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Characterization of a Novel Standardized Human Three-Dimensional Skin Wound Healing Model Using Non-Sequential Fractional Ultrapulsed CO_2 Laser Treatments

Yvonne Marquardt,[1†] Philipp M. Amann,[1†] Ruth Heise,[1] Katharina Czaja,[1] Timm Steiner,[2] Hans F. Merk,[1] Claudia Skazik-Voogt,[1‡] and Jens M. Baron[1*‡]

[1]*Department of Dermatology and Allergology, RWTH Aachen University, Aachen, Germany*

[2]*Department of Oral and Maxillofacial Surgery, Medical Faculty, RWTH Aachen University, Aachen, Germany*

Background and Objective: At present, there is no standardized *in vitro* human skin model for wound healing. Therefore, our aim was to establish and characterize an *in vitro*/*ex vivo* three-dimensional (3D) wound healing model, which we employed to analyze the effects of dexpanthenol on wound healing and gene regulation.

Materials and Methods: The novel human 3D skin wound healing model using scaffold and collagen 3D organotypic skin equivalents was irradiated with a non-sequential fractional ultrapulsed CO_2 laser. These standardized injured full-thickness skin equivalents enable qRT–PCR, microarray, and histological studies analyzing the effect of topically or systemically applied compounds on skin wound healing.

Results: These human laser-irradiated skin models were found to be appropriate for *in vitro* wound healing analysis. Topical treatment of skin wounds with a 5% dexpanthenol water-in-oil emulsion or two different 5% dexpanthenol oil-in-water emulsions clearly enhanced wound closure compared to laser-irradiated untreated control models. To find out whether this positive effect is caused by the active substance dexpanthenol, laser-irradiated skin models were cultured in calciumpantothenate containing medium (20 μg/ml) compared to skin equivalents cultured without calciumpantothenate. 3D models cultured in calciumpantothenate revealed considerably faster wound closure compared to the control models. Quantitative RT–PCR studies showed enhanced mRNA expression of MMP3, IL1α, keratin-associated protein 4–12 (KRTAP4–12), and decreased expression of S100A7 in laser-irradiated skin models cultured in medium containing calciumpantothenate.

Conclusion: This novel standardized human 3D skin wound healing model proves useful for topical pharmacological studies on wound healing and reveals new insights into molecular mechanisms of dexpanthenol-mediated effects on wound healing. In addition, these novel 3D model systems can be used to monitor *ex vivo* effects of various laser systems on gene expression and morphology of human skin. Lasers Surg. Med. 47:257–265, 2015.

Key words: Bepanthen; calciumpantothenate; full-thickness organotypic skin equivalents; human skin reconstitution model; pharmacogical skin model

INTRODUCTION

Pantothenic acid is essential to maintain normal epithelial function and homeostasis. It is a component of coenzyme A, which serves as a co-factor for a variety of enzyme-catalyzed reactions that are important in the metabolism of carbohydrates, fatty acids, proteins, sterols, steroid hormones, porphyrins, and gluconeogenesis (for review see [1]). Dexpanthenol, the stable alcoholic analog of pantothenic acid, is widely used in dermatological therapy. It shows good skin penetration and high local concentrations [1], helps to stabilize skin barrier function [2,3], prevents skin irritation [4], stimulates skin regeneration [5], and promotes wound healing [6].

So far, there is no established standardized *in vitro* human skin model that approximates physiological *in vivo* conditions in wound healing. There are different wound healing models that were carried out *ex vivo* [7], *in vivo* in animal studies [8,9] or human clinical trials [10,11]. Punch biopsy, ultrasound or cautery burns have been used to induce small epidermal-only or full-thickness wounds in epithelial skin models leaving rather unstandardized skin injuries. Lasers, used in dermatology such as Er:YAG or CO_2 lasers, provide an alternative and more attractive method for experimental wounding, since they allow rapid and controlled skin ablation [12]. Three-dimensional (3D) organotypic skin equivalents are also currently applied in several *in vitro* studies including dermato–toxicological and pharmacological testing [13–16].

†Yvonne Marquardt and Philipp M. Amann contributed equally to this work (first authorship shared).

‡Claudia Skazik-Voogt and Jens M. Baron are equally contributing senior authors.

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*Correspondence to: Jens Malte Baron, MD, Department of Dermatology and Allergology, RWTH Aachen University, Pauwelsstrasse 30, Aachen 52074, Germany

E-mail: JensMalte.Baron@post.rwth-aachen.de

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Therefore, our aim was to establish a standardized laser-irradiated *in vitro*/*ex vivo* 3D wound healing model, using full-thickness human skin equivalents, and analyze the effects of dexpanthenol on wound healing and gene regulation.

MATERIALS AND METHODS

Isolation of Normal Human Epidermal Keratinocytes (NHEK) and Normal Human Dermal Fibroblasts (NHDF)

Normal human keratinocytes were isolated from foreskin and biopsies of adult skin and foreskin obtained from healthy volunteers after cutaneous surgery. NHDF were obtained from the same donor. After separation of the epidermal sheet from the dermis by dispase (BD Biosciences, Franklin Lakes, NY), trypsin (Lonza, Basel, Switzerland) digestion, and neutralizing with Trypsin Neutralization Solution (TNS) (Lonza), a single cell suspension of NHDF was generated by incubating the dermis in collagenase 1 A (Sigma, Taufkirchen, Germany). This study was conducted according to Declaration of Helsinki Principles and approved by the ethical committee of the University Hospital, RWTH Aachen, Germany. A written informed consent was obtained from all participants/participating parents.

