Skin Engineering Lab

SOCIAL PURPOSE REPORT

2014 – 2024

La Colline

Swiss Riviera Beauty Treatment

E D I T O

Because the skin is an essential organ, being both a protector of our physical integrity and a vessel of our identity. Because the skin is what connects us to the world. Because it contributes to our well-being.

We are committed to carrying out research to help patients who have lost all or part of their skin recover and return to a normal life. Carried out by our La Colline Skin Engineering Laboratory, at the heart of the Irchel Campus of the University of Zurich, our program aims to expedite and improve the quality of skin grafting techniques.

This is our social purpose.

INDEX

1.

BACKGROUND

History of the Project

By M. Ghislain Pfersdorff, CEO.

The Skin Engineering Platform was founded in spring 2014 following a meeting between representative of La Colline and of the Division of Plastic Surgery and Hand Surgery of the University Hospital Zurich end of 2013. La Colline was looking for a research project in the field of skin engineering and/or reconstruction with a high social value and a realistic chance to be translated in a short time to a clinical application for its social purpose. The sponsoring contract was prepared by Unitectra for the University of Zurich and the legal office of La Colline with the main goal to sponsor a PhD Fellowship in the domain of Skin Engineering and Regeneration. Thank to this generous grant we could start the research and development activities in our skin engineering research group in October 2014 (Fig. 1). The primary endpoint was defined as the process establishment for the production of keratinocytes for burn patients (cultivated epidermal autografts, CEA) at the University Hospital Zurich.

As one of the first actions involving La Colline, a scientific fellowship for a young researcher, specialized in skin regeneration, was established. Our “La Colline Fellowship” was integrated in the reconstructive medicine department of the University Hospital Zurich in partnership with the Institute of regenerative Medicine (IREM) of the University Zurich with Dr Laura Frese.

Parallel to this task a research programme was launched to accompany the clinical work and to look for possible improvements and developments (process optimization). These experiences and studies would also become the cornerstone of the development of other tissue engineered tissue constructs for wound treatment and skin coverage.

Because of the La Colline Fellowship Grant and the successful research program, further funds from different private and public institutions could be raised. This is a perfect illustration of the serendipity created by our committment to our social purpose : it makes it easier for others to join, otherwise afraid of the risks of leadership and initiatives or constrained by their statutes. The project grew successfully, so that the fellowship program was extended. A new milestone was be reached in 2018 with the creation of the "La Colline Skin Engineering Lab". The lab was embedded in the laboratory premises of the Competence Center for Applied Biotechnology and Molecular Medicine (CABMM) on the Irchel campus of the University of Zurich.

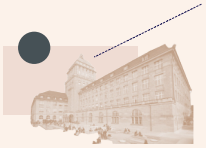
With these committments, Dr Frese and Dr Calcagni were able to optimize the manufacturing process and to develop a frozen protocol with a defined and standardised quality control. In 2023, the established research protocols were being translated into standardized protocols according to good manufacturing practise (GMP) so that they meet the regulatory requirements for a clinical phase I study.

2013

1ST MEETING

G. Pfersdorff &

Pr. Calcagni Pr. Horstrup Pr. Giovanolli



SKIN ENGINEERING RESEARCH GROUP

Universität Zürich[UZH]

12/02/2014 : First Innovation Committee
04/06/2014 : Second Innovation Committee
17/09/2014 : Third Innovation Committee
18/12/2014 : Fourth Innovation Committee

2014

1ST FELLOWSHIP PROGRAMME

with Dr. Frese

La Colline

INNOVATION POOL

Universitäts Spital Zürich

2015

PROCESS ESTABLISHMENT

NATIONAL FUNDING

04/03/2015 : Fifth Innovation Committee
24/06/2015 : Sicth Innovation Committee

2018

2ND FELLOWSHIP PROGRAMME

with Dr. Frese

La Colline

Skin Engineering Lab

The Skin Engineering Lab is founded within the CABMM @the Irchel Campus.

MOVEMENT TO CABMM PLATFORM

cabmm

2020

PROCESS OPTIMIZATION

3RD FELLOWSHIP PROGRAMME

with Dr. Frese

La Colline

2021

QUALITY CONTROL

FROZEN PROTOCOL

SCIENTIFIC ADVICE MEETING

swissmedic

2022

TRANSLATION TO GMP-STANDARDS

PREPARING PROTOCOLS FOR PHASE I STUDY

2023

4TH FELLOWSHIP PROGRAMME

with Dr. Frese

La Colline

Fig. 1: History of project development

Clinical Background

By Pr. Maurizio Calcagni

The skin provides an invaluable protective barrier for the human body. However, if the skin is seriously injured, e.g. by extensive burns or wound healing disorders (e.g. chronically open wounds), the patients suffer from the dramatic loss of their epidermal and dermal skin layers. The skin's natural ability to regenerate is no longer able to repair the defective skin. In addition, in severe burn patients, remaining unaffected skin areas are often not sufficient to provide an autograft coverage. For the treatment of this skin damage, cultivated patient-specific keratinocyte grafts have been clinically used since the 1970s. These can be produced in vitro from the patient's own cells (autologous) in order to avoid rejection reactions. An approx. 4cm² skin biopsy of the patient is used as the starting material for the production of these cell-based transplants. Keratinocytes can be isolated from this skin biopsy, which after successful expansion are transplanted to the patient in order to repair the defective skin layer (Fig. 2).

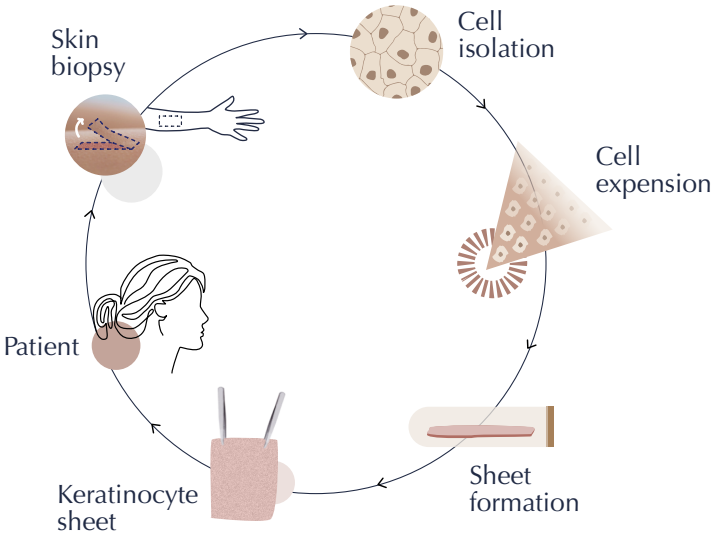


Fig. 2: Schematic representation of the keratinocyte graft production: After skin biopsy the epidermal cells are isolated and expanded. After reaching the required number of cells, the graft maturation follows, forming a multi cell layer. Thereafter the CEA can be harvested and transplanted onto the patient.

However, despite significant developments in recent years, successful and sustainable treatment, especially for deep and infected wounds, is still a challenge. In order to improve the clinical outcome, reliable, high-quality grafts with faster availability and a flexible time window for the transplant are required. In order to develop these new therapies, intensive basic scientific research is essential.

The main aim of the skin engineering group is to produce reliable, safe and traceable autologous keratinocyte sheets (cultivated epidermal autograft, CEA) to treat patients, all the while providing the surgeons with a window of flexibility in order to optimize the grafting date to the health of the patient. After establishing this optimized keratinocyte sheet production, we aim to translate and implement the protocol in accordance with the required necessary regulatory framework to treat severe burn patients with state-of-the-art keratinocyte sheets in the future

1.

BACKGROUND

Team

By Pr. Maurizio Calcagni and La Colline CEO M. Ghislain Pfersdorff

The team was built progressively and more collaborators joined the group as needed. Dr Salim Darwiche PhD was the first one I choose with the function of Project Manager. Together we choose Dr Laura Frese as La Colline Fellow and in charge of the laboratory work.

Dr Salim Darwiche is a bioengineer with extensive experience in tissue engineering and management of major, multicentre collaborations. Beside his collaboration in this project, he is Senior Scientist and Study Director at the Musculoskeletal Research Unit and Scientific Coordinator of the Centre for Applied Biotechnologies and Molecular Medicine of the Veterinary Faculty, University of Zurich.

