**Homotypic and heterotypic tissue recombinants from dermal and oral mucosa-derived fibroblasts and keratinocytes for skin replacement therapy**

**A. Scientific background**

The cost of treatments for dermal lesions like acute skin wounds, burns, and diabetic ulcers remains high [1-3], and alternative wound coverage and tissue repair methods that are both safe and effective are needed. Different therapeutic strategies and skin substitutes can be used to cover wounds and promote healing, but skin autographs or transplantations remain the most cost-effective method [4,1]. However, limited donor sites from which to harvest autografts are available in cases of skin injuries involving extended surface areas. While cultured epidermal autografts have been used for nearly three decades to treat extensive burn wounds [5,6], epithelial sheets lack supporting substructures, are fragile, difficult to handle, and prone to contractions [7]. One of the main disadvantages of the cultured epidermal autograft technology is its lack of consistent successful outcomes, with engraftment failures reported mainly on wounds devoid of dermal elements, even in the presence of properly cultured keratinocytes [8]. Furthermore, skin autografts are prone to scar formation [9], and the resulting disfigurements may have functional and cosmetic consequences, which reduce the quality of life of the treated patients [10,11]. These complications highlight the need for alternative wound-coverage materials.

Engineered combinations of skin cells and biopolymer scaffolds (or tissue recombinants) are available to treat and help heal acute or chronic dermal wounds [1]. These skin substitutes are made from synthetic materials or from autologous, allograft, xenogenic, or synthetic sources [3]; and, each of them has characteristic advantages and disadvantages. However, researchers are still trying to develop a fully functional skin substitute that can replace full-thickness skin fragments [12].

Tissue injuries initiate the processes (haemostasis, inflammation, proliferation, and remodelling [14]) that underlie the cascade of events needed for wound healing including chemotaxis, cell proliferation, neovascularization, extracellular matrix deposition, and scar formation [13]. However, some wounds, such as diabetic foot ulcers, do not follow this pattern and become chronic [15].

Oral mucosal wounds follow a process similar to that of skin wounds [16], but their healing is faster and leads to minimal or absent scars [17]. This may be due to local oral differences involving temperature, salivary flow, and/or microbiota, although skin segments transposed into the oral cavity maintain their morphologic features [18], and may lead to intraoral keloid formation [19]. Thus, tissue specific factors of the oral mucosa are probably involved in wound repair mechanisms. The harvesting of oral mucosa specimens for wound repair produces less disability than skin harvesting [20], and oral mucosa sheets have been used to repair wounds in the cornea, urinary tract, and intraoral mucosa [21,22]. Moreover, a few studies with oral mucosa specimens have suggested that this tissue source may help achieve rapid and scarless cutaneous wound repair [23-25].

Tissue recombinants are classified into homotypic and heterotypic [26]. Homotypic tissue recombinants taken from the same organ contain both epithelium and mesenchymal tissues that are expected to function as such in their new location [26,27]. By contrast, heterotypic tissue recombinants are derived from epithelium and mesenchymal tissues with different embryological origins and are expected to achieve epithelial development reprogramming in the target organ [28].

While a study by Chinnathambi et al [27] showed that heterotypic skin/oral mucosa recombinations of cultured oral fibroblasts and skin keratinocytes led to the formation of an epithelium with oral mucosa features, the mesenchymal-epithelial interactions of organotypic cultures remain unclear. However, these interactions are probably important during wound-healing, and a thorough understanding of their mechanisms may result in the development of novel treatment strategies [29,30]. We found no studies on skin wound healing using heterotypic tissue-engineered recombinants of oral mucosa and dermal tissues. However, mesenchymal-epithelial interactions are known to involve reciprocal signals [26] that may lead to a scarless and fast repair process. In addition, the benefits from each of these tissues may be combined in heterotypic oral mucosa/skin recombinant grafts.

We used a 3D collagen scaffold *in vitro*, based on the RAFT™ 3D Cell Culture System (Lonza) to generate full-thickness tissue engineered mucosal/dermal recombinants (Fig. 1). Our method can be used to produce homotypic or heterotypic tissue recombinants and to study mesenchymal-epithelial cell interactions *in vitro* and *in vivo*.

Our aims with this proposal are to assess the effects of the tissue origins of fibroblasts and epithelial cells on the epithelial-mesenchymal cell interactions of homotypic and heterotypic oral mucosa/skin recombinants and to evaluate the performance of these tissue recombinants during the *in vivo* treatment of cutaneous burns and diabetic wounds.

