A Novel Assay for Evaluating Wound Healing in a Full-Thickness in vitro Human Skin Model

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Abstract

Cutaneous wound healing involves interactions between dermal fibroblasts and epidermal keratinocytes as well as cell-extracellular matrix interactions. The current study describes wound healing experiments conducted in a full thickness in vitro human skin model (EpiDermFT™). This model exhibits stratified epidermal components and a fully developed basement membrane and resembles in vivo skin in regard to both morphology and barrier function. Small epidermal only wounds were induced in the model using a 3mm punch biopsy and subsequently evaluated at various recovery time points by two methods. Historically, EpiDermFT™ has been used to evaluate re-epithelization of the wound by: a) manually bisecting the tissues through the center of the wound, b) staining with hematoxylin and eosin, and c) quantifying migration from the wound origin. Accurate bisection of the wound is difficult and often leads to variability in assay results. Here we describe a novel method of visualizing wound re-epithelization in situ simplifying analysis and reducing introduction of variables inherent in tissue processing that could potentially confound data. Following wounding, tissues were fixed and immunostained with markers of epidermal differentiation as well as with a marker of fibroblasts allowing simultaneous visualization of migrating keratinocytes (keratin 14), differentiated suprabasal cells (involucrin), and dermal fibroblasts (vimentin) within the wound. Histological and immunohistochemical analysis showed keratinocyte migration at 2 days following wounding. In both methods, wounded tissues cultured without growth factors (2% human serum) had an increased healing rate. In contrast, wounded tissues cultured with growth factors (2% human serum) demonstrated a dramatic increase in healing. In conclusion, this novel method of evaluating re-epithelization by utilizing immunohistochemical markers of differentiation is a quicker and more reproducible method of analyzing wound healing.

Methods

EpiDermFT™ Tissue preparation: Normal human dermal fibroblasts (NHDF) were cultured in a collagen gel onto which normal human epidermal keratinocytes were seeded (Figure 1). The constructs were raised to the air/liquid interface and cultured for up to 14 days in serum-free medium to produce stratified, differentiated full-thickness skin equivalents (Figure 1).

Introduction of Wounds: Epidermal-only wounds were induced using a sterile 3 mm dermal biopsy punch (Miltex Inc., York, PA) (Figure 2). The epidermis was mechanically removed using forceps.

Histology: To examine the morphology of the in vitro reconstructed tissues, inserts containing tissues were fixed with 10% formalin, embedded in paraffin, and 5–7 µm thick cross-sections were cut. The sections were mounted on microscopic slides, stained with hematoxylin and eosin (H&E), and observed and photographed using an Olympus VS120 Virtual Slide Scanning System (Figure 5).

In situ Immunohistochemistry: Following treatment, EpiDermFT™ tissues were fixed in 10% formalin for 30 minutes followed by three washes in PBS containing 0.01% Triton X-100. Tissues were blocked for 1 hour with 10% normal goat serum/1% BSA in PBS. Primary antibody was diluted in 1% BSA/PBS and incubated at room temperature for 2 hours. Following incubation with primary antibody, tissues were washed two times (10 minutes each wash) in PBS containing 0.1% normal goat serum. Secondary antibody (goat anti-mouse 488, AlexaFluor, Molecular Probes) was diluted 1:400 in 1% BSA/PBS and incubated with the samples for 1 hour at room temperature. Following incubation with secondary antibody, tissues were rinsed PBS containing 0.1% normal goat serum and stained with DAPI (0.1 µg/ml). Tissues were mounted on a slide containing Immu-mount (Thermo Scientific) and coverslipped for visualization (Figures 3-4).

Confocal Microscopy: Images of immunostained EpiDermFT™ were captured using an Olympus Fluoview FV1000. All images were captured at 4X magnification. Images are shown as a z-stack projection through approximately 175 microns of the most apical tissue layers. were captured using an Olympus Fluoview FV1000. All images were captured at 4X magnification. Images are shown as a z-stack projection through approximately 175 microns of the most apical tissue layers.