Dr Laura Frese PhD is also a bioengineer with extensive experience with tissue culture in different fields. She is in charge in the laboratory for all the experiments and participate to the development and planning of future research. Her work is supported by a part-time lab technician.

Since May 2016, the project has been supported by regulatory experts as needed. They are central for the preparation of all the documents necessary for the regulatory institutions and the start of the clinical trial:

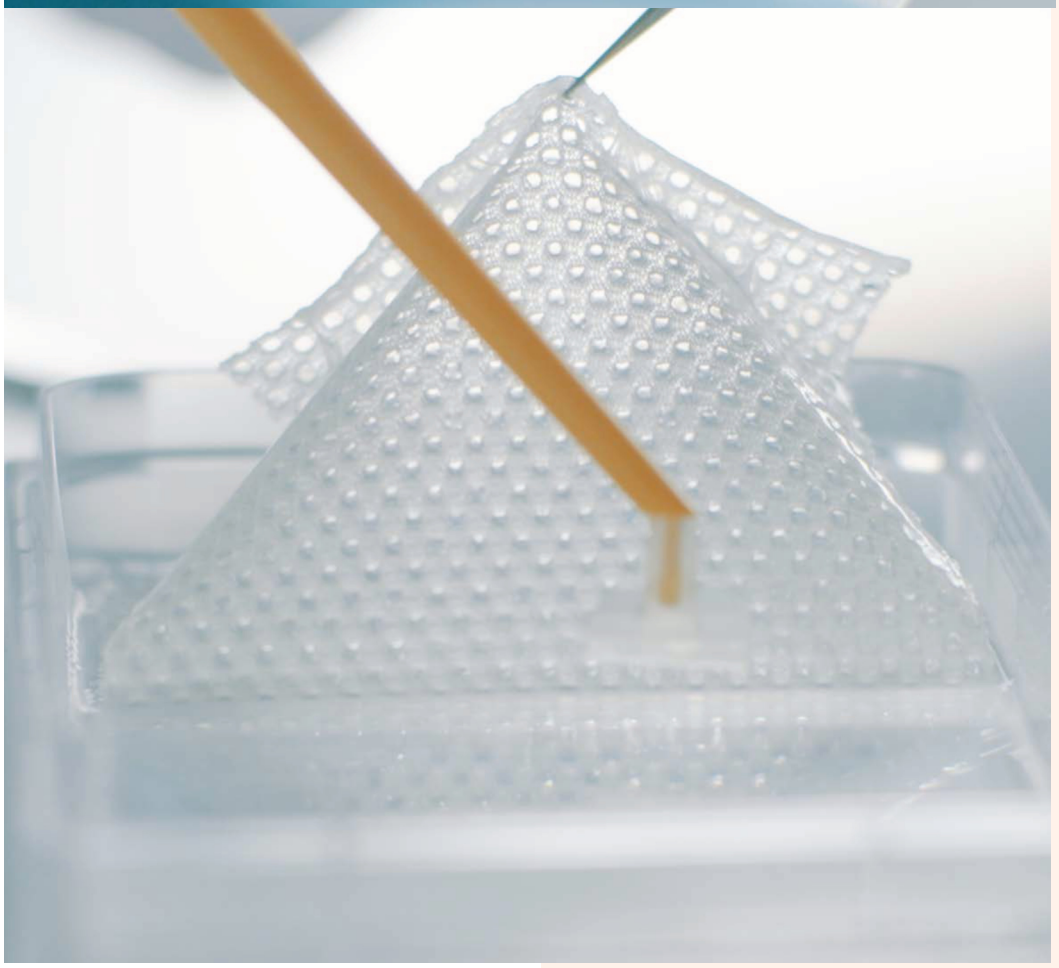
• 05/2016 - 01/2017: Dr Carrie Brubaker PhD. She is a bioengineer with additional training in project management in research and development with special expertise in regulatory matters in complex clinical trials.

• 01/2018 - 12/2018: Myrna Gunning. She is veterinarian and, with her expertise as a clinical research specialist, supported the team in creating the necessary regulatory documents.

• 10/2017 – 09/2019: Karolina Pal Kutas. She is a toxicologist and her responsibilities included drafting the standard operating procedures (SOP) in compliance with GMP.

• 02/2022 – now: Dr med Claudia Guebelin has taken on the task of supporting the team in creating the necessary documents for the regulatory authorities as a freelancer.

Enzymatic harvest of keratinocyte transplant: In the final step of the manufacturing process, the keratinocyte transplants are enzymatically detached from the culture dish before being transported to the operating theatre



For stable transport and better handling during transplantation, the keratinocyte transplants must be fixed on suitable carriers (here: Mepitel®, consisting of a flexible polyamide mesh coated with soft silicone)

2.

SCIENTIFIC ACHIEVEMENTS

Overview

By Pr. Maurizio Calcagni and Dr. Laura Frese, La Colline Fellow

The La Colline fellowship started in October 2014. Main goal of the project was the establishment of keratinocyte cultures at the University Hospital in Zurich for the treatment of patients with severe burn. There were secondary goals and in particular the improvement of some parameters of this process: reduce the time of culture and exclude all risky animal components (xeno-free). The last step was the translation from bench-to-bed for the treatment of burnt patients in our Intensive Care Unit for burns.

Within the first two years of the project, our team established a method to produce cultured autologous keratinocyte grafts, also called keratinocyte sheets, from human skin biopsies. The exclusion of animal components such as bovine serum or murine feeder cells, which are potential disease transmitters, was also successful. The methodology has been reliable in over 150 biopsies processed to date. Furthermore, we have established a protocol to reduce the culture time of about four days compared to other similar protocols. We are able to produce a live graft as early as 18 days after biopsy harvest, which is stable and can therefore be transplanted for the following 7 days (day 25 after biopsy harvest). This, we anticipate, would give surgeons a much wider window to graft their patients.

Parallel to this activity we explored also the influence of temperature on the cultures and the possibility to perform cell function tests through proteomic analysis. These studies are not only interesting for the improvement of the cell culture, but are also important for the quality control of theclinical work. In particular, the proteomic essays will eventually allow for non-invasive (no cell loss) analysis of cell viability and activity. With these achievements we can produce keratinocyte sheets of maximal quality and without risky xenogenic components. Currently we are working on the translation of our production protocol into the standards of GMP. We have incorporated our scientific achievements into the manufacturing process of the keratinocyte sheets. In this way, we managed to improve and optimize the keratinocyte sheet manufacturing protocol in terms of safety and efficacy.

With the achievement of these major milestones, we were able to hold a Scientific Advice Meeting in October 2021 with the regulatory authority in Switzerland, Swissmedic.

Crucial points in our manufacturing process have been discussed to ensure the safety and reliability of manufacture. We started the clinical translation through the production of all the documents necessary for the regulatory agencies (Swissmedic and the Regional Ethical Committee) approval. Furthermore, we are interfacing with the clinical team of the burn facility of the Plastic Surgery Department in the University Hospital of Zurich in order to build the clinical trial protocol. We have identified key items for the clinical trial design, such as adverse event anticipation and reporting, Clinical trial criteria of inclusion and exclusion, procedure of consent an. d patient enrollment as well as primary and secondary outcomes from this unblended Phase I clinical safety trial.

2.

SCIENTIFIC ACHIEVEMENTS

Publications

By Pr. Maurizio Calcagni and Dr. Laura Frese, La Colline Fellow

The scientific knowledge gained in this project to optimize keratinocyte transplants has been successfully published in two peer-reviewed journals and is available to the scientific community.

- *Optimizing large-scale autologous human keratinocyte sheets for major burns - Toward an animal-free production and a more accessible clinical application*

BACKGROUND & AIMS

Autologous keratinocyte sheets constitute an important component of the burn wound treatment toolbox available to a surgeon and can be considered a life-saving procedure for patients with severe burns over 50% of their total body surface area. Large-scale keratinocyte sheet cultivation still fundamentally relies on the use of animal components such as inactivated murine 3T3 fibroblasts as feeders, animal-derived enzymes such as trypsin, as well as media components such as fetal bovine serum (FBS). This study was therefore aimed to optimize autologous keratinocyte sheets by comparing various alternatives to critical components in their production.