**B. Research objectives & expected significance**

**Aim 1: To generate and characterize *in vitro* homotypic and heterotypic dermal and oral tissue recombinants.** The results of transplantation studies using whole tissues or combinations of sheets of epithelia and of connective tissues from different sources suggest that cell proliferation and differentiation are mostly directed by connective tissue signals [31]; however, epithelial intrinsic features are needed for expression of the full adult phenotype [32]. Interpreting results from studies on recombinant tissues is hard due to the many different cell types and confounding variables at play; but a more controlled organotypic culture that restricts cellular and other exogenous *in vivo* factors may minimize these problems and allow researchers to study specific interactions between two cell types. Moreover, organotypic co-culture analyses can help elucidate the mechanisms by which epithelial-mesenchymal cell interactions shape different tissues [26].

Thus, we will generate *in vitro* homotypic (dermal and oral) and heterotypic (dermal fibroblasts with oral keratinocytes or oral fibroblasts with dermal keratinocytes) tissue recombinants growing them under air-liquid interface culture conditions. We will histologically confirm the similarity of the generated tissue recombinants to the natural dermis and oral mucosa and compare their gene expression analysis profiles. The results from this aim will help to identify the mesenchymal-epithelial interactions that underlie the generation of tissue-engineered dermal and oral mucosa equivalents, as well as to understand their role in wound healing and scar formation.

**Aim 2: To evaluate the performance *in vivo* of homotypic and heterotypic dermal and oral tissue recombinants for the treatment of cutaneous burn wounds in a rat model.** Severe thermal and chemical burn wounds are characterized by destruction of skin structure and functionality, and specifically by the loss of the skin’s regeneration ability [33]. Satisfactory coverage materials promoting rapid and appropriate healing of burn wounds do not exists because the current materials are either ineffective, cause immunological rejections, take too long to be filled with a sufficient number of cells, or are too expensive [33]. We want to test homotypic or heterotypic dermal and oral tissue recombinants as potential skin substitutes to produce a full-thickness skin dressing for the treatment of severe cutaneous burns. Therefore, we will inflict third-degree burn wounds on the back of rats and cover the wounds with either homotypic or heterotypic dermal and oral tissue recombinants, or with cultured epidermal autografts as controls. We will perform both gross and microscopic examinations of the post-surgical skin wounds and will measure scar sizes. To understand the molecular mechanisms of wound healing after grafting, we will assess expression levels of genes involved in inflammation, fibrosis, and tissue remodeling, and we will compare them among the different treatment groups. The results from this aim will allow us to compare the burn wound healing characteristics between the animals treated with our tissue recombinants and those treated with the well-studied cultured epidermal autografts.

**Aim 3: To evaluate the homotypic and heterotypic tissue recombinants *in vivo* for the treatment of excisional wounds in a diabetic rat model.** Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both [34]. As of 2014, 422 million people had DM worldwide, a number four times higher than the recorded number in 1980 [35]. Impaired wound healing is a frequent complication of patients with DM that often results in chronic wounds requiring skin replacement therapy [36]. Thus, we will generate soft tissue wounds with exposed bone on the heads of diabetic rats and cover them with homotypic or heterotypic dermal or oral tissue recombinants. We will assess wound healing by both gross and microscopic examinations, and will evaluate the underlying molecular mechanisms by determining gene expression profiles of the different wounds. The results of this aim will allow us to determine the feasibility and efficacy of the proposed tissue recombinants for the treatment of full thickness skin defects under diabetic conditions.

**C. Detailed description of the proposed research**

**1. Working hypothesis**

Wounds in the oral cavity heal faster and display minimal scar formation compared with cutaneous wounds [37]. The current evidence suggests that the superior repair by the oral mucosa is due to intrinsic characteristics of both oral keratinocytes (characterized by enhanced proliferation and migration) and oral fibroblasts (which possess a fetal-like phenotype) [37,28]. Therefore, we hypothesized that tissue recombinants consisting of oral mucosal fibroblasts and/or keratinocytes will combine the intrinsic properties of the oral mucosa and the skin to produce a scarless wound healing that is faster than that achieved by pure dermal tissue recombinants. Moreover, the heterotypic oral mucosa/dermal recombinants may generate a graft that overcomes the skin tissue engineering impediment caused by the epigenetic memory within oral mucosal cells [28]. In addition, our proposed detailed examination of genetic elements and factors responsible for the accelerated and scarless oral wound healing may identify candidate molecules to enhance skin wound healing.

**2. Research design & methods**

**Aim 1: To generate and characterize *in vitro* homotypic and heterotypic dermal and oral tissue recombinants.** We have gained extensive knowledge and experience with oral mucosa and skin derived fibroblasts and keratinocytes in 2D cell cultures [38-41]. We recently established a reliable and highly reproducible method to generate full-thickness tissue-engineered oral mucosa and/or dermal recombinants on 3D collagen scaffolds *in vitro*, based on the RAFT™ 3D Cell Culture System (Lonza).

As a first step for our proposed project, we will produce and characterize homotypic (dermal and oral) and heterotypic (dermal fibroblasts with oral keratinocytes and oral fibroblasts with dermal keratinocytes) tissue recombinants.