Cell Culture

Cultivation of NHEK and NHDF was done as described previously [14].

Scaffold Skin Equivalents

Scaffold skin equivalents were prepared using Hyalograft 3D (Fidia Advanced Biopolymers, Abano Terme, Italy) as already described [17]. In brief, for construction of dermal equivalents circular 8 mm punches of Hyalograft 3D were transferred into 24 well cell culture inserts (Millipore, Billerica, MA) and inoculated with 3×10^5 NHDF per cm^2 in Tissuecol (Baxter, Deerfield, IL). Dermal equivalents were submersed in DMEM with 10% FBS, 50 mg/ml L-ascorbic acid (Sigma), 1 ng/ml rhTGF-b1 (R&D Systems, Wiesbaden, Germany), 50 mg/ml heparin (Sigma), 1 ng/ml basic fibroblast growth factor (bFGF; Progen, Heidelberg, Germany), and 5 ng/ml platelet-derived growth factor (PDGF-BB; Sigma). After 3–5 days NHEK were seeded on top of the dermal equivalents at a density of 1×10^6 per cm^2 in equal volumes of DMEM (Life Technologies, Carlsbad, CA) and KGM (Lonza) supplemented with 5% FCS, 50 mg/ml L-ascorbic acid (Sigma) and 5 μg/ml aprotinin (Applichem, Chicago, IL). On the following day, skin equivalents were lifted to the air liquid interphase and cultivation was continued by raising the calcium concentration of the culture medium to 1.2 mM. Cultivation of scaffold skin models was continued and medium change was done every other day.

Collagen Skin Equivalents

Collagen skin equivalents were performed as described previously [13]. In brief, to construct the dermis of the skin equivalent, collagen gels were prepared by mixing 8 vol of ice-cold bovine collagen I solution (Vitrogen; Cohesion Technologies, Palo Alto, CA) with 1 vol of X10 concentrated Hank's balanced salt solution (Gibco/Invitrogen, Darmstadt, Germany). After neutralization with 1 M NaOH, 1 volume of NHDF suspended in FCS was added. The final concentration of NHDF in this gel solution was 1×10^5 cells/ml. Four milliliters of this gel solution was poured into each polycarbonate membrane insert (0.4 μm pore size; Thermo Fisher Scientific, Langenselbold, Germany). Then, inserts were placed in 6-well plates. Following complete polymerization, gels were covered with DMEM containing 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin and incubated in a humidified atmosphere at 37°C and 5% CO_2. After 2–5 days, approximately 2×10^6 NHEK were seeded on each dermis equivalent. The originated skin equivalents were cultured in equal volumes of DMEM and keratinocyte growth medium with 5% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 50 mg/ml ascorbic acid. After 3–4 days of submerged culture, the skin equivalents were lifted to the air-liquid interface and the calcium concentration in the medium was raised to 1.2 mM. Cultivation of the collagen skin models was continued and medium change was done every other day.

Skin Lesion Models

Mechanical, thermal, and irradiation induced lesions were examined on complete stratified and organized skin equivalents. Skin Biopsy Model: Skin models were injured by 3 mm punch biopsies (PFM Medical, Cologne, Germany). During the biopsy procedure models were removed from the culture inserts. Electric Cautery Model: Thermal injuries were performed with a low temperature cautery (Faromed, Berlin, Germany). Cauter was preheated for 8 seconds and lesions were set using a Type E platin–iridium tip (Faromed, Berlin, Germany) for 1 second. Laser Irradiation Model: 3D skin models were irradiated with the non-sequential ultrapulsed fractional carbon dioxid (CO_2) laser: Encore, Lumenis, Dreieich, Germany (energy 80 mJ, 100 Hz, 1 pass). During laser irradiation, culture medium was removed. Radiation intensity was kept under equal conditions by fixing the laser head on a tripod.

After treatment, models were cultivated with fresh culture medium and harvested on day 0, 3, 5, or 7 to perform histology and gene expression analysis. An untreated model was maintained as a negative control at given times. All experiments were repeated once for every time point.

Topical Therapy of Skin Lesions

"Injured" 3D skin equivalents were subsequently treated topically with various 5% dexpanthenol containing ointments (Bepanthen® Cream, Bepanthen® Wound Healing Ointment, and Bepanthen® Eye and Nose Ointment from Bayer, Leverkusen, Germany), a 2% fusidic acid containing ointment (Fucidine® Cream, Leo, Neu–Isenburg, Germany) or white vaseline for three days. A second set

of experiments was performed comparing 3D models cultured in medium containing 20 μg/ml calciumpantothenate (Sigma–Aldrich, Missouri) in comparison to cell culture medium without any supplement of calciumpantothenate or dexpanthenol. Therefore, skin models prepared for these experiments were cultured in Derma Life K (Life Line, Frederick, MD) supplemented with 50 mg/ml L-arcorbic acid (Sigma). After lifting the skin equivalents to the air-liquid interface, skin models were continuously cultured in Derma Life K without TGFα. Calcium concentration of the medium was raised to 1.2 mM. 3D models were studied on day 3 and 5 after skin lesion using histological and mRNA expression analysis. Experiments were repeated twice for every time point.