METHODS

Human skin samples were retrieved from remnant operative tissues. Cell isolation efficiency and viability were investigated by comparing the efficacy of porcine-derived trypsin and animal-free enzymes (Accutase and TrypLESelect). The subsequent expansion of the cells and the keratinocyte sheet formation was analyzed, comparing various cell culture substrates (inactivated murine 3T3 fibroblasts, inactivated human fibroblasts, Collagen I or plain tissue culture plastic), as well as media containing serum or chemically defined animal-free media.

RESULTS

The cell isolation step showed clear cell yield advantages when using porcine-derived trypsin, compared to animal-free alternatives. The keratinocyte sheets produced using animal-free serum were similar to those produced using 3T3 feeder layer and FBS-containing medium, particularly in mechanical integrity as all grafts were liftable. In addition, sheets grown on collagen in an animal-free medium showed indications of advantages in homogeneity, speed, reduced variability, and differentiation status compared to the other growth conditions investigated. Most importantly, the procedure was compatible with the up-scaling requirements of major burn wound treatments.

CONCLUSION

This study demonstrated that animal-free components could be used successfully to reduce the risk profile of large-scale autologous keratinocyte sheet production, and thereby increase clinical accessibility.

• *Thermal conditioning improves quality and speed of keratinocyte sheet production for burn wound treatment*

BACKGROUND & AIMS

Cultured patient-specific keratinocyte sheets have been used clinically since the 1970s for the treatment of large severe burns. However, despite significant developments in recent years, successful and sustainable treatment is still a challenge. Reliable, high-quality grafts with faster availability and a flexible time window for transplantation are required to improve clinical outcomes.

METHODS

Keratinocytes are usually grown in vitro at 37°C. Given the large temperature differences in native skin tissue, the aim of the authors' study was to investigate thermal conditioning of keratinocyte sheet production. Therefore, the influence of 31°C, 33°C and 37°C on cell expansion and differentiation in terms of proliferation and sheet formation efficacy was investigated. In addition, the thermal effect on the biological status and thus the quality of the graft was assessed on the basis of the release of wound healing-related biofactors in various stages of graft development.

RESULTS

The authors demonstrated that temperature is a decisive factor in the production of human keratinocyte sheets. By using specific temperature ranges, the authors have succeeded in optimizing the individual manufacturing steps. During the cell expansion phase, cultivation at 37°C was most effective. After 6 days of culture at 37°C, three times and six times higher numbers of viable cells were obtained compared with 33°C and 31°C. During the cell differentiation and sheet formation phase, however, the cells benefited from a mildly hypothermic temperature of 33°C. Keratinocytes showed increased differentiation potential and formed better epidermal structures, which led to faster biomechanical sheet stability at day 18. In addition, a cultivation temperature of 33°C resulted in a longer lasting and higher secretion of the investigated immunomodulatory, anti-inflammatory, angiogenic and pro-inflammatory biofactors.

CONCLUSION

These results show that by using specific temperature ranges, it is possible to accelerate the large-scale production of cultivated keratinocyte sheets while at the same time improving quality. Cultivated keratinocyte sheets are available as early as 18 days post-biopsy and at any time for 7 days thereafter, which increases the flexibility of the process for surgeons and patients alike. These findings will help to provide better clinical outcomes, with an increased take rate in severe burn patients.

3.

FUNDING

The generous grant offered by La Colline was fundamental to launch the project by funding a PhD Fellowship. We could select Dr Laura Frese PhD as Fellow for the laboratory work and start the development. The decision to employ a post-doc was strategic to have a jump-start of the scientific activities and reach as fast as possible good results. Her expertise led to immediate establishment of the cell cultures and the fast development of the keratinocyte sheets.

The La Colline Fellowship Grant was crucial to trigger other funds from different private and public institutions (Fig. 3). The University Hospital decided to support this project with a contribution of the InnovationsPool of 722'000 CHF for two years with the option to extend it to three years. This contribution financed the acquisition of instruments, the development of production processes and the bench-to-bed part of the project with all the validation and pre-clinical studies until the start of the production. Moreover, it was fundamental for the preparation of all the GMP documents for the regulatory institutions (SwissMedic and the Regional Ethic Committee).

The two studies on temperature and proteomics essays that required very expensive consumables, were submitted to some private Foundations for additional private funding and we could raise a total amount of 45'000 CHF. The funds raised by the Propter Homines Foundation, Georg and Bertha Schwyzer-Winiker Foundation, Evi Diethelm-Winteler Foundation and the Foundation for Scientific and Technical Research (SFNTF) will be used to implement the clinical study phase I. Figure 3 summarize the funds granted to this project and the distribution of the expenses:

Period	Institution	Total CHF	Personnel	Consumables / Laboratory
2014-2017	1st Fellowship La Colline	210'000	192'000	18'000
2014-2020	Innovationspool University Hospital Zurich "Etablierung der Herstellung von Keratinozyten-Sheets"	722'000	80'000	642'000
2015	Karitative Stiftung Gerberten "Investigating the effect of hypo-thermal conditioning on the quality and growth potential of in vitro cultured keratinocytes for skin grafting"	25'000	-	25'000
2016	Heubergstiftung "Proteomic investigation in keratinocytes culture"	20'000	-	20'000
2018-2020	2nd Fellowship La Colline	270'000	207'000	63'000
2020	Stiftung Propter Homines «Haut-zellen für Brandverletzte»	75'000	-	75'000
2021	Karitativ Stiftung Georg und Bertha Schwyzer-Winiker Stiftung	200'000	-	200'000
2021	Karitative Stiftung Evi Diethelm-Winteler	150'000	-	150'000
2021	Stiftung für Naturwissenschaftliche und Technische Forschung (SFNTF)	250'000	-	250'000
2021-2023	3rd Fellowship La Colline	292'000	229'000	63'000

Fig. 3. Summary of the funds granted to the project "Establishment of keratinocytes culture"

4.

OUTLOOK

With the achievement of the project goal, the GMP translation and implementation of our keratinocyte sheet production method in accordance with swiss regulatory framework, a clinical trial phase I will be the next step. This study primarily seeks to determine the effect of Kerasheet on re-epithelialization of burned skin areas that have undergone grafting with keratinocytes in patients with extensive burns.

Furthermore, the expansion of the range of autologous keratinocyte products from our own production within the University of Zurich and the University Hospital Zurich will have an impact on a larger population of patients needing wound-healing enhancement.

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RESEARCH ARTICLE

Optimizing large-scale autologous human keratinocyte sheets for major burns—Toward an animal-free production and a more accessible clinical application

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Abstract

Background and Aims: Autologous keratinocyte sheets constitute an important component of the burn wound treatment toolbox available to a surgeon and can be considered a life-saving procedure for patients with severe burns over 50% of their total body surface area. Large-scale keratinocyte sheet cultivation still fundamentally relies on the use of animal components such as inactivated murine 3T3 fibroblasts as feeders, animal-derived enzymes such as trypsin, as well as media components such as fetal bovine serum (FBS). This study was therefore aimed to optimize autologous keratinocyte sheets by comparing various alternatives to critical components in their production.

Methods: Human skin samples were retrieved from remnant operative tissues. Cell isolation efficiency and viability were investigated by comparing the efficacy of porcine-derived trypsin and animal-free enzymes (Accutase and TrypLESelect). The subsequent expansion of the cells and the keratinocyte sheet formation was analyzed, comparing various cell culture substrates (inactivated murine 3T3 fibroblasts, inactivated human fibroblasts, Collagen I or plain tissue culture plastic), as well as media containing serum or chemically defined animal-free media.

Results: The cell isolation step showed clear cell yield advantages when using porcine-derived trypsin, compared to animal-free alternatives. The keratinocyte sheets produced using animal-free serum were similar to those produced using 3T3 feeder layer and FBS-containing medium, particularly in mechanical integrity as all grafts were liftable. In addition, sheets grown on collagen in an animal-free medium showed indications of advantages in homogeneity, speed, reduced variability, and differentiation status compared to the other growth conditions investigated. Most

Laura Frese and Salim Elias Darwiche first authorship.

importantly, the procedure was compatible with the up-scaling requirements of major burn wound treatments.

Conclusion: This study demonstrated that animal-free components could be used successfully to reduce the risk profile of large-scale autologous keratinocyte sheet production, and thereby increase clinical accessibility.

