications. Biomed. J. Sci. Tech. Res 2018:2(3–5.
39. Buskermolen JK, Reijnders CM, Spiekstra SW, Steinberg T, Kleverlaan CJ, Feilzer AJ, Bakker AD, Gibbs S. Development of a full-thickness human gingiva equivalent constructed from immortalized keratinocytes and fibroblasts. Tissue Eng Part C Methods 2016:22(8): 781–791. [PubMed: 27406216]
40. Bucchieri F, Fucarino A, Marino Gammazza A, Pitruzzella A, Marcianò V, Paderni C, De Caro V, Gabriella Siragusa M, Lo Muzio L, T Holgate S. Medium-term culture of normal human oral mucosa: A novel three-dimensional model to study the effectiveness of drugs administration. Current pharmaceutical design 2012:18(34): 5421–5430. [PubMed: 22632390]
41. MacNeil S, Shepherd J, Smith L. Production of tissue-engineered skin and oral mucosa for clinical and experimental use. Methods Mol Biol 2011:695(129–153.
42. Oryan A, Sahvieh S. Effectiveness of chitosan scaffold in skin, bone and cartilage healing. International Journal of Biological Macromolecules 2017:104(1003–1011.
43. Takei T, Sakai S, Yoshida M. In vitro formation of vascular-like networks using hydrogels. Journal of bioscience and bioengineering 2016:122(5): 519–527. [PubMed: 27117917]
44. Golinski PA, Groger S, Herrmann JM, Bernd A, Meyle J. Oral mucosa model based on a collagen-elastin matrix. J Periodontal Res 2011:46(6): 704–711. [PubMed: 21781121]
45. Pena I, Junquera L, Meana A, García E, García V, De Vicente JC. In vitro engineering of complete autologous oral mucosa equivalents: Characterization of a novel scaffold. Journal of periodontal research 2010:45(3): 375–380. [PubMed: 20337894]
46. Suzuki A, Kato H, Kawakami T, Kodama Y, Shiozawa M, Kuwae H, Miwa K, Hoshikawa E, Haga K, Shiomi A. Development of microstructured fish scale collagen scaffolds to manufacture a tissue-engineered oral mucosa equivalent. Journal of Biomaterials Science, Polymer Edition 2020:31(5): 578–600. [PubMed: 31928320]
47. Terada M, Izumi K, Ohnuki H, Saito T, Kato H, Yamamoto M, Kawano Y, Nozawa-Inoue K, Kashiwazaki H, Ikoma T. Construction and characterization of a tissue-engineered oral mucosa equivalent based on a chitosan-fish scale collagen composite. Journal of Biomedical Materials Research Part B: Applied Biomaterials 2012:100(7): 1792–1802. [PubMed: 22807349]
48. Tra WM, Van Neck JW, Hovius SE, Van Osch GJ, Perez-Amodio S. Characterization of a three-dimensional mucosal equivalent: Similarities and differences with native oral mucosa. Cells Tissues Organs 2012:195(3): 185–196. [PubMed: 21494020]
49. Kang PL, Huang HH, Chen T, Ju KC, Kuo SM. Angiogenesis-promoting effect of lipus on hadscs and huvecs cultured on collagen/hyaluronan scaffolds. Materials Science and Engineering: C 2019:102(22–33.
50. Bédard P, Gauvin S, Ferland K, Caneparo C, Pellerin È, Chabaud S, Bolduc S. Innovative human three-dimensional tissue-engineered models as an alternative to animal testing. Bioengineering (Basel, Switzerland) 2020:7(3): 115.
51. Lam L, Lee RSB, Ivanovski S. 16 - periodontal soft tissue reconstruction. In: Tayebi L and Moharamzadeh K editors. 16 - periodontal soft tissue reconstruction. Biomaterials for oral and dental tissue engineering; 2017/01/01/: Woodhead Publishing; 2017.
52. Srokowski EM, Woodhouse KA. 2.20 decellularized scaffolds. In: Ducheyne P editor. 2.20 decellularized scaffolds. Comprehensive biomaterials ii; 2017/01/01/; Oxford: Elsevier; 2017.