RNA Isolation

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including on-column digestion of DNA with RNase-free DNase I. The RNA was quantified by photometric measurement (NanoDrop Technologies, Wilmington, DE) and its integrity was analyzed on a 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT–PCR)

Purified RNA was reversed transcribed with the SS VILO Mastermix (Life Technologies) according the manufacturer's instructions. TaqMan experiments were carried out on an ABI Prism 7,000 sequence detection system (Applied Biosystems, Weiterstadt, Germany) using Assays-on-Demand gene expression products for human MMP3 (HS00233962_m1), CXCL16 (HS00222859_m1), KRTAP10–3 (HS0418669_gH), IL1α (HS00174092_m1), S100A7 (HS00161488_m1), CXCL1 (HS00236937_m1), KRTAP4–12 (HS00258949_s1), and MMP9 (HS00234579_m1) according to the manufacturer's recommendations. An Assay-on-Demand product for HPRT (Hs99999909) was used as an internal reference to normalize the target transcripts. All measurements were performed in triplicate in separate reaction wells.

Analysis of Gene Expression Using Exon Expression Arrays

Purified mRNA was analyzed on the GeneChip Human Gene 2.0 ST array as reported previously [10]. Expression values of each probe set were determined and laser irradiated samples were compared to laser irradiated samples following dexpanthenol-treatment probes using the Gene-Spring GX 11.0.2 software (Agilent Technologies, Frankfurt am Main, Germany).

Light Microscopy and Immunofluorescence

For light microscopy, 4 μm cryosections of skin equivalents were embedded in Tissue Tec OCT and stained with hematoxylin and eosin. Sections were examined by a photomicroscope (DMIL, Leitz, Wetzlar, Germany). For immunofluorescence, 4 μm cryosections were fixed for 10 minutes in acetone at 4°C. First antibody (Ki67, Dako, Glostrup, Denmark) was diluted with Antibody Diluent (Dako, Carpinteria, CA) and incubated at room temperature for one hour. Following washing steps with PBS, sections were incubated in fluorochrome—conjugated secondary antibody Alexa Fluor 488 IgG H + L (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cell nuclei were stained with DAPI (Applichem, Darmstadt, Germany). After a final washing step, sections were mounted in Fluorescent Mounting Medium (Dako, Glostrup, Denmark) and coverslipped. Sections were stored in the dark at 4°C, examined by a photomicroscope (DMIL, Leitz, Wetzlar, Germany) equipped with epifluorescence illumination and digitally photodocumentated (DISKUS, Hilgers, Königswinter, Germany).

RESULTS

To establish a standardized laser irradiated *in vitro* 3D wound healing model, we developed two different full-thickness human skin equivalents, a collagen skin equivalent and a scaffold skin equivalent. In both full-thickness human skin equivalents we found full developed epidermal and dermal structures with stratum corneum, basal layer, and basal membrane (Fig. S1).

There are different methods carried out to induce lesions in organotypic human skin models. We decided to test punch biopsies, electric cauterization, and irradiation by non-sequential ultrapulsed fractional carbon dioxid (CO_2) laser on our 3D skin equivalents as wound healing models. The skin biopsy models (collagen model, 5d, 3 mm punch biopsy) showed a large ablation of the epidermis and a prolapse of the dermis directly after mechanical injury (Fig. 1a shows a representative model). Thermal lesions were set with a Type E platin–iridium tip for 1 second. The electric cautery model (collagen model, 5d) showed a large thermal injury affecting epidermal and dermal structures directly after burn (Fig. 1b). Respectively, these methods lead to varying skin lesions and are therefore difficult to standardize.

Furthermore, 3D skin equivalents were irradiated with the non-sequential ultrapulsed fractional carbon dioxid (CO_2) laser. Radiation intensity was kept under equal conditions by fixing the laser head on a tripod resulting in multiple standardized skin injuries. The laser irradiation model (collagen model, 5d) showed a clearly defined lesion of the epidermis and dermis directly after injury (Fig. 1c). The dermis was nearly recovered and the basal layer was restored 5 days after laser irradiation. After 7 days, re-epithelization of the human skin model was completed (Fig. 1d and e).