**Cell isolation and culture**- We will harvest rat palatal mucosa and dorsal skin specimens and will subject them to enzymatic treatment to separate the epithelial and connective tissue compartments. We will then cut the connective tissue into fragments and place them in culture medium to allow cell outgrowth. We will apply a trypsin solution to the epithelial tissues to dissociate single cells and will seed those onto a feeder layer of lethally irradiated 3T3-J2 fibroblasts. We will confirm the presence of the right cell types by real-time PCR analysis of specific cell markers [37].

**Construction of homotypic and heterotypic tissue recombinants**- We will construct tissue recombinants using the RAFT™ 3D Cell Culture System (Lonza)following the manufacturer's protocol with slight modifications. Briefly, we will suspend oral mucosal/dermal fibroblasts into a pre-chilled mixed collagen solution of the RAFT™ kit and incubate the suspension at 37°C to form a hydrogel. Then, we will place RAFT™ absorbers on top of the hydrogel in a laminar flow hood at room temperature (RT) for 15 min. After removing the absorbers, we will immediately load the hydrogels with medium containing oral mucosal/dermal keratinocytes and will allow the recombinants to grow under air-liquid interface culture conditions for 10 days.

**Assessment of epithelial-mesenchymal interactions**- We will visualize general morphological features of the recombinants by histological examination and will compare the features to those of native tissues. We will assess the phenotype of the recombinants, the expression of epithelial differentiation, and the basement membrane markers through immunohistochemical analysis. We will evaluate differential gene expression profiles by whole-transcriptome analysis with total RNA sequencing (RNA-seq).

Ideally, an engineered tissue equivalent should approximate the properties of the skin but possess the advantages of the oral mucosa, such as lower levels of TGF-beta1, a proinflammatory pro-fibrotic cytokine implicated in the etiology of hypertrophic scars [42].

**Aim 2: To evaluate the performance *in vivo* of homotypic and heterotypic dermal and oral tissue recombinants for the treatment of cutaneous burn wounds in a rat model.** In the second step of the proposed project we will inflict cutaneous burn wounds in a rat model and evaluate the efficacy of treatment with homotypic and heterotypic dermal and oral tissue recombinants *in vivo* comparing the outcomes to those of cultured epidermal autografts.

Isogenic colonies of inbred animals show histocompatibility between donors and recipients after skin graft transplantations [43]. Therefore, in our experiments, we will use inbred Lewis rats (Envigo, Israel) as isogenic donors (for harvesting palatal mucosa and dorsal skin specimens) and additional animals of the same strain as a burn model recipients.

**Burn wound creation**- We will inflict a round-shaped third-degree cutaneous burn wound on each rat’s dorsum as described by Lee *et al* [23]. Briefly, we will shave a patch of dorsal skin hair and generate a thermal wound on the rat’s dorsum by softly applying a round-bottom stainless steel rod pre-heated in boiling water (100°C) for 20 seconds.

**Keratinocyte cell sheets**- Cultured epidermal autografts have been used for the treatment of extensive burn wounds for nearly three decades [5,6]. To compare burn treatment outcomes of this widespread technique to those of oral mucosa/dermal recombinants, we will grow rat keratinocyte cell sheets according to the method implemented at the Division of Plastic & Reconstructive Surgery at the Sheba Medical Center (Tel Hashomer Hospital). Briefly, we will seed oral mucosa/dermal keratinocytes into cell culture dishes with a feeder layer of lethally irradiated 3T3-J2 fibroblasts and incubate them for approximately 10 days until confluence. Before placing the sheets on the burn wounds, we will separate the keratinocyte cell sheets from the culture dish using an enzymatic treatment with a dispase solution.

**In vivo testing of recombinants**- We will detach the dermal/oral mucosa recombinants from the culture dishes and place them on the burn wounds, fixing them with 5-0 absorbable sutures and then overlaying them with a medical adhesive film. In another group of rats, we will cover the wounds with keratinocyte cell sheets.

**Gross and microscopic examinations of postsurgical wounds**- We will regularly capture gross photographs of each wound in a standardized manner at day 1 and twice a week post-surgery, and we will measure the wound and scar formation sizes. We will euthanize animals at different days after wounding. Tissues from the wound will be subjected to histological processing and we will observe them under a microscope to evaluate the amount of reepithelization, inflammatory reactions, and fibrosis in them. We will assess the characteristics of the healed connective tissue and epithelium and compared them between the different treatment modalities. In addition, we will assess tissue vascularization using immunohistochemical techniques.

**Gene expression profiling**- We will perform whole-transcriptome profiling of the healing tissues at different time points using RNA-seq.