53. Tissue model epioral™ & epigingival[tm]
54. Skinethic hge / gingival epithelium
55. Skinethic hoe / oral epithelium
56. Gintuit (allogeneic cultured keratinocytes and fibroblasts in bovine collagen)
57. Sarker M, Chen XB, Schreyer DJ. Experimental approaches to vascularisation within tissue engineering constructs. J Biomater Sci Polym Ed 2015:26(12): 683–734. [PubMed: 26053971]
58. Liu X, Chen W, Zhang C, Thein-Han W, Hu K, Reynolds MA, Bao C, Wang P, Zhao L, Xu HHK. Co-seeding human endothelial cells with human-induced pluripotent stem cell-derived mesenchymal stem cells on calcium phosphate scaffold enhances osteogenesis and vascularization in rats. Tissue Eng Part A 2017:23(11–12): 546–555. [PubMed: 28287922]
59. Cheung JW, Jain D, McCulloch CA, Santerre JP. Pro-angiogenic character of endothelial cells and gingival fibroblasts cocultures in perfused degradable polyurethane scaffolds. Tissue Eng Part A 2015:21(9–10): 1587–1599. [PubMed: 25631100]
60. Farris AL, Rindone AN, Grayson WL. Oxygen delivering biomaterials for tissue engineering. J Mater Chem B 2016:4(20): 3422–3432. [PubMed: 27453782]
61. Baiguera S, Ribatti D. Endothelialization approaches for viable engineered tissues. Angiogenesis 2013:16(1): 1–14. [PubMed: 23010872]
62. Costa-Almeida R, Granja PL, Soares R, Guerreiro SG. Cellular strategies to promote vascularisation in tissue engineering applications. Eur Cell Mater 2014:28(51–66; discussion 66–57.
63. Min S, Ko IK, Yoo JJ. State-of-the-art strategies for the vascularization of three-dimensional engineered organs. Vasc Specialist Int 2019:35(2): 77–89. [PubMed: 31297357]
64. Allah NUM, Berahim Z, Ahmad A, Kannan TP. Biological interaction between human gingival fibroblasts and vascular endothelial cells for angiogenesis: A co-culture perspective. Tissue Engineering and Regenerative Medicine 2017:14(5): 495–505. [PubMed: 30603504]
65. Tien J Tissue engineering of the microvasculature. Compr Physiol 2019:9(3): 1155–1212. [PubMed: 31187896]
66. Morin KT, Tranquillo RT. In vitro models of angiogenesis and vasculogenesis in fibrin gel. Exp Cell Res 2013:319(16): 2409–2417. [PubMed: 23800466]
67. Bae H, Puranik AS, Gauvin R, Edalat F, Carrillo-Conde B, Peppas NA, Khademhosseini A. Building vascular networks. Sci Transl Med 2012:4(160): 160ps123.
68. A highly perfused tissue-engineered construct for gingival tissue regeneration using degradable/polar/hydrophobic/ionic polyurethanes and a dynamic co-culture system. date.
69. Monteiro N, He W, Franca CM, Athirasala A, Bertassoni LE. Engineering microvascular networks in led light-cured cell-laden hydrogels. ACS Biomaterials Science & Engineering 2018:4(7): 2563–2570. [PubMed: 33435119]
70. Parthiban SP, He W, Monteiro N, Athirasala A, França CM, Bertassoni LE. Engineering pericyte-supported microvascular capillaries in cell-laden hydrogels using stem cells from the bone marrow, dental pulp and dental apical papilla. Sci Rep 2020:10(1): 21579. [PubMed: 33299005]
71. Bertassoni LE. Progress and challenges in microengineering the dental pulp vascular microenvironment. Journal of Endodontics 2020:46(9, Supplement): S90–S100. [PubMed: 32950200]
72. Avolio E, Alvino VV, Ghorbel MT, Campagnolo P. Perivascular cells and tissue engineering: Current applications and untapped potential. Pharmacology & therapeutics 2017:171(83–92.
73. Heller M, Frerick-Ochs EV, Bauer HK, Schiegnitz E, Flesch D, Brieger J, Stein R, Al-Nawas B, Brochhausen C, Thuroff JW et al. Tissue engineered pre-vascularized buccal mucosa equivalents utilizing a primary triculture of epithelial cells, endothelial cells and fibroblasts. Biomaterials 2016:77(207–215.
74. Lee J, Shin D, Roh J-L. Use of a pre-vascularised oral mucosal cell sheet for promoting cutaneous burn wound healing. Theranostics 2018:8(20): 5703. [PubMed: 30555575]
75. Tra WMW. Tissue-engineered mucosa: Clinical tool or in vitro model? [dissertation]. Erasmus University Rotterdam; 2014.