To characterize this model for wound healing and pharmacological approaches, we decided to determine the effects of dexpanthenol on this *in vitro* test system. Laser irradiated 3D scaffold and collagen equivalents (10d) were subsequently treated topically with various 5% dexpanthenol containing ointments for three days. A non-irradiated non-treated (Fig. 2a and S2) and a laser irradiated non-treated (Fig. 2b, S2) 3D skin model were

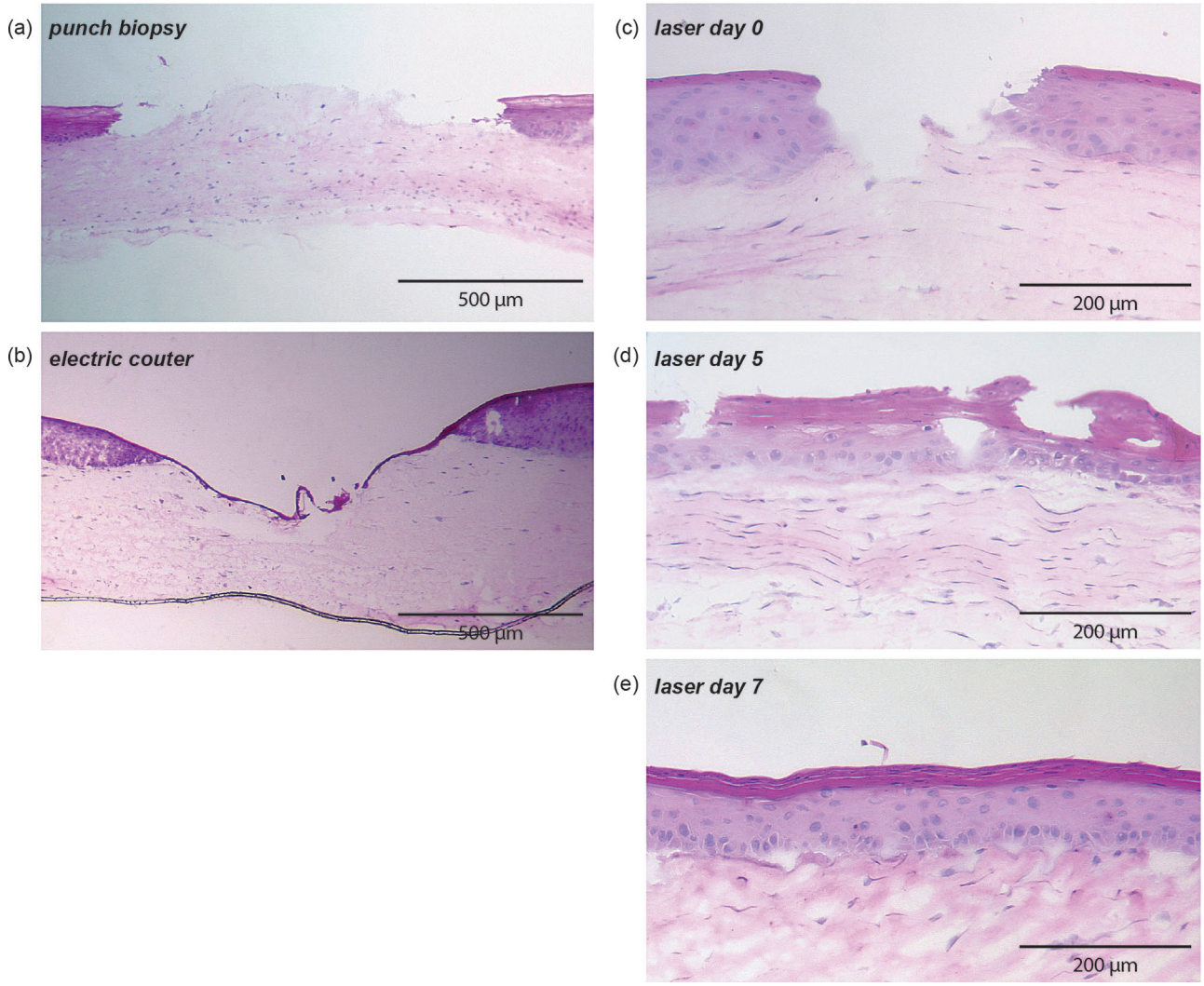

Fig. 1. Skin lesion and laser irradiated 3D skin models (**a**) Skin biopsy models were injured by 3 mm punch biopsies. (**b**) Electric cautery models were injured with low temperature cautery. Cauter was preheated and lesions were set using a Type E platin–iridium tip. This figure shows representative skin lesion models. (**c**–**e**) 3D skin models were irradiated with a non-sequential ultrapulsed fractional carbon dioxid (CO_2) laser. Radiation intensity was kept under equal conditions by fixing the laser head on a tripod. After irradiation, models were cultivated with fresh medium and harvested directly after treatment (day 0), day 5, or day 7.

used as control. Topical treatment with Bepanthen® Cream, Bepanthen® Wound Healing Ointment, and Bepanthen® Eye and Nose Ointment (Fig. 2d and f, S2) showed a clearly enhanced wound closure compared to laser irradiated untreated control models or skin model treated with white vaseline (Fig. 2c) or 2% fusidic acid containing ointment (Fig. S2).

To verify whether this positive effect on wound closure is caused by the active substance dexpanthenol and not only due to the ointment base, we cultured laser irradiated skin models with medium containing calciumpantothenate (20 µg/ml) compared to laser irradiated skin equivalents cultured without any supplement of calciumpantothenate for 3 or 5 days after skin injury (Fig. 3a). Already after 3 days we observed a difference in wound closure. In the skin models cultured with calciumpantothenate, the lesion showed smaller dimension and the epidermis started to close in comparison to the control model without calciumpantothenate. After 5 days it was clearly visible that calciumpantothenate revealed faster wound closure. In the skin model cultured with calciumpantothenate the re-epithelisation was nearly completed whereas the control model still displayed a large skin lesion. Furthermore, immunofluorescence staining revealed that Ki67 protein expression is upregulated in laser irradiated 3D skin equivalents cultured in calciumpantothenate compared to control models 3 days after skin lesion (Fig. 3b).

