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Development of an *in vitro* three-layered skin wound healing model for pre-clinical testing

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Abstract: Skin wound healing involves many cell types in a stepwise process of tissue regeneration. Reepithelialization is an essential characteristic of successful healing. In tissue engineering, mimicking the complex process of injury repair *in vitro* is challenging and requires the development of advanced skin models. In this study, a simple and reproducible method for wounding three-layered skin models on membranes with different pore sizes (0.4, 1, 3 µm) was established. The model allows the investigation of reepithelialization processes in a more complex environment. Hemalaun-eosin (HE) and 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) staining proved sufficient removal of the epidermis directly after wounding. An increasing pore size of the culture membrane delayed the reepithelialization time. Transepithelial electrical resistance (TEER) measurements provided non-invasive monitoring of reepithelialization, showing increasing values 4 days after wounding for the skin models on 0.4 and 1 µm membranes but not for those on 3 µm membranes. Cytokine quantification of interleukin (IL)-6 and IL-8 complemented the TEER results with increasing levels directly after wounding for all skin models. This skin wounding model could be used to simulate different wounding scenarios and test wound matrix materials. Furthermore, it could be adapted by adding immune cells to resemble the *in vivo* setup more closely.

Keywords: reepithelialization; three-layered skin model; wound healing

1 Introduction

Skin wound healing is a complex process aiming to restore tissue integrity and functionality. The healing process follows different stages, starting with inflammation, proliferation, and remodelling [1]. Different cells produce matrix proteins and release cytokines following injury. The signalling between various skin cells is crucial for sufficient wound healing. Adipocytes have been implicated in wound healing mechanisms due to their endocrine functions, which affect immune cell infiltration and tissue repair [2].

Developing improved skin models for wound healing research is necessary to understand the underlying mechanisms and uncover sufficient treatment strategies. *In vitro* skin models offer controlled experimental systems for testing wound dressings and can lead to a reduction in animal experiments. For studying reepithelialization processes, the development of reproducible wounding assays is required. Currently, there are highly heterogeneous approaches to *in vitro* wound healing models and wounding methods such as 2D scratch assays [3] and thermal injury [4]. However, 2D cell monoculture systems are oversimplified and do not represent the physiological composition of the human skin as they lack cross-talk of different cell types. 3D models incorporating more cell types can better mimic the native skin structure and can help gain more significant insights into skin wound healing processes. Most 3D skin models solely incorporate the epidermal [5] or epidermal-dermal layers [6], neglecting the subcutaneous part, which is relevant for studying the healing of deep wounds [7].

This study introduces an easy-to-apply and reproducible wounding method for three-layered skin models to investigate reepithelialization of the model's epidermis.

2 Methods

The three-layered skin model was constructed according to Schmidt et al. [8]. After 14 days of maturation, the skin model was turned upside down, and the dermal and subcutaneous parts were wounded with a 2 mm biopsy punch and the wounded part was aspirated. Then the epidermis was scratched with a biopsy punch, followed by enzymatic dissociation of the epidermal part using a filter paper soaked with 0.1 % trypsin-EDTA solution for 4 min at 37 °C. The epidermis was

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carefully peeled off with tweezers while not puncturing the insert membrane. The enzymatic reaction was stopped by fetal calf serum incubation. Wounded models were cultured in AM1-Air medium (Amsbio, AM1 medium + 1.44 mM $CaCl_2$ + 73 µg/mL ascorbic acid-2-phosphate + 1 % penicillin-streptomycin) and monitored for 11 days post-injury. Wounded skin models on 0.4 µm membranes were fixed using 4 % paraformaldehyde for 4 h. After embedding tissues in paraffin, sections of 5 µm were generated and stained with a standard HE staining procedure.

MTT (1mg/mL) staining of the epidermis on inserts with different pore sizes (0.4, 1, and 3 µm, ThinCert™, Greiner Bio-One) was done on days 1, 4, 6, 8, and 11 after wounding. Non-wounded and wounded skin models were analyzed by TEER measurement using a 12-well EndOhm chamber and EVOM power source (1000 Hz, WPI) considering a membrane area of 1.13 cm^2. Cytokine release of IL-6 and IL-8 was quantified by ELISA (PeproTech) according to the manufacturer's instructions. Data were analyzed for significance by two-way analysis of variance followed by Šídák post-hoc test for multiple comparisons.

3 Results and discussion

To study skin healing processes *in vitro*, a reproducible wounding method for three-layered skin models needs to be developed. Our model was initially designed for substance testing and incorporates an artificial membrane between the epidermis and dermis [8]. The wounding method was adapted from Kiesewetter et al. [5] to fit the three-layered skin model with an intact artificial membrane after wounding (see Figure 1). We could set defined wounds in the three-layered skin model with this modified method. Successful removal of the epidermal, dermal, and subcutaneous parts was proved by HE staining. Non-wounded skin models on day 14 of skin model maturation showed characteristic skin morphology with a stratified epidermis, fibroblast-containing dermis, and a subcutis with roundish adipocytes. On day 1 after wound setting, the epidermis was shown to be removed while the insert membrane remained intact.