76. Rodrigues Neves C, Buskermolen J, Roffel S, Waaijman T, Thon M, Veerman E, Gibbs S. Human saliva stimulates skin and oral wound healing in vitro. Journal of Tissue Engineering and Regenerative Medicine 2019:13(6): 1079–1092. [PubMed: 30968584]

77. Roffel S, Wu G, Nedeljkovic I, Meyer M, Razafiarison T, Gibbs S. Evaluation of a novel oral mucosa in vitro implantation model for analysis of molecular interactions with dental abutment surfaces. Clinical implant dentistry and related research 2019:21(25–33.

78. Klausner M, Ayehunie S, Breyfogle BA, Wertz PW, Bacca L, Kubilus J. Organotypic human oral tissue models for toxicological studies. Toxicol In Vitro 2007:21(5): 938–949. [PubMed: 17383851]

79. Dobrowolski D, Orzechowska-Wylegala B, Wowra B, Wroblewska-Czajka E, Grolik M, Szczubialka K, Nowakowska M, Puzzolo D, Wylegala EA, Micali A et al. Cultivated oral mucosa epithelium in ocular surface reconstruction in aniridia patients. Biomed Res Int 2015:2015(281870.

80. Fernández-Valadés-Gámez R, Garzón I, Liceras-Liceras E, España-López A, Carriel V, Martin-Piedra M, Muñoz-Miguelsanz M, Sánchez-Quevedo MC, Alaminos M, Fernández-Valadés R. Usefulness of a bioengineered oral mucosa model for preventing palate bone alterations in rabbits with a mucoperiostial defect. Biomed Mater 2016:11(1): 015015. [PubMed: 26894556]

81. Yang G, Mahadik B, Choi JY, Fisher JP. Vascularization in tissue engineering: Fundamentals and state-of-art. Progress in Biomedical Engineering 2020:2(1): 012002. [PubMed: 34308105]

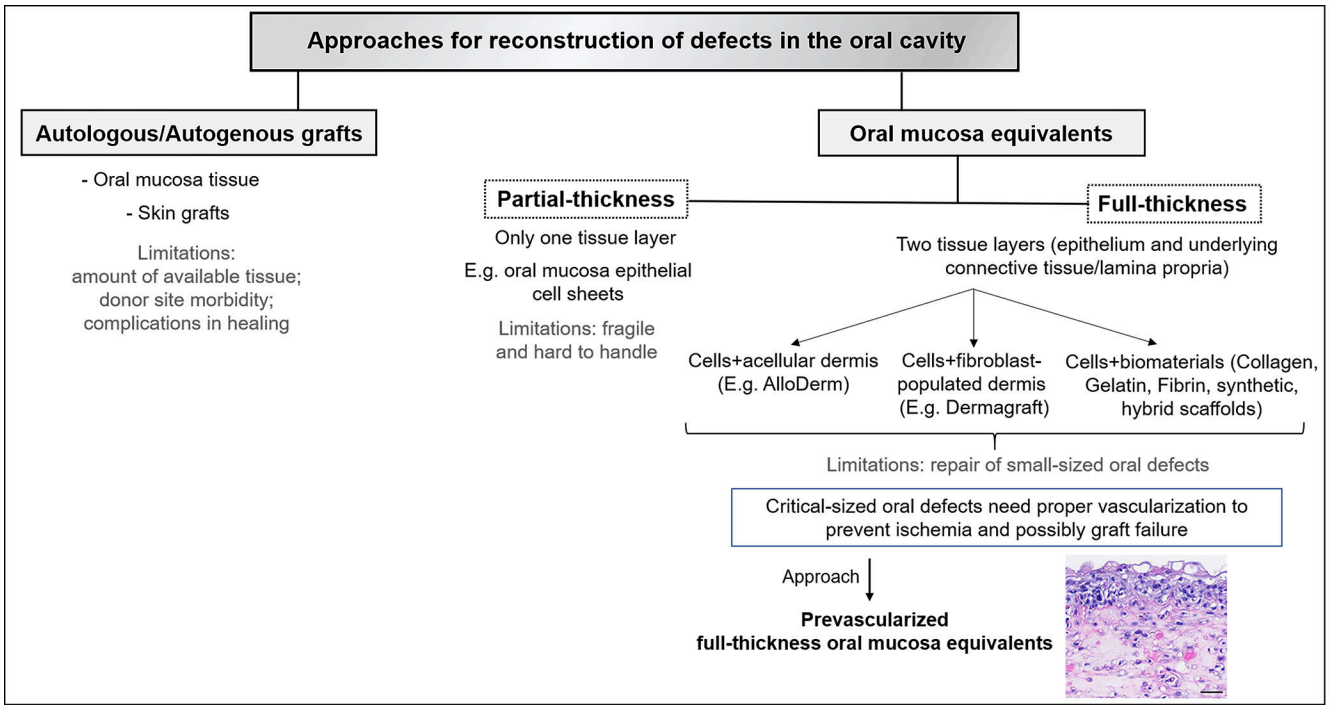

Figure 1.