To confirm these dexpanthenol-mediated stimulatory effects on wound closure, we investigated the influence of calciumpantothenate on gene expression in laser

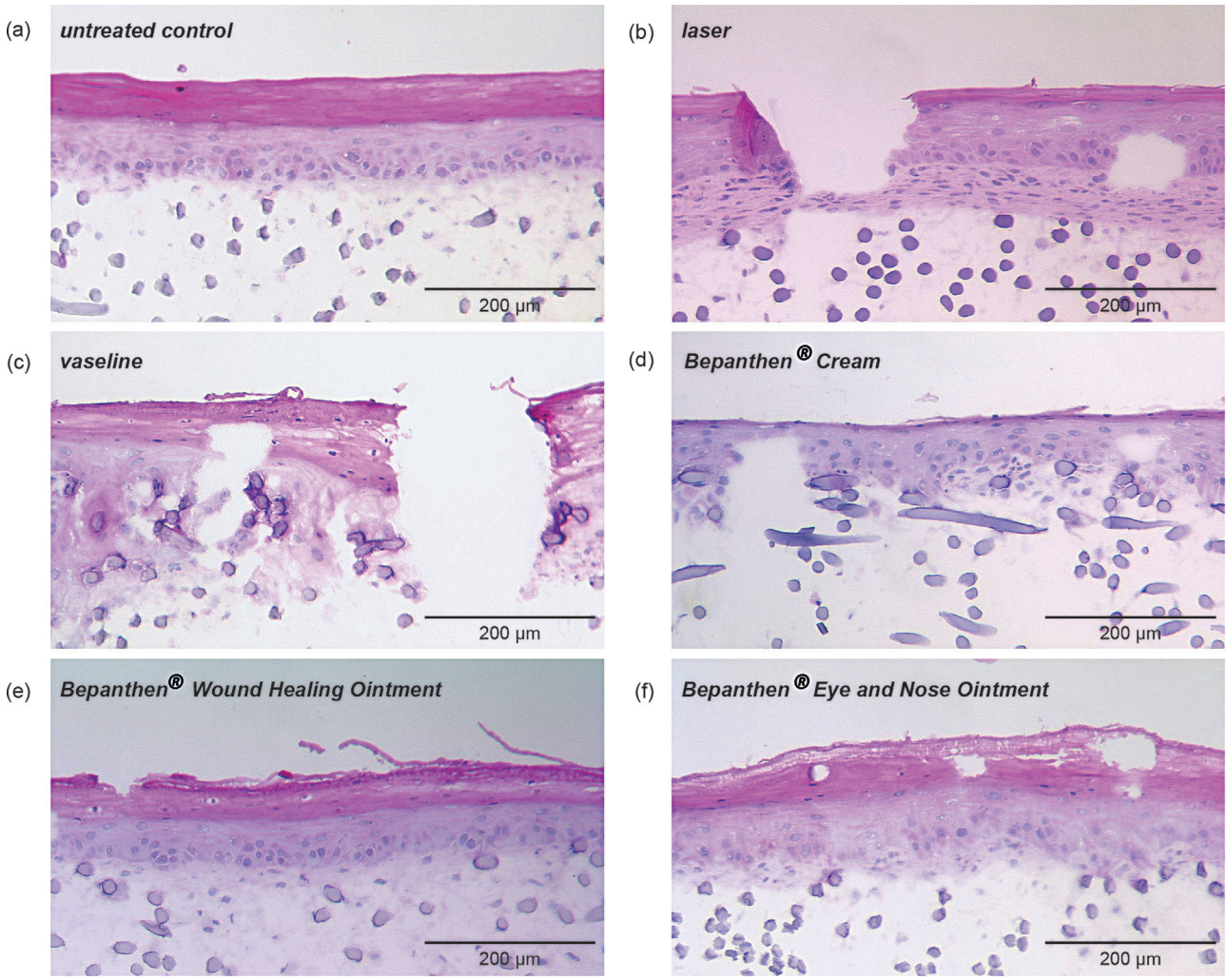

Fig. 2. Topical treatment with dexpanthenol enhanced wound closure in laser irradiated 3D skin models Non-irradiated (**a**) and irradiated non-treated (b) 3D skin models were used as control. Laser irradiated scaffold 3D skin equivalents were subsequently treated topically with white vaseline (**c**) or various 5% dexpanthenol containing ointments: Bepanthen® Cream (**d**), Bepanthen® Wound Healing Ointment (**e**), and Bepanthen® Eye and Nose Ointment (**f**) for 3 days.

irradiated 3D skin models cultured with calciumpantothenate using an Affymetix gene array and quantitative real-time PCR. Gene Array analysis showed a moderate up-regulation of MMP3, CXCL16, KRTAP10–3, IL1α, and S100A7 (Fig. 4a) 5 days after skin injury. These data were confirmed with qRT–PCR (Fig. 4b). Additionally, we observed a moderate increase of mRNA expression of MMP9 up to 147% and high up-regulation of KRTAP4–12 up to 370% after 5 days (Fig. 4b). MMP3, CXCL16, KRTAP10–3, IL1α, CXCL1, and KRTAP4–12 mRNA expression was increased 3 days after skin lesion whereas S100A7 (up to only 8%) and MMP9 (up to 24%) were strongly decreased (Fig. 4b).