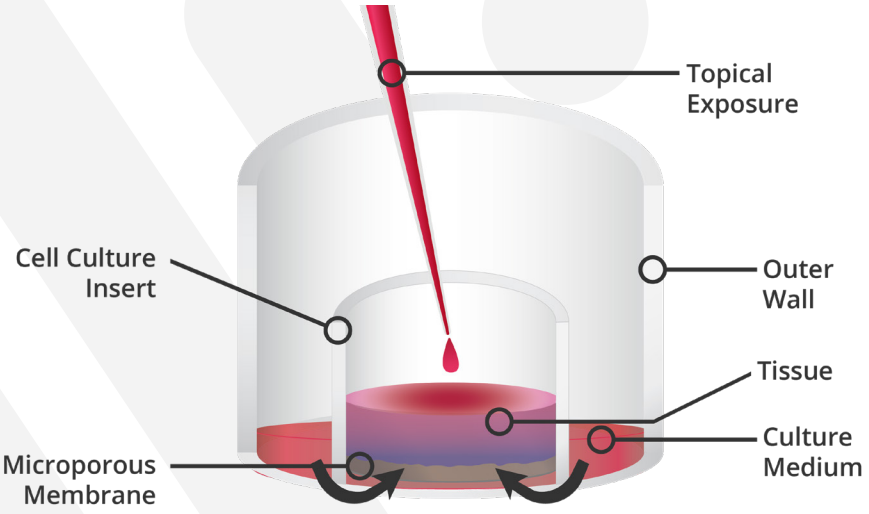

Figure 1: Schematic illustration of air/liquid interface (ALI) culture on microporous membrane inserts. Because the apical tissue surface is not submerged in culture medium, test articles and other exogenous stimuli (e.g. UVR, virus, bacteria, etc.) can be topically applied, as occurs in vivo.

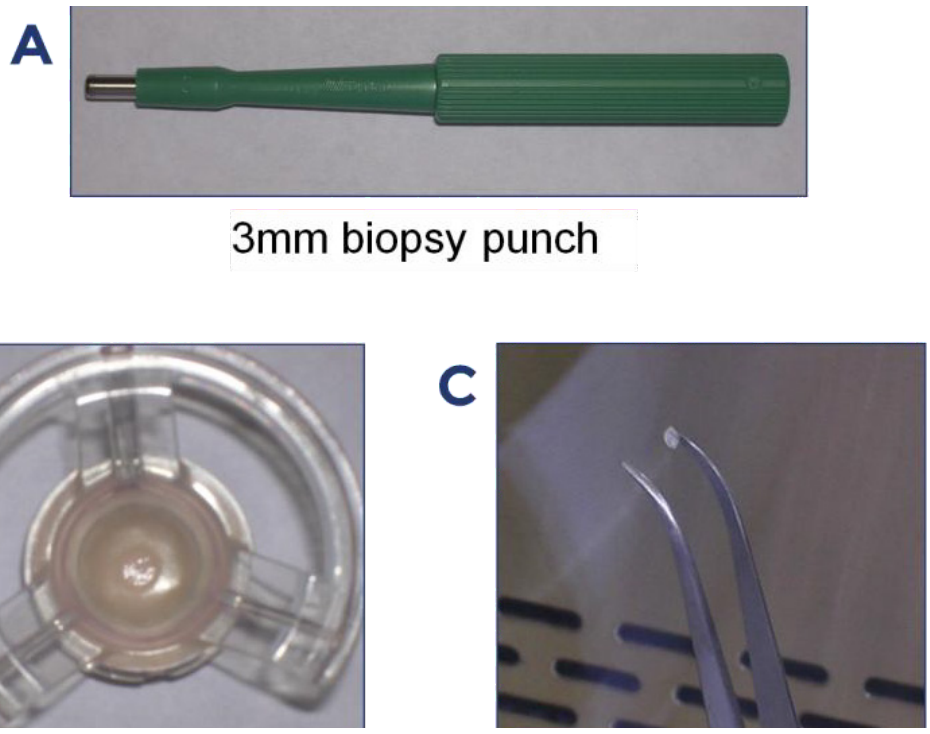

Figure 2: Wounding of EpiDermFT™. **(A)** A 3 mm punch biopsy is used to introduce a wound in the center of the tissue through the epidermis only. **(B)** EpiDermFT™ following wounding. **(C)** A fine tipped forceps is used to remove the cut section of epidermis.