KEYWORDS

autograft, epidermal skin engineering, keratinocytes, process development, xenofree

1 | INTRODUCTION

Autologous keratinocyte sheets constitute an important component of the burn wound treatment toolbox available to a surgeon. They can be used to promote wound closure and re-establish epidermal barrier protection. That is why the use of autologous keratinocyte sheets, grown from a patient's own skin cells, has been successfully applied for many years and is widely considered a life-saving procedure.[1-3] This becomes particularly important in patients with severe burns over 50% of their total body surface area (TBSA),[3] in which the use of split-thickness skin grafts is simply not possible, despite it being a gold-standard in skin reconstruction.[4,5] Indeed, any autologous skin cell therapy would require harvesting a sample of intact skin, which is in short supply in these cases.

With a reported average TBSA of 1.88 m^2 in male patients and 1.66 m^2 in female patients,[6] at least 1 m^2 of epidermal coverage would have to be restored as soon as possible in these polytraumatized major burn patients (>50% affected TBSA, with second- or third-degree burns). The challenge of upscaling epidermal coverage, starting from a 4 cm^2 skin biopsy to produce sheets covering 1 m^2 of TBSA, is significant. The technology of autologous keratinocyte sheet creation has relied on the methodology first described by J. Rheinwald and H. Green in the 1970s.[7,8] This method, while adapted over the years and used in a variety of clinical studies,[3,9-11] still fundamentally relies on the use of animal components such as inactivated murine 3T3 fibroblasts as feeders, animal-derived enzymes such as trypsin, as well as media components such as fetal bovine serum (FBS).

The murine 3T3 feeder cells, used to enhance cell proliferation,[12] must be inactivated using arresting mutagenic agents such as gamma irradiation or Mitomycin-C[13,14] and can risk introducing remnants of murine components in the transplanted human sheets.[15] Trypsin, used to isolate and dissociate cells, is extracted from the porcine pancreas, which carries the risk of contamination with adventitious agents, such as certain viruses that are widespread among pigs.[16] FBS can also be problematic, potentially carrying bovine spongiform encephalopathy (BSE) and animal viruses that could cause disease transmission.[17] The use of FBS requires extensive and expensive testing to ensure the clinical-grade serum is used and to secure against batch-to-batch variability in efficacy.[18]

Many advances in the field of cell culture have brought alternatives to the use of trypsin,[19] as well as chemically defined, serum-free media alternatives that would not require the use of murine feeder layers.[20-23] In fact, protein coatings such as Collagen IV have also been used as substrates to promote keratinocyte proliferation,[24] but the scalability of such systems has not been shown. Establishing a method of cultivating autologous keratinocyte sheets, while ensuring scalability, as well as an improved safety profile, would make autologous keratinocyte sheets much more economically accessible to burn units worldwide.

This study was therefore aimed to optimize autologous keratinocyte sheets by comparing various alternatives to critical components in their production. Specifically, cell isolation efficiency and viability (Figure 1, phase A) was investigated by comparing various enzymes (animal-derived and animal-free origins). The subsequent expansion of the cells and the keratinocyte sheet formation (Figure 1, phase B and C) was investigated, comparing various cell culture substrates, as well as media containing animal-derived components or chemically defined media. The ultimate goal of the comparative study was to reduce the risk profile of large-scale autologous keratinocyte sheet production to increase clinical accessibility.

2 | MATERIAL AND METHODS

2.1 | Source of human keratinocytes

For the present study, human keratinocytes were isolated from skin biopsies of patients undergoing plastic surgery ($n_{total} = 29$) at the Division of Plastic Surgery and Hand Surgery of the University Hospital Zürich, Switzerland. The skin tissue was obtained by full-thickness ($n = 5$) or split-thickness ($n = 24$) collection from female patients of 42.4 ± 12.1 years of age. Samples were retrieved from remnant operative tissues from the breast or abdomen region, which would otherwise be discarded. This was done following procedures approved by the local ethics committee (KEK-ZH-2014-0197). The assays described below were performed using a subpopulation of samples from the 29 total biopsies harvested for this study. For each assay, the subpopulation of biopsy samples used is specified.

2.2 | Enzymatic isolation of keratinocytes

Samples from 5 female donors, 41.7 ± 13.9 years of age, were used to test three enzymes for the isolation of keratinocytes. The skin was microdissected in equal pieces of approx. 10 × 10mm and digested with dispase solution (12 U/mL PBS) for 15–18 hours at 4°C, as previously described.[25] Thereafter, the epidermis and dermis were mechanically

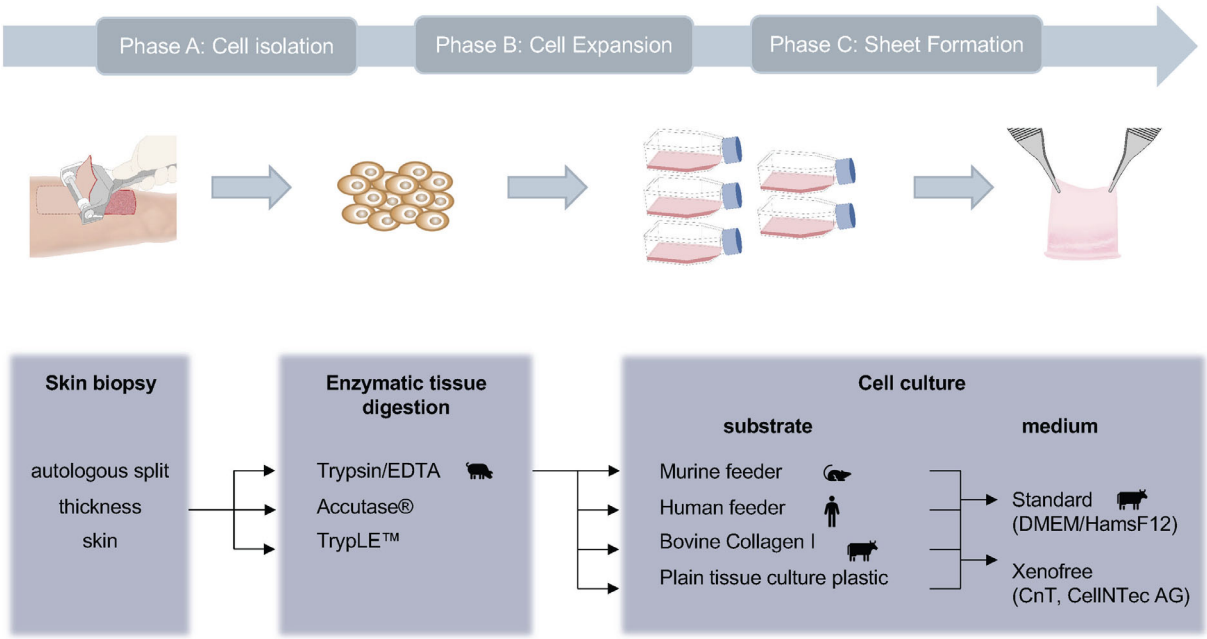

FIGURE 1 Study design. After human skin biopsy collection, various animal-derived and xenofree enzymes were investigated to obtain their best yield and the highest cell isolation efficiency (Phase A). For the expansion of the cells, as well as for the sheet formation (Phase B and C), various cell culture substrates, as well as media, were investigated to produce autologous keratinocyte sheets with mechanical and structural integrity. (Pictures C. De Simio)

separated. To investigate the use of xeno-free enzymatic alternatives compared to the commonly used porcine trypsin, StemPro Accutase 1 × (Gibco), and TrypLE Select Enzyme 1 × (Gibco) were tested in parallel to trypsin/EDTA (Gibco). Therefore, the epidermal layer was separated into three equal pieces and digested with trypsin/EDTA 5 × (0.25% Trypsin, 0.1% EDTA), Accutase, or TrypLE for 25 minutes at 37°C to get the epidermal cell fraction. With Accutase and TrypLE, the cell isolation efficiency was also tested with an extended incubation time of 60 minutes. In order to halt the digestion with trypsin/EDTA, a 3.75 mg/mL soybean trypsin inhibitor was added (1:1). In the case of Accutase or TrypLE, the enzymes were simply diluted with PBS (1:1). Acridine orange was used to stain the entire cell population, and 4′,6-Diamidin-2-phenylindol (DAPI) was used to specifically stain the DNA of dead cells. The number of isolated cells, as well as their viability, was determined using a cell counter (NucleoCounter NC-200, Chemometec).