DISCUSSION

Wound healing is a dynamic, interactive process that passes in three phases and overlap in time: inflammation, tissue formation, and tissue remodeling [18]. Studies, analyzing wound healing, use mainly mechanical methods for wound induction, which are laborious and difficult to standardize [12]. In the previously published work of Ferraq et al. [12], Er:YAG laser treatment of human skin *in vivo* achieved a rapid standardized epidermal ablation, which was similar in terms of epidermal regeneration and barrier formation to our results. In our experiments, we used a non-sequential fractional ultrapulsed CO_2 laser. Hereby, we were able to set multiple standardized injuries with defined dimensions in human full-thickness 3D skin equivalents, enabling the investigation of wound healing on molecular and histological level. This *ex vivo* laser model using human skin allows the generation of different time courses to analyze the gene regulation both on RNA and protein level at same time points. These time course analysis are due to ethical reasons hardly possible in clinical *in vivo* studies in humans. The induced epidermal wounds were uniform in size and rapidly obtained as

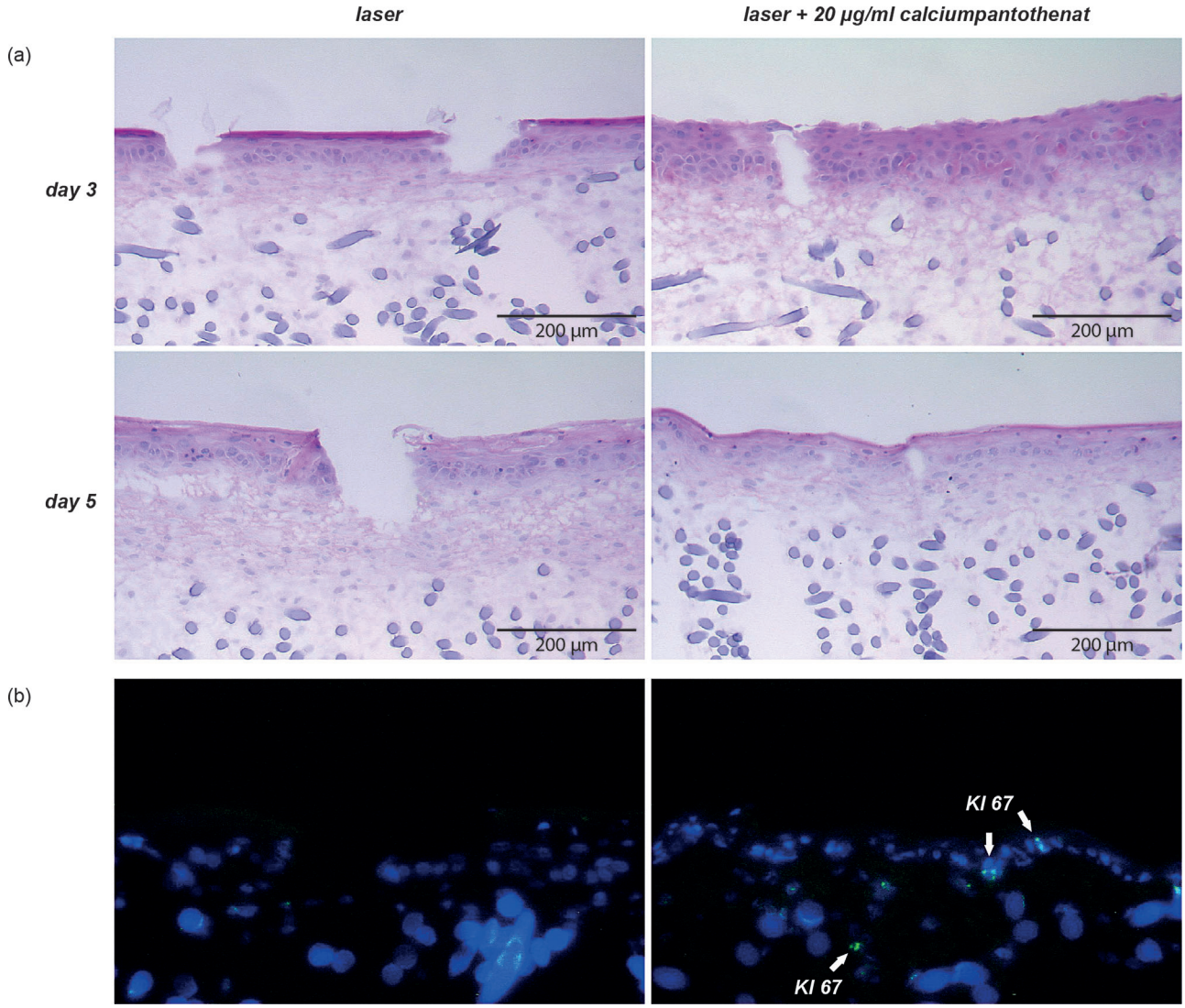

Fig. 3. Calciumpantothenate revealed faster wound closure and Ki67 upregulation in laser irradiated 3D skin models compared to the non-treated irradiated control (**a**) Laser irradiated 3D skin equivalents cultured in calciumpantothenate containing medium (20 µg/ml) for 3 or 5 days after skin lesion were compared to laser irradiated 3D skin equivalents cultured without any supplement of calciumpantothenate. (**b**) Immunofluorescence examination of Ki67 counterstained with DAPI was performed.

compared to the *in vivo* wounds induced by Er:YAG laser treatment [12]. Both human skin models, collagen, and scaffold skin equivalents, were found to be appropriate for *in vitro* wound healing analysis. After one week, re-epithelization of the laser-treated human skin model was completed in both skin model types similar to epidermal regeneration in humans *in vivo* [19]. Therefore, this 3D skin model could also serve as a model to analyze the impact of different laser systems on skin physiology and morphology.