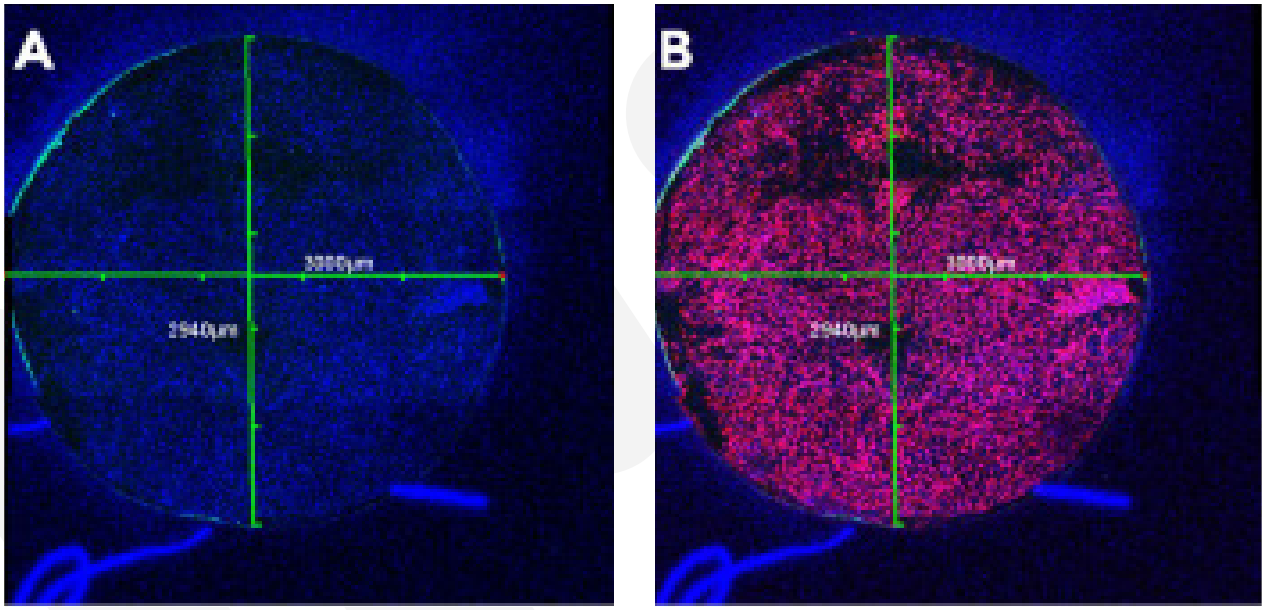

Figure 3: in situ staining of wounded EpiDermFT™. EpiDermFT™ tissues wounded with a 3 mm punch biopsy were immunostained with (A) Keratin 14 (AbCam, ab7800) or (B) vimentin (AbCam, ab7752), a marker of fibroblasts immediately following wounding. Keratin 14 was not detectable in the newly introduced wound whereas fibroblasts were readily visible in the underlying dermis. The width of the wound was measured using Olympus Fluoview v4.1 software.