2.3 | Keratinocyte 2D culture substrates

Murine 3T3 feeder cells (generously provided by Prof. Dr. Karl Frei, University Hospital Zurich) were inactivated with 10 μg/mL Mitomycin C (Sigma) for 2 hours at 37°C. Thereafter, the inactivated 3T3 were plated at 5,000 cells/ cm^2 in order to form a murine feeder layer for keratinocyte culture. In parallel, as allogenic human feeder cells, human foreskin fibroblasts (HFF, ATCC SCRC-1041) were tested in order to avoid the use of xenogenic feeder cells. The HFF were also inactivated using Mitomycin C, and thereafter, seeded with 5,000/cm^2, consistent with the murine ones (3T3). The use of the extracellular matrix component Collagen I was also examined instead of feeder cells as a substrate to avoid the risks associated with xenogeneic and allogeneic cell co-cultures. To that end, clinical-grade bovine Collagen I (Symatese) was used in a concentration of 6 μg/cm^2 to coat tissue culture plastic prior to keratinocyte seeding. While the source of Collagen was bovine, the processing ensures that Collagen batches are free of agents that could transmit spongiform encephalopathies. The fourth and last condition tested was plain tissue culture polystyrene without any coating or feeder cell layer.

For this assay, enzymatically isolated epidermal cells keratinocyte cells from biopsies of 18 female patients (age 42.3 ± 11.5 years) were then plated at a density of 30,000 viable cells/cm^2 on the four substrates, namely a feeder layer of inactivated murine cells (3T3), a feeder layer of inactivated human fibroblasts (HFF), Collagen I and plain tissue culture plastic (T75, EasYFlasks, Nunc). Cells of all conditions were incubated at 37°C in a humidified atmosphere (5% CO_2).

2.4 | Keratinocyte culture media

With regard to xenogenic medium supplements such as, for example, bovine serum, the culture medium has to be optimized in terms of safety and reliability. In this study, a standard keratinocyte medium, adapted from Rheinwald and Green,[8] was utilized as a benchmark. The standard medium, used in the current production facility, was composed of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (3:1), supplemented with 10% fetal bovine serum (Gibco, cat.No.10270), 0.5 μg/mL hydrocortisone (Solu-Cortef, Pfizer), 9.8 ng/mL cholera enterotoxin (Sigma), 42 ng/mL epidermal growth

factor (Sigma), 7.5 μg/mL insulin (Sigma), and 1 mM glutamine (Gibco). This medium also contained antibiotic supplementation of 100 IU/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), and 24 μg/mL gentamicin (Sigma). In contrast, a xenofree medium was also investigated, namely the chemically defined and xenofree CnT-Pr medium (CELLnTEC advanced cell systems AG). According to the manufacturer, the formulation elements for the expansion and differentiation of the human keratinocytes include 21 amino acids, 19 minerals and trace elements, including selenium, manganese, and zinc, 14 vitamins, including folate, niacinamide, and biotin, glucose, pyruvate, and buffers. Furthermore, up to six additional recombinant growth factors and cofactors, including insulin, hydrocortisone, EGF, and FGF, are supplements of the medium (two additional growth factors were considered CellNTec trade secrets and were not disclosed to the authors). The medium does not contain serum, cholera toxin, or phenol red.

Both media were changed every 2 to 3 days following keratinocyte seeding, until cells reached 80% to 90% confluency. The influence of medium and substrate regarding cell morphology and growth behavior was observed using an inverted light microscope (ZEISS Axiovert 40 CFL and ZEISS Axioplan II; Carl Zeiss AG). Additionally, the days required until confluence was reached were recorded for both media used and with all four culture substrates (3T3, HFF, Collagen, plain).

2.5 | Keratinocyte sheet formation

For this assay, cells from biopsies of 6 female patients (age 39.2 ± 9.7 years) were isolated using trypsin/EDTA digestion method and brought to confluence in a primary expansion. Then keratinocyte sheet formation was initiated after cell passaging (Passage 1) by plating the cells at a density of 5,000 viable cells/cm^2 on the four different coatings (3T3, HFF, Collagen, plain) in T115 peel off cell culture flasks (TPP), using standard and xenofree media in parallel. When the cells reached 80% to 90% confluency after 3 to 5 days, the keratinocyte differentiation was initiated by adding the appropriate differentiation medium. In the sheet production approach described by Rheinwald and Green,[7,8] the standard medium was further used for the sheet formation, with the addition of 1.2 mM $CaCl_2$. When using the xenofree medium for cell expansion, a corresponding xenofree differentiation medium was used for sheet production, namely CnT-Pr-2D (CellNTec), supplemented with 1.2 mM $CaCl_2$. After 21 days of culture, the sheets were harvested enzymatically by treating with 0.25% (w/v) dispase II (Life Technologies) at 37°C for 10 minutes. After flushing the sheets with phosphate-buffered saline (PBS), the skin grafts were carefully detached and lifted up.

2.6 | Histological characterization

In order to qualitatively evaluate the tissue organization, sheet samples were fixed with 4% formalin for 1 hour at RT, transferred into an agarose plug (Lonza, Switzerland), and carefully spread using two tweezers. Stepwise dehydration through a series of graded alcohols was performed, followed by paraffin embedding (Paraplast, Leica, Biosystems Switzerland AG, Muttenz) and slicing into 3 μm sections. For assessing the tissue composition of the keratinocyte sheets, Hematoxylin Eosin staining was performed. All sections were analyzed by a single operator using an inverted light microscope (ZEISS Axiovert 40 CFL and ZEISS Axioplan II; Carl Zeiss AG). HE stained samples were assessed for the overall architecture, including the layering, presence of a keratinized layer, as well as keratohyalin granules and qualitative cell/ECM compactivity. The thickness of the sheets was evaluated using the Image J software (n = 3; 4 measurements per donor).

2.7 | Immunohistochemical characterization

The expression profile of the cells within the sheets was analyzed in order to confirm keratinocyte cell identity (keratin K5/K8), as well as show the early (Desmoglein 3) and late (Filaggrin) differentiation markers. Specifically, the paraffin-embedded tissue sections (5 μm) were deparaffinized and rehydrated through a graded ethanol series. For K5/K8 staining, the deparaffinized sections were unmasked for 20 minutes at 37°C with protease (0.05% in distilled water), for Desmoglin 3 with proteinase K (DAKO) for 5 minutes. Paraffin sections for Filaggrin staining were transferred to Target Retrieval Solution High pH 9 (K8004, Dako Denmark A/S, Glostrup, Denmark) in a Dako PT Link (PT100/PT101, Dako Denmark A/S) for 20 minutes at 97°C. Anti-Keratin K5/K8 (Progen 61031, 1:10), anti-Desmoglein 3 (Clone 3G133, Abcam, ab14416, 1:100), as well as anti-Filaggrin (Abcam, ab17808, 1:100) was used following a standard immunohistochemistry staining protocol on a Dako Autostainer Link48 Instrument (Dako Denmark A/S). The visualization system consisted of a secondary Cy2 conjugated AffiniPure antibody (Jackson Immuno Research, goat antimouse) for K5/K8 detection and for Desmoglein and Filaggrin detection Dako K4001 or Dako K4008 EnVision HRP/DAB system, respectively, and Hematoxylin as a counterstain. The expression profiles of Keratin, Desmoglein 3, and Filaggrin were qualitatively assessed.

2.8 | Statistical analysis

Quantitative data are presented as mean ± SD. For statistical comparison of the differences regarding enzymatic cell isolation efficiency, culture duration on different substrates, as well as thickness of keratinocyte sheet after cultivation on different substrates and in different culture systems, one-way ANOVA combined with a posthoc TUKEY analysis was performed (SPSS 17.0, IBM, Somers, New York). Results were significantly different for a P-value <.05.