Many manufactures of laser systems recommend topical treatments (e.g., vaseline) after laser therapy to reduce skin inflammation and various clinical laser studies apply petrolatum as control topical therapy [20–22]. We wanted to show that the laser *ex vivo* wound models could be used for pharmacological approaches and to investigate whether topical treatments such as dexpanthenol are more effective than vaseline. Therefore, we treated the laser-injured skin equivalents with different 5% dexpanthenol emulsions. Wound closure was clearly enhanced in both human skin models, collagen and scaffold skin equivalents, as compared to topical treatment with vaseline or the untreated control. Also the addition of calciumpantothenate to the cell culture led to an improved wound healing. In a previously published *in vitro* mono-layer wound healing model, cultured fibroblasts showed increased proliferation after incubation with calciumpantothenate [23]. Consistently, in this study, we observed an increased protein expression of the proliferation marker Ki67 in the calciumpanthothenate treated 3D models. Experiments

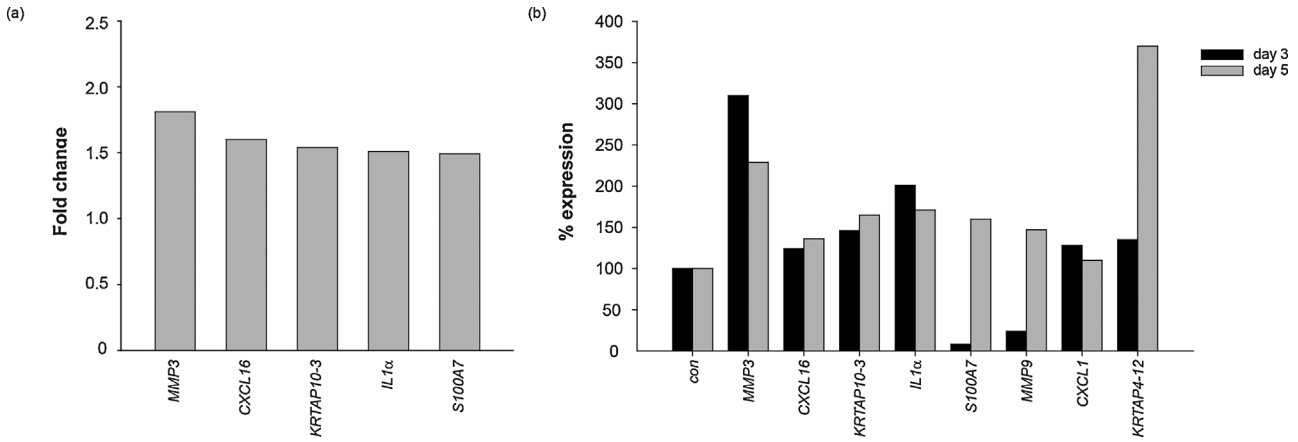

Fig. 4. Calciumpantothenate regulates gene expression in laser irradiated 3D skin equivalents Laser irradiated 3D skin equivalents were cultured in calciumpantothenate containing medium (20 μg/ml) and compared to laser irradiated 3D skin equivalents cultured without any supplement of calciumpantothenate. (**a**) Affymetrix microarray gene analysis. 3D skin models were harvested 5 days after lesions and gene expression was measured using Affymetrix® Gene Chip Human Exon 1.0 ST array. (**b**) Quantitative real-time PCR analysis. 3D skin models were harvested 3 or 5 days after injury and TaqMan real-time PCR analysis was performed.

of other groups revealed an increased fibroblast proliferation rate by calciumpantothenate [24–26]. This novel laser-irradiated 3D wound healing skin model enables standardized histological and RT–PCR studies analyzing the effect of topically or systemically applied compounds on skin wound healing. It also allows examining effects of new compounds on epidermal and dermal structures and on wound healing *in vitro*. These findings suggest that this *in vitro* model is able of analyzing aspects of the complex wound healing process helping us to determine the role of cell types such as dermal fibroblasts or epidermal keratinocytes in wound healing.