Overview of common approaches for the reconstruction of oral cavity defects. Histological image was extracted from Lee et al., 2017 [15], which is under the terms of the Creative Commons CC BY license.

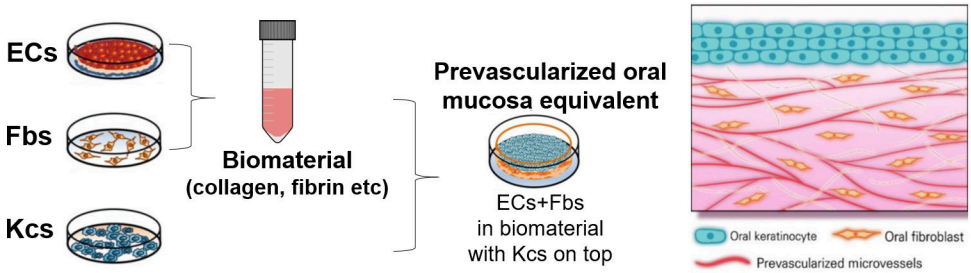

Figure 2.

Summarized representation of an experimental protocol to generate prevascularized oral mucosa equivalents. Adapted and rearranged from [74], which is under the terms of the Creative Commons Attribution (CC BY-NC 4.0). ECs: endothelial cells; Fbs: fibroblasts; Kcs: keratinocytes.

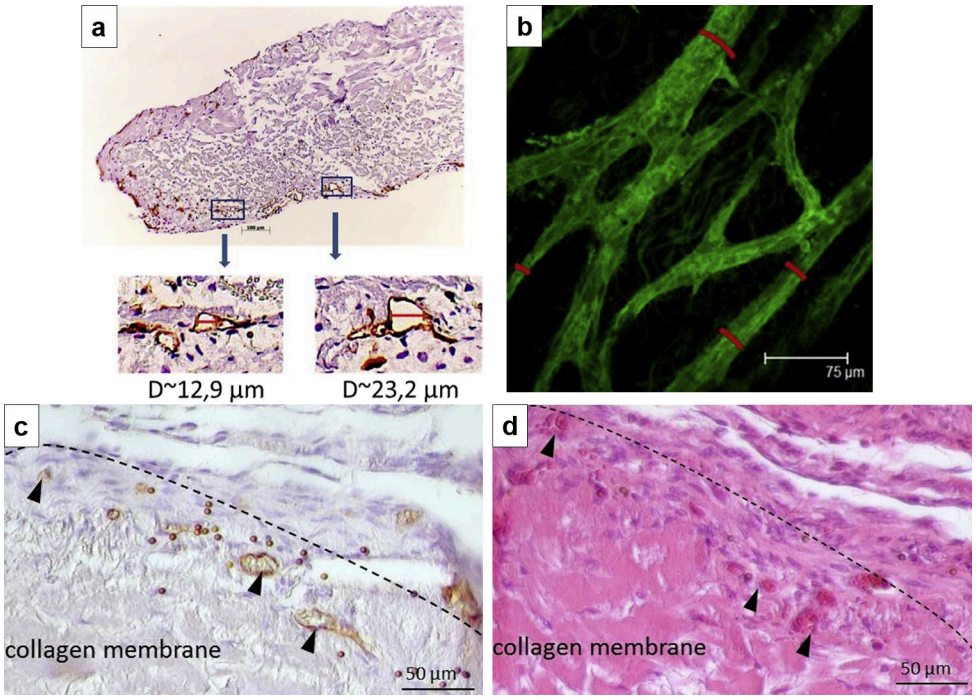

Figure 3.