The MTT staining of the epidermis in the insert confirmed a successful removal of the epidermis on day 1 after wounding for all pore sizes of 0.4, 1, and 3 µm (see Figure 2A). A complete reepithelialization with metabolically active keratinocytes was observed from day 4 on for 0.4 µm pore size. Skin models cultured on inserts with 1 µm pore size showed complete wound closure from day 6, and skin models on membranes with 3 µm pore size experienced nearly no reepithelialization over the analysis period of 11 days.

The course of reepithelialization was monitored by the TEER method (see Figure 2B). A rebuilding epidermal barrier was detected 6 days after wounding on 0.4 µm membranes. Directly and on day 1 after wounding, the TEER values decreased compared to the non-wounded control. Throughout wound healing, the TEER values increased until they were higher than the control on day 11. Skin models cultured on 1 µm pore-sized membranes revealed a similar course of TEER values to those cultured on 0.4 µm membranes. The skin models cultured on 3 µm pore-sized membranes showed significantly lower TEER values from day 4 after wounding than those on 0.4 and 1 µm membranes. The reaction of the skin model to the wounding on the molecular level was analyzed by quantifying the release of IL-6 and IL-8 (see Figure 2C). An increase in cytokine concentration for both cytokines compared to the non-wounded control was observed on days 1, 4, and 6 after wounding for skin models on 0.4 µm membranes. Skin models on 3 µm membranes showed significantly higher IL-6 release on day 4 after wounding than those on 1 µm membranes. For IL-8, the cytokine concentration was significantly higher for skin models on

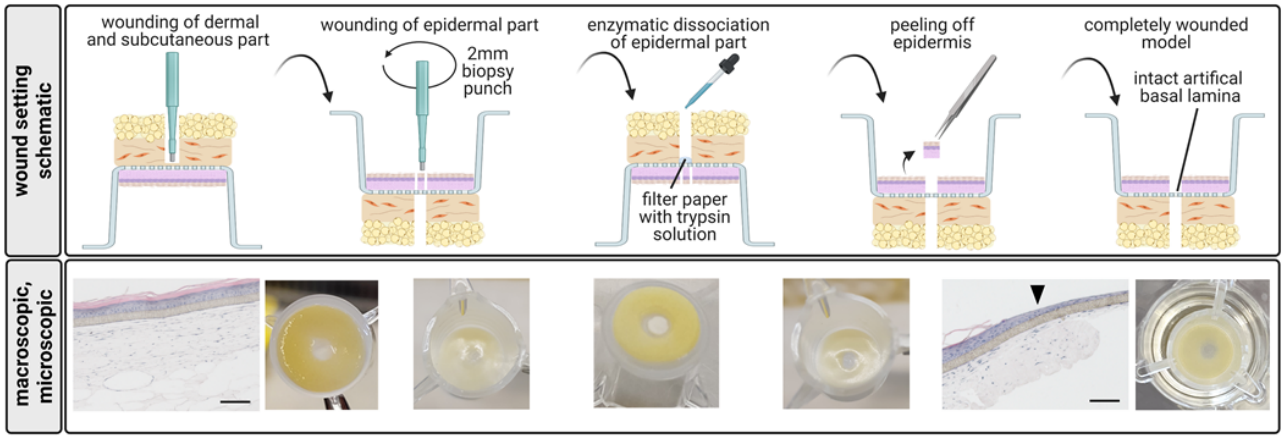

Figure 1: Schematic, macroscopic and microscopic representation of the wound setting method. The black arrow indicates the original wound margin, scale bar: 100 µm.

3 µm membranes on days 1, 4, 6, and 11 compared to those cultured on 0.4 µm membranes.

In the present study, we established a method for setting defined wounds in a three-layered skin model cultured in inserts with different pore sizes (0.4, 1, 3 µm). Reepithelialization is an essential part of skin wound healing [9] and was successfully monitored for the skin models cultured on 0.4 µm and 1 µm pore-sized membranes over 11 days after wounding. Three-layered skin models resemble the native skin structure more closely, making them more relevant as a humanized test system compared to animal models.

We demonstrated that the epidermis of our human skin model on 0.4 µm and 1 µm membranes can regrow, as keratinocytes migrated into the wound and completely covered it until day 4 and day 6 of monitoring, respectively. These results are consistent with the findings of other groups reporting healing times of around 7 days for epidermal [5, 10] and 3-5 days for keratinization of *in vivo* wounds [11]. Healing times greatly vary according to the wounding method and skin model setup. For further investigation of the reepithelialization process, measuring the TEER value of wounded skin models offers a non-destructive monitoring method as introduced by other groups [5]. In this study, the TEER values allowed clear differentiation of the reepithelialization process between the different skin models. Bigger pores slowed down the reepithelialization process in our *in vitro* skin model. The altered reepithelialization times with different membrane pore sizes could be used to simulate different skin wound healing models *in vitro*, like acute physiologically healing wounds and slow healing or chronic wounds [12]. It was already observed by Clement et al. that the microstructure of culture surfaces could affect the behavior of keratinocytes in *in vitro* skin models [13]. Different pore sizes offer the opportunity to recreate different wound scenarios *in vitro* and broaden the application of our three-layered skin wound healing model.