3 | RESULTS

3.1 | Enzymatic isolation of keratinocytes

The isolation of the epidermal fraction from human skin biopsies was successful using trypsin/EDTA, as well as using animal-free

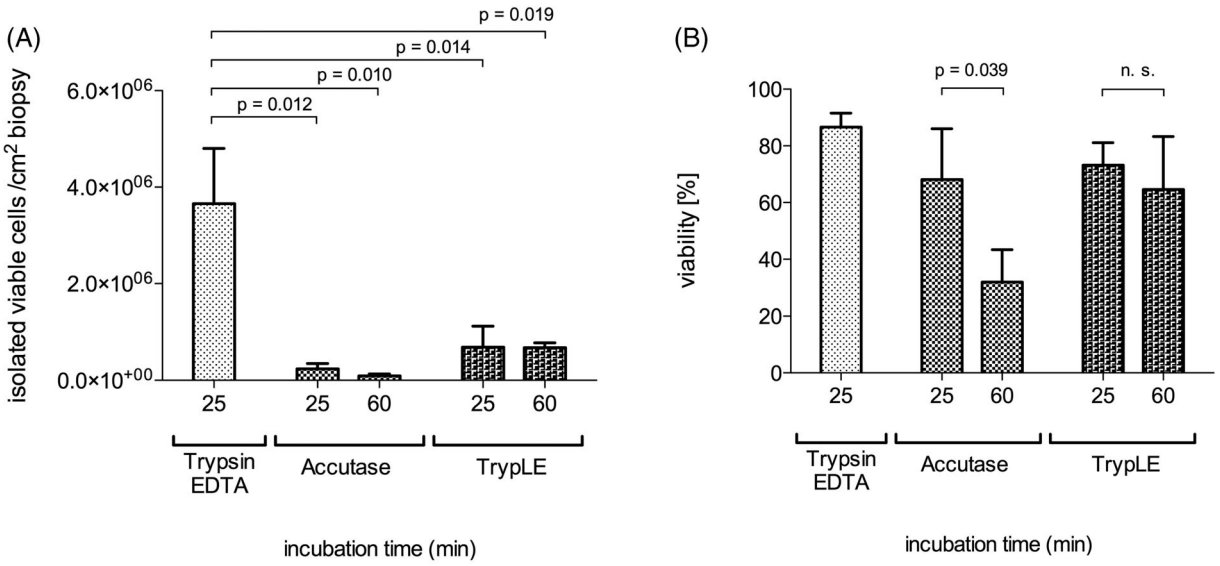

FIGURE 2 Optimization of human keratinocyte enzymatic isolation comparing Trypsin/EDTA, as well as animal-free alternatives Accutase and TrypLE Select were investigated using the standard incubation time of 25 minutes at 37°C. For increasing the isolation efficiency of Accutase, as well as TrypLE Select, the incubation time was extended up to 60 minutes. The number of isolated viable cells A, as well as the viability B, of the isolated cells, were determined after the standard incubation time of 25 minutes at 37°C or, for the animal-free alternatives, an extended incubation time of 60 minutes (Mean ± SD, n = 5)

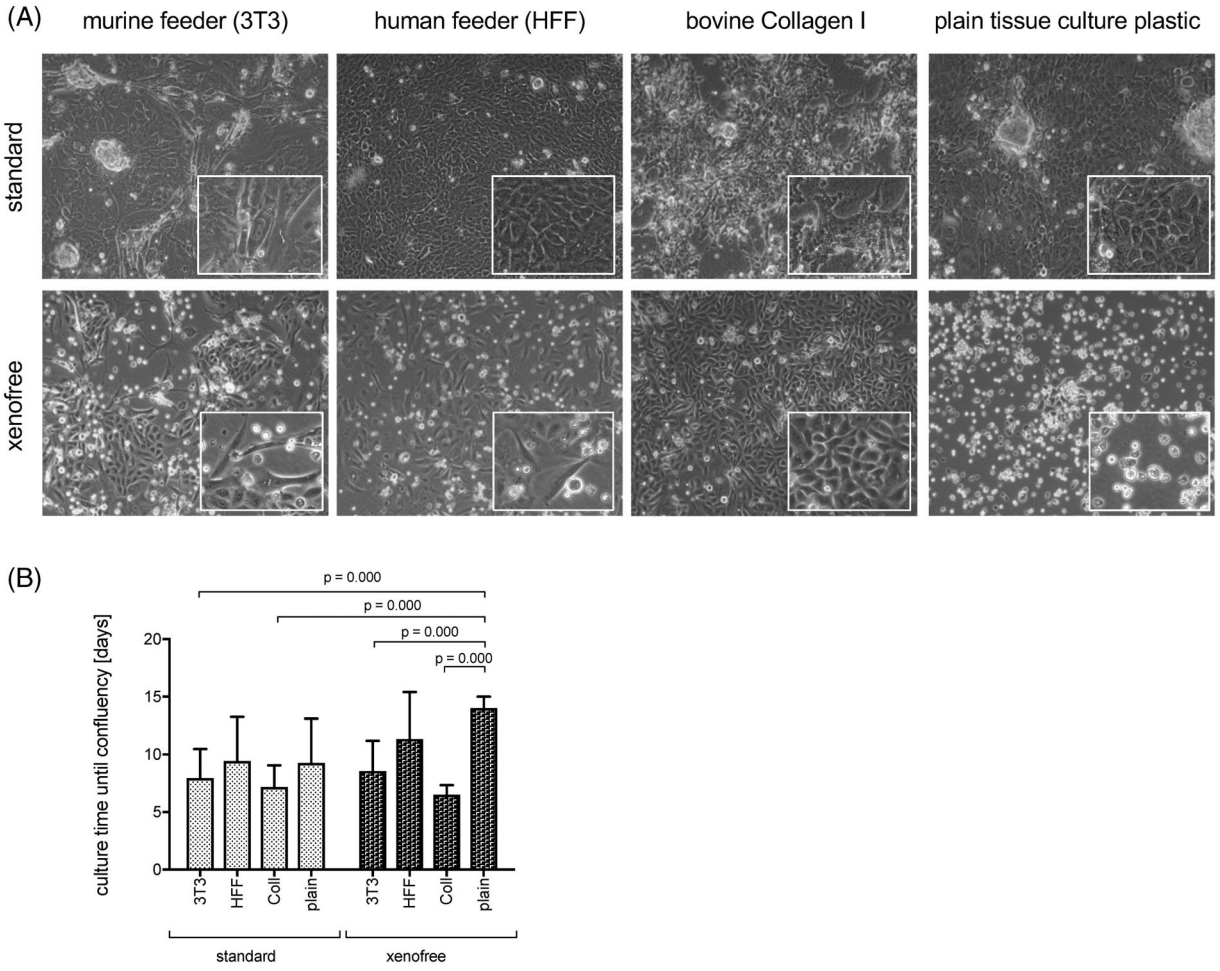

FIGURE 3 Substrates for keratinocyte cultivation. Four substrates were tested, including murine 3T3 fibroblasts (3T3), human foreskin fibroblasts (HFF), Collagen I (Coll), and tissue culture plastic (plain). Either the standard medium containing FBS or the chemically defined xenofree medium was used. The influence of medium and substrate was examined in terms of A, morphology and B, culture duration until confluency. Monolayer cultures A, are shown in 10 × magnification with an additional 2-fold zoom image section. B, Bars indicate mean ± SD

alternatives such as Accutase and TrypLE Select. However, the isolation efficiency was notably different among the three, with trypsin/EDTA outperforming TrypLE by 5 times and Accutase by 12 times (Figure 2A). Using an extended digestion time of 60 minutes, compared to the standard 25 minutes, did not lead to significant improvements in cell isolation efficiency with Accutase or TrypLE. The longer digestion time did, however, notably affect cell viability, particularly with Accutase (Figure 2B).

3.2 | Proliferation on 2D substrates

Cells seeded on the four different substrates (3T3, HFF, Collagen I, plain tissue culture plastic) and cultivated in two different media (standard medium containing FBS and chemically defined animal-free medium) attached and proliferated in all tested conditions (Figure 3A). Nevertheless, using the standard medium, the epidermal keratinocyte cultures notably had some cross-contaminating fibroblasts (Figure 3A, upper row). Using the xenofree medium, which is designed as a selection medium specifically favoring keratinocyte proliferation, the cultures showed no fibroblasts and were more homogenous (Figure 3A, lower row).