(a) Immunohistological images of the collagen membrane stained with the endothelial marker CD31 showing lumen of capillary-like structures, stained in brown, in the superficial layers of the seeded membrane. (b) Confocal laser scanning microscopy images of CD31 green stained capillary structures. (c, d) Representative images showing the integration of the prevascularized mucosa equivalent removed from mice 10 days after transplantation: (c) CD31-stained blood vessels inside the mucosa equivalent at superficial areas of the collagen membrane (dashed line), (d) connection of blood vessels to the vessel system of the mouse by H&E staining of erythrocytes inside the blood vessels. Adapted from Heller et al., 2016 [73] with permission from Elsevier.

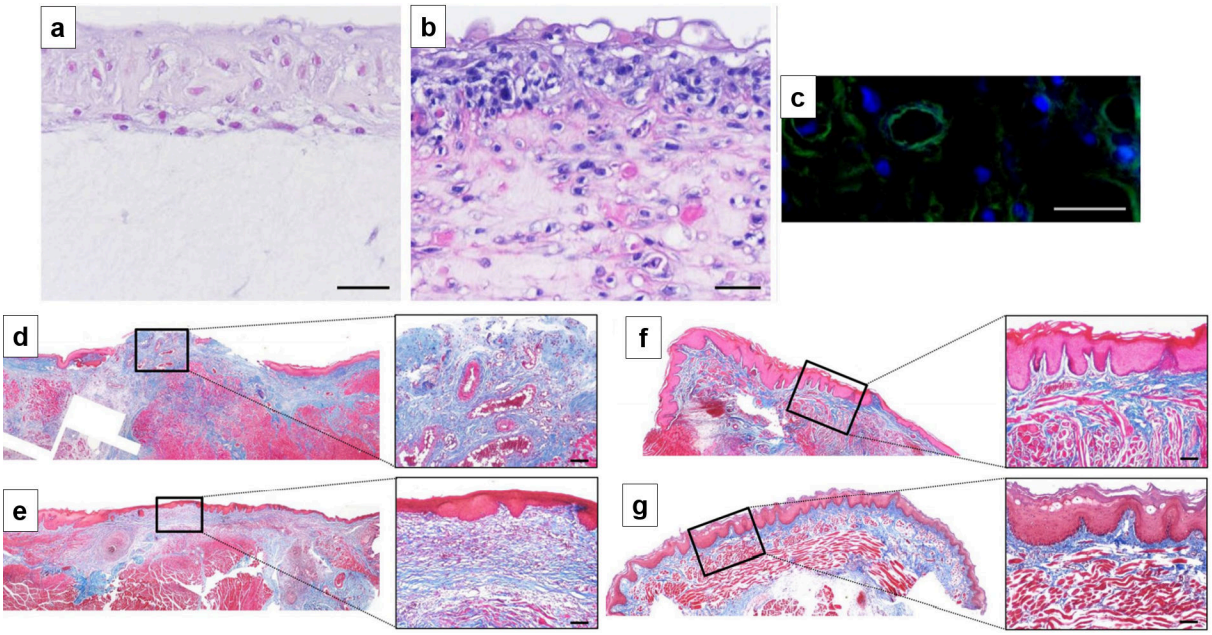

Figure 4.

H&E staining of non-vascularized (a) and prevascularized (b) cell sheets showing their morphology. (c) Immunofluorescence staining showing capillary structures stained in green by CD31. (d-g) Masson's trichrome staining showing the effect of the cell sheets in wound healing 28 days after implantation in rats, representing: (d) wound control, (e) wound treated with non-vascularized sheets, (f) wound treated with prevascularized sheets, and (g) normal unwounded buccal mucosa. Bars indicate 50 μm. Adapted from Lee et al., 2017 [15], which is under the terms of the Creative Commons CC BY license.