The molecular mechanisms resulting in the proliferative effect of pantothenate were previously investigated by global gene expression analysis (microarray analysis) in cultured human dermal fibroblasts [23] and in a clinical trial *in vivo* [10]. Previously, effects of dexpanthenol were monitored on gene expression in different phases of wound healing (24, 72, and 120 hours) after initial wounding. Consistent to our results, *in vivo* data in humans revealed a downregulation of S100 calcium binding protein A7A (S100A7, psoriasin) mRNA and protein in samples treated topically with dexpanthenol [10]. S100A7 is known to function as a transglutaminase substrate/cornified envelope precursor, signal transduction protein, chemokine, and antibacterial epidermal protein [27]. It is highly expressed in wound granulation tissue and is produced by wound-associated keratinocytes [28]. Although S100A7 is constitutively expressed in human skin, it was shown that it is additionally inducible and subsequently detectable in all epidermal layers in chronic wounds [29]. Dexpanthenol reduces cutaneous bacteria recolonization [30]. It was assumed that this decreased colonization leads to down-regulation of antibacterial proteins such as psoriasin [8]. Interestingly, expression of S100A7 was restored 5 days after dexpanthenol treatment in our wound healing models. S100A7 seems to be differently regulated in different phases of wound healing and dexpanthenol exerts its downregulatory effects on S100A7 in early phase of wound healing.

Array data of human skin samples treated with dexpanthenol revealed an enhanced expression of KRTAP4–12 [10]. This is consistent to our results in the scaffold 3D skin models which also showed an enhanced expression after dexpanthenol treatment. Besides, KRTAP10–3 was also upregulated in our experiments. KRTAPs are a major component of the hair fiber. Changes in epithelial keratin expression during healing of rabbit corneal wounds and delayed wound healing in keratin 6a knockout mice have been reported [31,32] suggesting also a putative role of KRTAPs in wound healing.

A function of MMPs, produced by fibroblasts, is to degrade and remove damaged structural extracellular matrix proteins such as collagen [33,34]. Recent evidence suggests that they also influence other wound healing responses, such as inflammation and re-epithelialization. Several studies in healing and non-healing wounds demonstrated alterations in MMP expression. It has been stated that the MMP3 (stromelysin-1) expression decreases in patients with impaired wound healing [35]. Wound healing is slowed in mice lacking MMP3. Targeted disruption of the MMP3 gene in mice caused a delay in excisional wound healing due to a failure in wound contraction [36]. This was not due to altered re-epithelialization as keratinocyte migration was not affected [34]. Previous results indicate that MMP3 is responsible for fibroblast contraction and initiating wound contraction [37]. It was shown that Aloe vera compounds induce MMP3 during skin wound repair in rats [38]. We also observed an up-regulation of MMP3 after dexpanthenol treatment. Consistent to our results are previously published *in vivo* array data showing a 5-fold up-regulation

of MMP3 in human skin treated 3 days with dexpanthenol [10].

Furthermore, a study of Melerzanov et al. (2014) revealed that laser irradiation itself regulates MMP expression [39]. It was shown that expression of MMP1, MMP2, and MMP12 decreased at different time points in monolayers of the keratinocyte cell line HaCaT [39]. Therefore, another explanation for the reduced MMP3 expression in our laser model could be the laser irradiation itself. However, dexpanthenol moderates these effects.

Further, expression of MMP9 has been reported in epidermal cells of acute wounds in rodents [40,41]. A comparative study analyzed the dynamic changes of MMP9 expression in skin wounds of diabetic and nondiabetic rats, and showed that high expression and activity of MMP9 contributed to delayed healing at the late stage of wound healing (after the seventh day) much more than at the early stage (before the third day) [42]. Furthermore, high MMP9 expression level in diabetic wound exudates was predictive of poor wound healing [43,44]. Recently, wound healing was promoted and wound closure was enhanced by downregulation of MMP9 [45]. Consistently, we found that MMP9 was downregulated 3 days after dexpanthenol treatment. However, MMP9 expression was increased 5 days after treatment. We assume that dexpanthenol has positive effects on the regulation of extracellular matrix factor synthesis in early phase of wound healing.

In conclusion, we demonstrated that CO_2 laser treatment is an efficient and rapid method for providing a model of epidermal wounding on human skin equivalents which serve as a standardized *in vitro* model for human wound healing. Topical treatment with different formulations containing 5% dexpanthenol as well as supplementation of cell culture medium with calciumpantothenate enhanced wound closure and modulated gene expression in skin equivalents treated with non-sequential fractional ultrapulsed CO_2 laser. This novel 3D wound healing skin model system is useful to find biomarkers for wound healing in skin as well as to investigate cellular events associated with abnormal wound healing (e.g., chronic, non-healing wounds). Data about gene regulation on both RNA and protein level at different time points are easy to gain. It allows reliable data and can be applied for comparative studies analyzing the effect of topically or systemically applied compounds on wound healing hereby avoiding animal experiments or clinical trials in humans. In addition, this novel 3D model system can be utilized in future to monitor *ex vivo* effects of various laser systems on skin physiology, morphology, and gene expression.

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