**Aim 3: To evaluate the homotypic and heterotypic tissue recombinants *in vivo* for the treatment of excisional wounds in a diabetic rat model.** During the third step of our proposed project, we will induce DM conditions in a rat model and create a soft tissue excisional wound with exposed bone in the rats’ cranium. We will cover the wounds with homotypic or heterotypic dermal or oral tissue recombinants and we will assess wound healing.

The rodents’ skin is unique in that it has a panniculus carnosus layer (a thin muscle layer that is only found in the platysma of the neck in humans), which produces rapid wound contraction following injury [44]. In contrast, human wounds heal via re-epithelialization and granulation tissue formation. We will create a soft tissue wound on the head of rats, because the head is devoid of the panniculus carnosus layer and should reflect human wound healing characteristics better than other areas.

**Induction of diabetes**- We have gained extensive knowledge and experience with a streptozotocin (STZ)‐induced diabetes rat model [45-47]. We will induce diabetes in rats using a single intra-peritoneal administration of STZ. We will evaluate blood glucose levels using a glucometer and use diabetic rats with blood glucose levels >250 mg/dL for the relevant experiments.

**Creation of soft tissue excisional wounds**- We will generate circular soft tissue defects with exposed bone on the head of normoglycemic or hyperglycemic rats by removing the cutaneous tissue and the periosteum of the cranium and then either leave the wounds to heal spontaneously without treatment or graft them with different dermal/oral mucosa recombinants.

**Wound healing analysis**- We will perform gross and microscopic examinations of postsurgical wounds and gene expression profiling as described. We will assess epidermal regeneration, fibroblast proliferation, granulation tissue thickness, presence of inflammatory cells, and angiogenesis. We will also measure the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α and that of growth factors such as VEGF and TGF-1β.

The application of tissue-engineered oral mucosa/dermal recombinants to improve wound closure

may promote tissue repair and regeneration. The results of this investigation may identify new therapeutic opportunities for the treatment of extensive cutaneous wounds associated with DM.

**3. Preliminary results**

***In vitro* culture of dermal/oral mucosa fibroblasts and keratinocytes**- We successfully harvested rat dorsal skin and palatal tissue fragments from Lewis rats (Envigo, Israel) and separated them into connective tissue and epithelium specimens using dispase. We then cut the connective tissue into smaller fragments and placed them in culture medium to promote fibroblast outgrowth. We dissociated the epithelial tissues into single cells using trypsin. We seeded keratinocytes onto a feeder layer of lethally irradiated 3T3-J2 fibroblasts in a Keratinocyte Growth Medium (Lonza).

**Construction of tissue recombinants**- We constructed tissue recombinants based on the RAFT™ 3D Cell Culture System (Lonza)according to the manufacturer's protocol with slight modifications. Briefly, we dispensed fibroblasts into a pre-chilled mixed collagen solution of the RAFT™ kit and incubated them at 37°C to form a hydrogel. Then, we placed RAFT™ absorbers on top of the hydrogel in a laminar flow hood at RT for 15 min. After removing the absorbers, we immediately loaded the hydrogels with medium containing keratinocytes and allowed the recombinants to grow under air-liquid interface culture conditions for up to 10 days.

**4. Resources**

As part of the Faculty of Medicine, our School of Dental Medicine is located on the main campus of Tel Aviv University, in Israel. The school aims to provide an exceptional research environment for both basic (pre-clinical) and clinical investigations. Researchers have access to state-of-the-art instrumentation and service centers, including the service centers of the School of Medicine and of the Faculty of Life Sciences. The service centers provide common resources for biochemical, biomedical, and molecular biology experiments, as well as dedicated proteomics and deep-sequencing equipment. Additional available resources include a dedicated microscopy core facility and two animal facilities including a Specific Pathogen Free Rodent Unit.

At the Weinberg laboratory, one of the research laboratories of the Goldschleger School of Dental Medicine, we focus on oral cell biology and tissue engineering. Our laboratory is fully equipped to complete this project, it includes tissue culture facilities, bright field and fluorescence microscopy tools, and all the necessary equipment to perform molecular analyses.

**4. Potential pitfalls**

We do not anticipate major challenges during cell culture or construction of homotypic and heterotypic tissue recombinants because we have reliably produced them in our lab. One of the challenges we foresee will be during the *in vivo* testing of our recombinants for cutaneous burn and diabetic wound healing because we know that skin grafting is a delicate technique. To overcome this challenge, we will collaborate with Professor Josef Haik, a plastic surgeon and the director of the Division of Plastic & Reconstructive Surgery at the Sheba Medical Center (Tel Hashomer Hospital). We are aware that we may not find our skin recombinant treatment technique to be superior to the standard technique for cutaneous burn and diabetic wound healing. However, while we cannot guarantee that heterotypic recombinants have superior properties than homotypic dermal or gingival recombinants, our results will extend the evidence in the field of tissue engineering and will help to identify the best treatment modality for clinical applications and future studies.

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