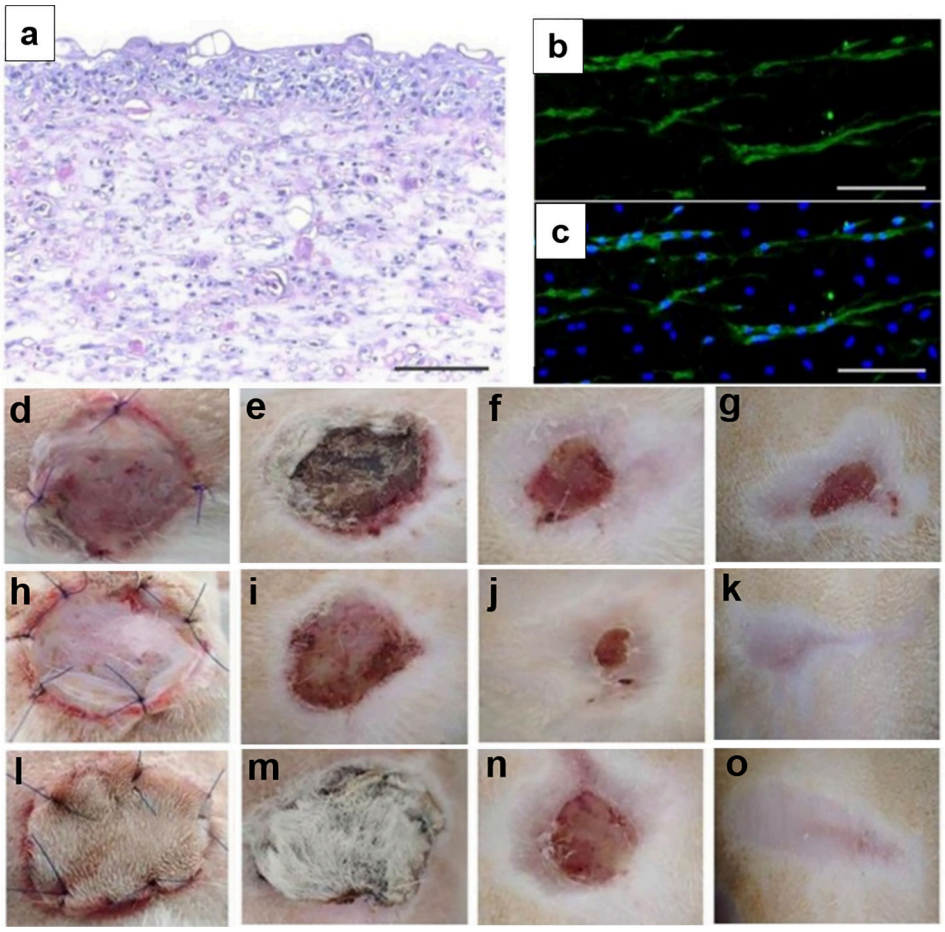

Figure 5.

Representative images of prevascularized cell sheets stained with H&E (a) showing cell sheet morphology, and immunostaining of microvessels in green by CD31 (b, c), with nuclei counter-stained with DAPI (blue). Bars indicate 100 μm. Healing progress of the burn wounds treated with control sheets (d-g), prevascularized cell sheets (h-k), and skin grafts (l-o), on days 0, 7, 21 and 28, respectively. Adapted from Lee et al., 2018 [74], which is under the terms of the Creative Commons Attribution (CC BY-NC).

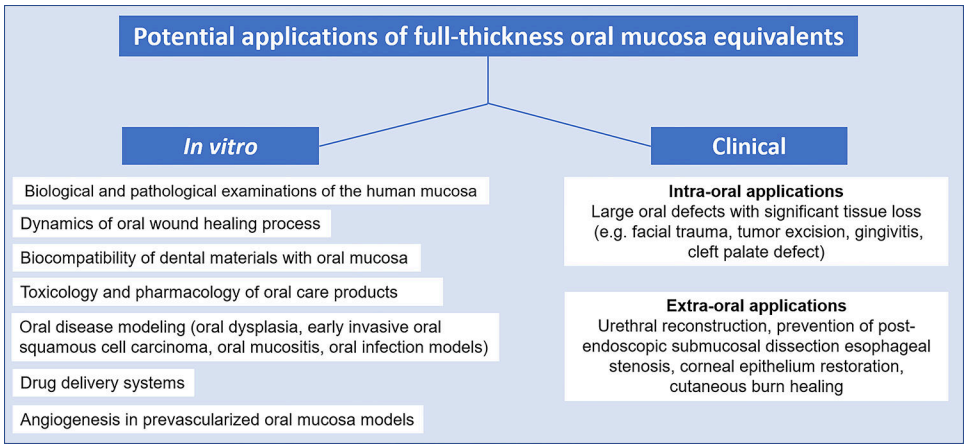

Figure 6.
Overview of potential applications of full-thickness oral mucosa equivalents

Table 1.

Overview of studies on oral mucosa equivalents vascularization.