The proliferation time until cell confluency averaged 9.5 ± 3.5 days for those cultivated in standard medium and 8.1 ± 2.8 days for the cells in xenofree medium (Figure 3B). Under both medium conditions, the fastest time until confluency was recorded when cultured on Collagen I (7.2 ± 1.9 days in standard medium and 6.5 ± 0.8 days in xenofree medium). Notably, the lowest donor-to-donor variability in proliferation rate was

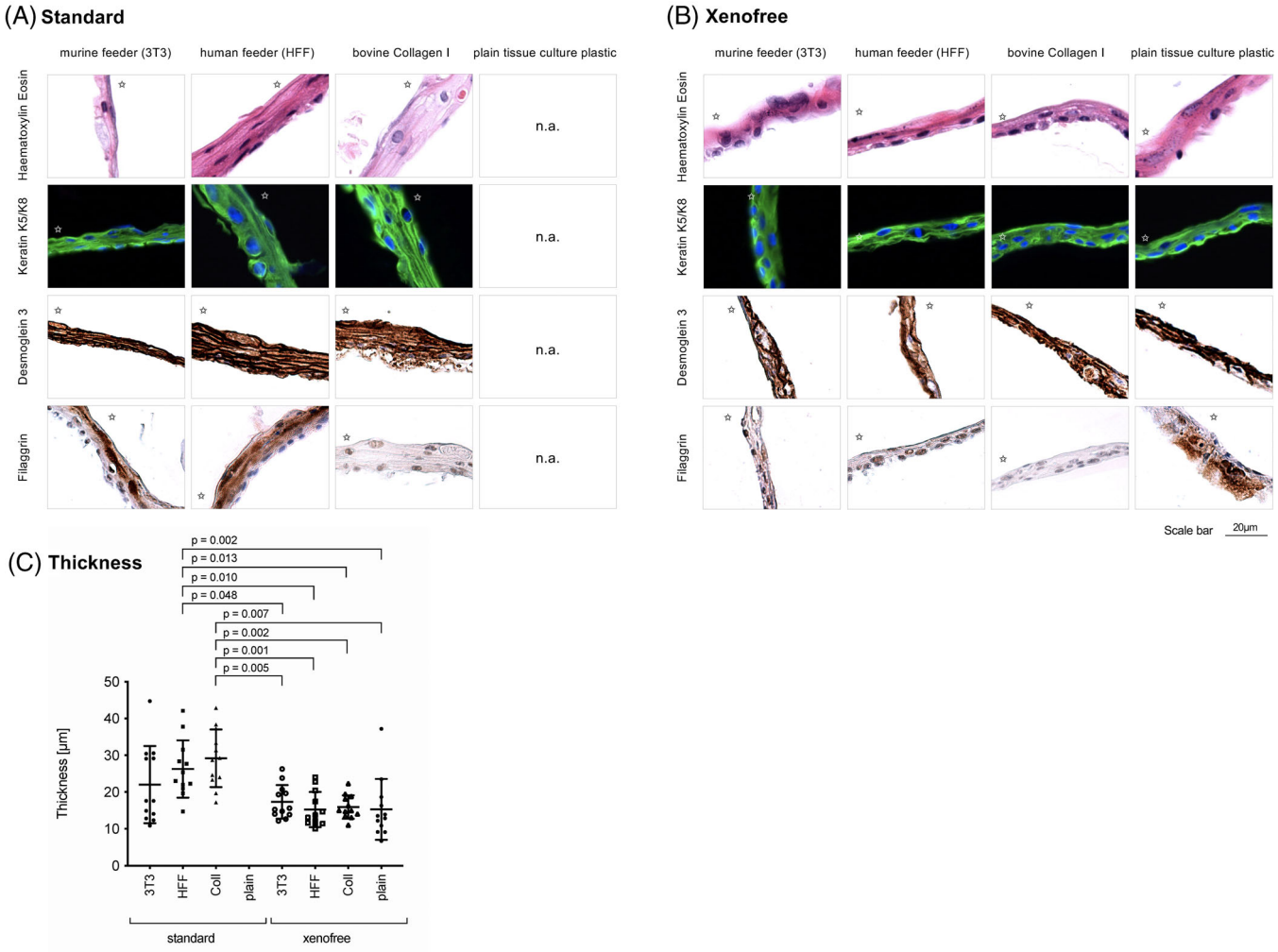

FIGURE 4 Keratinocyte sheet formation and maturation depending on culture medium and substrate. Keratinocyte sheets cultivated in either standard A, or xenofree medium B, were harvested after 21 days of culture. Hematoxylin Eosin staining reveals tissue structure (A, B first row). Expression of Keratin K5K8 (green) is shown, as well as cell nuclei (DAPI in blue) (A, B second row). The early differentiation marker Desmoglein 3 is shown in brown (A, B third row), and the late differentiation marker Filaggrin is shown in brown (A, B fourth row). Sheets could not be formed on plain tissue culture plastic using standard medium (A, fourth column; n.a. indicates not available). Keratinocyte thickness was quantified, taking two measurements per sample, with six samples per group (mean ± SD and individual sample point scatter shown in C). Scale bar for all panels indicates 20 µm, the asterisk ⋆ marks the apical side of the keratinocyte sheet

observed with cells cultured in xenofree conditions on Collagen with a SD of 0.89 days, a third of the variability using the benchmark condition (standard medium, 3T3 feeder), which had a SD of 2.55 days.

3.3 | Keratinocyte sheet formation

Keratinocyte differentiation and keratinocyte sheet formation was investigated, comparing various medium-substrate culture

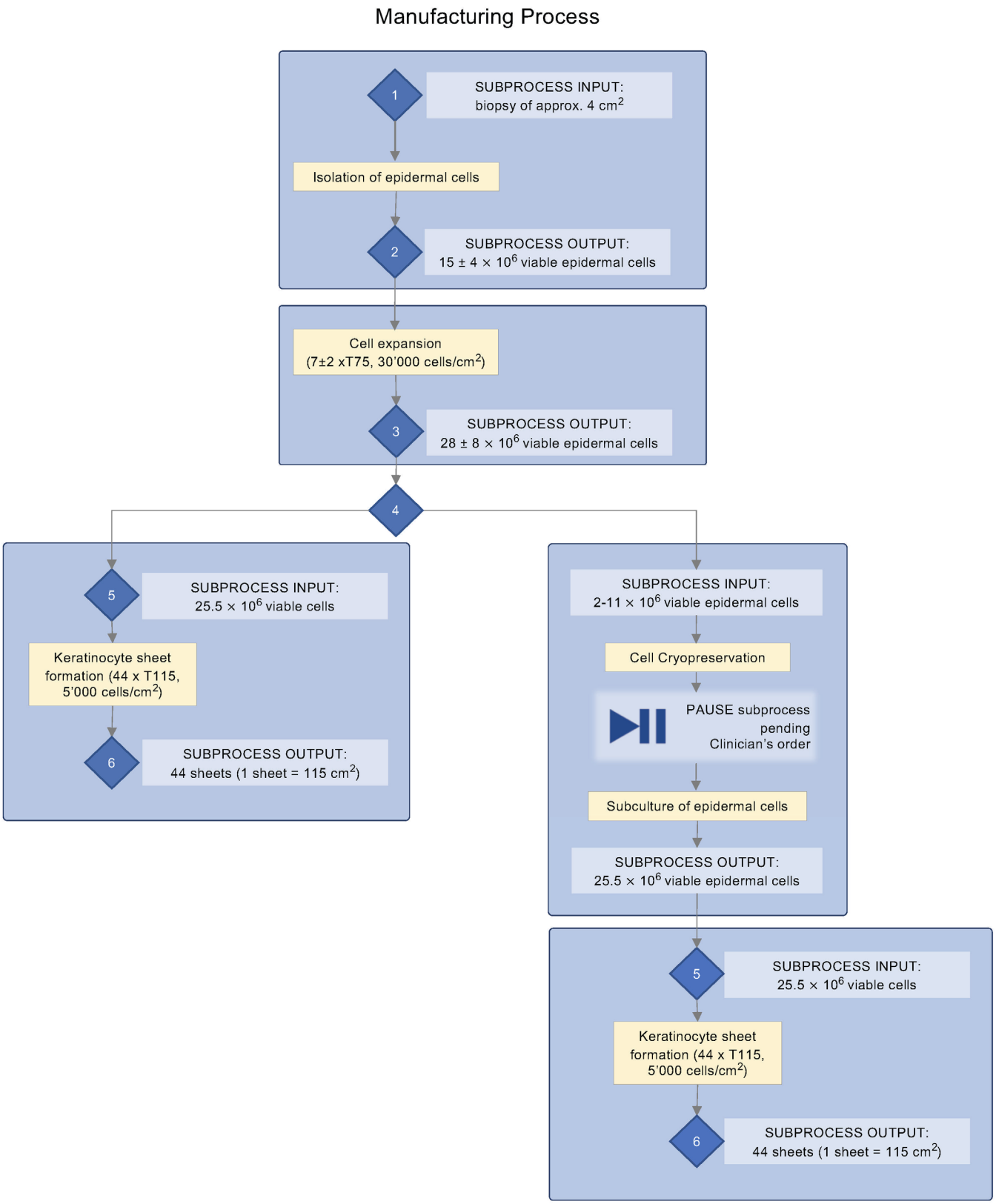

FIGURE 5 Large scale calculation for the keratinocyte sheet production procedure for major burn wound treatments, namely using a 4 cm^2 skin biopsy to produce 1 m^2 of keratinocyte sheets in a 21-day period

combination groups (Figure 4). Except when grown on plain tissue culture plastic using standard medium (Figure 4A, column "plain"), all other conditions yielded a successfully formed sheet of cells that had sufficient mechanical integrity to be lifted (Figure 4A, B).