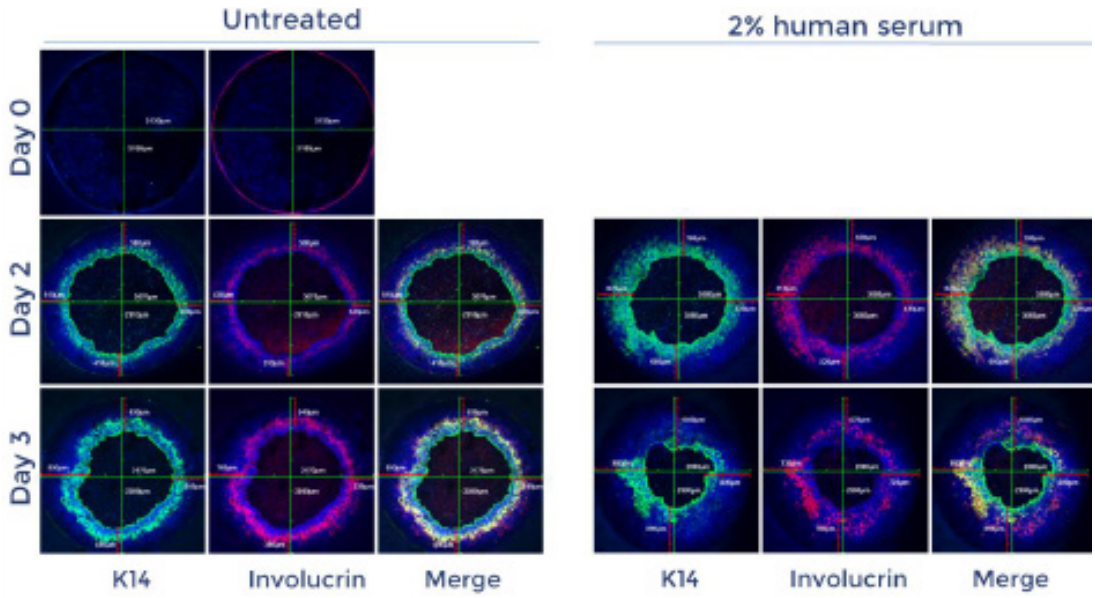

Figure 4: In situ labeling of markers of differentiation following punch biopsy. EpiDermFT™ tissues were collected and stained 0-3 days post-wounding with keratin 14 (AbCam, ab7800) (a marker of proliferating basal keratinocytes) and involucrin (AbCam, ab53112) (a marker of the cornified envelope). Tissues were left untreated or were fed with EFT-400-MM media containing 2% human serum to promote re-epithelization. K14 positive cells were found to migrate toward the center of the wound from day 2-3 however the rate of re-epithelization was greater in tissues treated with 2% human serum. Re-epithelization was measured using Olympus Fluoview software v4.1. Measurements taken from the edge of the wound origin to the leading edge of re-epithelization are shown as red brackets. Measurements of the original wound are shown as green brackets.

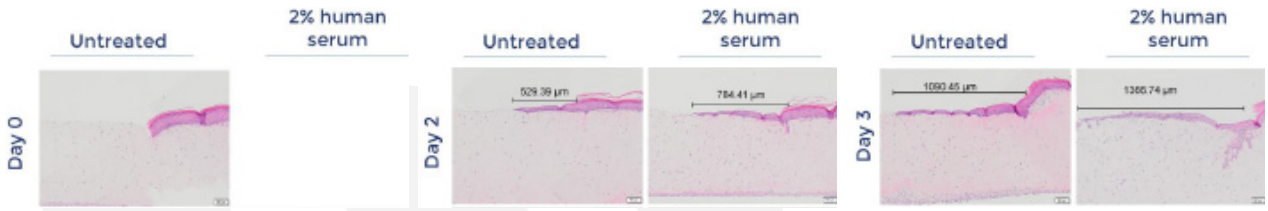

Figure 5: Histological evaluation of human serum on wound repair following punch biopsy. Wounded EpiDermFT™ tissues cultured in growth factor free medium demonstrated epithelial healing at day 2.. 2% human serum increased the rate of epidermal healing and the accumulation of dermal fibroblasts observed as early as day 3.

Summary

- Epithelial healing is observed in EpiDermFT™ within 1-3 days following punch biopsy with full closure by 6 days.
- Human serum increases the rate of epithelial wound healing and fibroblast accumulation following biopsy wounding.
- In situ labeling of EpiDermFT™ tissue using differentiation specific markers allows for a rapid, reproducible and quantitative evaluation of wound healing.
- These results demonstrate that EpiDermFT™ is a useful in vitro skin model for investigating dermal-epidermal interactions during wound healing and evaluation of the role of specific growth factors or new therapeutics in the dermal wound healing process.