Cells	Scaffold material	Cell seeding density and ratio	Structure	*In vitro* methods to assess vascularization	Ref.
Epithelial cells and fibroblasts isolated from human gingiva + Dermal microvascular endothelial cells (HDMECs) isolated from human juvenile foreskin	- Porcine type I and III collagens - Bilayered collagen membranes: 1. Native collagen membrane (Geistlich Bio-Gide®) 2. Cross-linked collagen membrane (Geistlich Bio-Gide® Pro)	Endothelial cells and fibroblasts (ratio 1:1) in co-culture (2×10^5, 4×10^5, 8×10^5 each cell type/scaffold). Epithelial cells 2×10^5/scaffold.	Full-thickness oral mucosa equivalents	**Capillary-like structures:** PECAM/CD31 staining of endothelial cells in the construct: Fluorescence (CLSM) and IHC **Secretion of angiogenic factors:** Bio-Plex Pro human angiogenesis assay (PDGF-BB, IL-8, Angiopoietin-2 and VEGF)	[1]
Human embryonic stem cells (hESC) derived-endothelial cells, vascular smooth muscle cells (vSMCs), fibroblasts and hESC-oral keratinocytes	Fibrin-based matrix (Fibrin in conjugation with poly (ethylene) glycol)	hESC-Fibs: hESC-vSMCs: hESC-ECs (1:2:40) hESC-Kcs: 0.5×10^6 cells/cm^2	Full-thickness oral mucosa equivalents	**Network of blood vessels and the presence of vSMCs:**- H&E -Immunofluorescence. Expression of fibronectin, VE-Cadherin, von Willebrand factor by ECs and calponin by vSMCs	[2]
Epithelial cells and fibroblasts isolated from oral mucosa (Sprague-Dawley rats) + Endothelial progenitor cells (isolated from peripheral blood (Sprague-Dawley rats)	Fibrin matrix (autologous plasma fibrin-Sprague-Dawley rats)	Endothelial cells and fibroblasts (ratio 1:1) in co-culture (5×10^5 cells/scaffold)	Full-thickness cell sheets	**Capillary-like structures:** endothelial cells monoculture: tube formation assay in Matrigel; Fluorescence anti-CD31 and calcein AM **Capillary-like structures:** CD31 staining of endothelial cells in the cell sheets: Fluorescence	[3, 4]
Human oral mucosal fibroblasts + Human oral keratinocytes isolated from gingiva or oral mucosa, to construct keratinized or nonkeratinized epithelium + Human umbilical vein endothelial cells (HUVECs)		Fibroblasts: 1×10^6 cells/scaffold-well (24-well plate) HUVECs: 0.5, 1 or 2×10^5cells/scaffold Kcs: 1, 3 or 6×10^5 cells/scaffold	Full-thickness oral mucosa equivalents layer-by-layer (LbL) cell coating technology	**Capillary-like structures:** CD31 staining of endothelial cells in the construct: Fluorescence (CLSM) and IHC	[5]
Human gingival fibroblasts (HGFs) (HGF-1 cell line, ATCC CRL-2014) + Human umbilical vein endothelial cells (HUVECs) (HUV-EC-C, ATCC CRL-1730)	Polyurethane hydrogels (degradable/polar/hydrophobic/ionic polyurethane [D-PHI]	HUVECs and HGFs (ratio 1:1) 80,000 cells/D-PHI scaffold or cells/D-PHI scaffold HUVECs and HGFs at ratios 2:1, 1:1, or 1:2 (HUVEC:HGF) Perfused co-cultures in bioreactor	Lamina propria	**Secretion of angiogenic factors:** ELISA assay: VEGF, TGF-β1, and FGF-2 **Capillary-like structures:** CD31 staining of endothelial cells in the construct: Fluorescence	[6]
Fibroblasts isolated from human gingiva + Dermal microvascular endothelial cells (HDMECs) isolated from human juvenile foreskin	Bilayered collagen membrane: Native collagen membrane (Geistlich Bio-Gide®)	Endothelial cells and fibroblasts in co-culture were seeded: 4×10^5 then 2×10^5cells/scaffold, (HDMECs then fibroblasts) -2×10^5 then 4×10^5cells/scaffold, (fibroblasts then HDMECs)	Lamina propria	**Capillary-like structures:** PECAM/CD31 staining of endothelial cells in the construct: Fluorescence (CLSM) **Secretion of angiogenic factors:** ELISA assay: VEGF, IL-8, and bFGF	[7]

CLSM: Confocal laser scanning microscopy; IHC: Immunohistochemistry; H&E: Hematoxylin and Eosin.