As the wound-healing process is orchestrated by the release of cytokines, we measured the release of IL-6 and IL-8 to characterize the molecular response of our three-layered skin model before and after wounding. Both IL-6 and IL-8 are pro-inflammatory cytokines involved in the acute inflammation phase of wound healing by recruiting leukocytes and enhancing keratinocyte proliferation [14]. In our skin model on 0.4 µm membranes, IL-6 and IL-8 values increased significantly until day 6 after wounding compared to non-wounded skin models, indicating a response to the injury. Spiekstra et al. also demonstrated that the interaction of keratinocytes and fibroblasts significantly influences the cytokine response of the skin model by a paracrine loop [15]. Our skin model also includes fibroblasts and adipocytes, which makes it more physiologically relevant by allowing crosstalk between the different cell types. The skin models cultured on 3 µm membranes showed higher cytokine levels besides only being significantly higher on day 4 after wounding. Therefore, the cytokine release for bigger pore sizes does not allow a clear distinction of the wound healing response. Depending on the wounding method, this suggests more complex underlying mechanisms for cytokine release upon injury. Since wound healing processes include the action

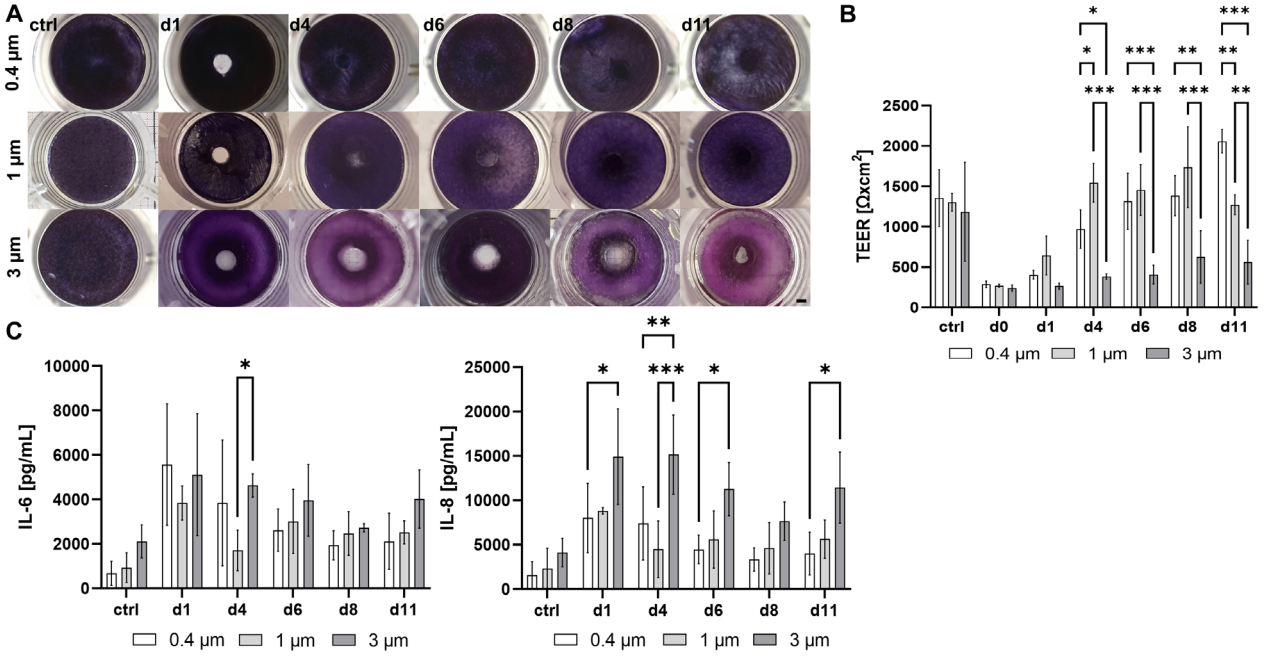

Figure 2: Course of reepithelialization of skin models on different membranes visualized by MTT staining, scale bar: 1 mm (A) and quantified by TEER (B). Cytokine release (IL-6 and IL-8) on days 1, 4, 6, 8 and 11 (C), n=3, *p < 0.05, ** 0.01, *** 0.001.

of immune cells *in vivo* [16], cytokine release measurements can accompany our results of the wound healing response *in vitro*. The group of Kreimendahl et al. showed that the integration of immune cells like macrophages can significantly enhance wound healing in an *in vitro* skin model [17]. Therefore, our model should be re-evaluated by integrating immune cells, which influence the results in the respective context.

In conclusion, we developed a simple and reproducible wounding method for three-layered skin models with an intact artificial basal lamina to study reepithelialization. Additionally, we demonstrated the impact of membranes with different pore sizes on reepithelialization times *in vitro*. Our wounded three-layered skin model represents a platform for mimicking different wound healing scenarios *in vitro*. Future studies can enable the evaluation of potential therapeutics and wound matrix materials to develop strategies to promote tissue regeneration.

Author Statement

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