Analyzing the keratinocytes histologically and immunohistochemically, the output with regard to the presence of keratinocytes and the early differentiation stage was comparable between the 3T3, HFF, and Collagen I conditions. The expression of K5K8 confirmed the presence of keratinocytes within all sheets. The cells in all sheets were Desmoglein 3 positive, a marker of early-stage differentiation. However, differences occurred in the late-stage differentiation marker Filaggrin. A slightly more advanced and stratified Filaggrin expression was found in the two feeder conditions (3T3, HFF) in combination with the standard medium. In contrast, a lower and more heterogeneous expression pattern was observed in the xenofree samples. The lowest Filaggrin expression was seen in Collagen groups.

Regarding structure, the sheets cultivated with the standard medium were thicker and less compacted compared to the sheets cultivated in xenofree media, which were more compacted with cells closer to each other and less interstitial ECM. This observation was also confirmed by the thickness measurements (Figure 4C), with xenofree cultured sheets about 25% to 50% thinner than those cultured in standard medium ($P < .05$ comparing standard medium HFF or Col sheet thicknesses with all xenofree sheet thicknesses). However, keratinocyte sheets cultured in standard medium showed a more heterogeneously distributed thickness with higher variances compared to xenofree cultured sheets, which exhibited an increased uniformity in thickness. Notably, even though sheets grown in the xenofree medium may have been thinner overall, they were nevertheless mechanically stable and liftable.

4 | DISCUSSION

This study successfully compared the use of alternative, animal-free components in critical steps of the autologous keratinocyte sheet production process: keratinocyte cell isolation, cell expansion, and sheet formation. On the one hand, the cell isolation step showed clear cell yield advantages when using porcine-derived trypsin, compared to animal-free alternatives. On the other hand, the medium and substrate comparative study demonstrated the feasibility of expanding keratinocytes and producing sheets using a chemically defined, animal-free medium and a Collagen coating, which would replace murine feeder layers without compromising scalability. The produced keratinocytes were largely similar to those produced using the standard method (3T3 feeder layer and FBS-containing medium), particularly in mechanical integrity, as all grafts were liftable. In addition, sheets grown on Collagen in an animal-free medium showed indications of advantages in homogeneity, speed, reduced variability, and differentiation status compared to the other growth conditions investigated. Most importantly, the procedure was compatible with the upscaling requirements of major burn wound treatments, namely using a 4 cm^2 skin biopsy to produce 1 m^2 of keratinocyte sheets in a 21-day period (Figure 5).

The knowledge about the risks of disease transmission due to the use of animal components has increased within the last decades, causing the requirements of the regulatory authorities to drastically change and become stricter.[26] Replacing as many components of animal origin as possible with xenofree ones is preferable for clinical applications nowadays, thus guaranteeing safer and more standardized therapies.[27] The move toward animal-free components would also reduce the risk profile of cell therapy production, potentially reducing the need for extensive testing, thereby increasing economic accessibility to life-saving procedures for major burn victims.

All three enzymatic candidates, the porcine-derived trypsin, as well as the two animal-free alternatives, Accutase and TrypLESelect, were able to isolate cells from the human skin biopsies. However, the yield was significantly different, which would greatly impact the scale-up potential for major burns. Notably, the precision of biopsy size measurement could be improved in this assay. However, due to skin contraction, attempting a more precise measurement of the skin biopsy size may jeopardize the integrity or the sterility of the fragile samples, and this methodological variability alone would not account for the differences observed in cell yield. Indeed, based on the cell isolation efficiency found, Accutase and TrypLE would isolate just enough cells from a 4 cm^2 skin biopsy to produce an estimated 0.05 and 0.12 m^2 of keratinocyte sheets, respectively, instead of the 1 m^2 estimated yield using trypsin. While Accutase and TrypLESelect have been described to be gentler for other applications such as cell passaging, for example, this study shows the use of trypsin efficiently isolated cells from the biopsy. The risks associated with its porcine origin, however, may be mitigated by the supplier's gamma radiation treatment, which would reduce the risk of disease transmission.[28]

Working with cell-based products, the medium component that is the most challenging to replace due to its composition is the serum. Some reports highlighted the need for serum to ensure epidermal cell stratification.[29] Serum contains numerous components and factors that influence the growth and differentiation of cells and their specific functionality. However, the composition of serum is poorly defined and has a tremendous lot-to-lot variability.[30] Because of safety concerns due to the possible transfer of animal proteins[31,32] and pathogens to patients,[33] the clinical application requires extensive testing prior to releasing a cell-based product. In recent years, various serum-free and chemically defined media were developed[34,35] to cultivate human epidermal cells. Data from this present study demonstrated that the standard serum-containing medium formulation could be substituted with the animal-free CnT-Pr medium from CELLnTEC advanced cell systems AG. In fact, using the CnT-Pr medium showed an improved homogeneity in cultured cells, as well as homogeneity in keratinocyte sheet structure and a notably reduced variability in thickness. Using an animal-free, chemically defined medium for large-scale autologous keratinocyte sheet production would have notable advantages, particularly in removing the need for serum batch validation and associated potential variability.

The use of substrates to support epidermal cell cultivation has been widely described. The use of feeders, particularly for serum-free cultivation of keratinocytes, can be a necessity in order to avoid

unwanted effects such as poor cell adhesion, slow cell proliferation, or insufficient epidermal stratification.[29,36] Concerns surrounding the use of inactivated murine 3T3 fibroblasts as feeder cells have prompted groups to investigate alternative feeder systems, some involving human cells.[27] The preparation of feeder layers requires cell inactivation using mutagenic agents such as Mitomycin-C or gamma-irradiation. However, the risks involved with trace Mitomycin-C remnants and DNA disruption,[37,38] as well as risks using gamma-irradiation and immunologic activation[31,39] drive the need for a departure from the use of feeder layers. A possible alternative to feeder layers, such as a fibroblast-embedded three-dimensional fibrin dermal matrix-like human plasma, has also been proposed.[40] However, the availability of autologous plasma from severe burn patients suffering from extensive and acute skin loss is very limited. Coating tissue culture surfaces with extracellular matrix components, such as Collagen I, have also been explored as alternatives to feeder layers.[36] This present comparative study demonstrated that human keratinocyte sheets with sufficient mechanical integrity could be grown in serum-containing and serum-free media onto a Collagen-coated surface. In fact, the serum-free Collagen substrate group required the shortest time to reach cell confluence. In the treatment of burn patients, the factor time plays a very crucial role since failure to treat patients fast enough may result in sepsis, multiple organ failure, and in the end, the death of the patient.[41] In an effort to eliminate the need for an animal-derived extracellular matrix coating, a plain tissue culture plastic group was added. However, keratinocytes could not form a sheet on plain tissue culture plastic when grown in serum-containing media and in the serum-free group, the time to the confluence was doubled compared to the Collagen group. The Collagen used in this study was of bovine origin and was manufactured following the safety guidelines and current knowledge regarding the risk associated with Transmissible Spongiform Encephalopathy (TSE) and Bovine Spongiform Encephalopathy (BSE). Due to its clear advantages in supporting keratinocyte expansion and sheet formation, the potential risk associated with its use can be mitigated by procuring certified clinical-grade products. Its risk profile would also be much lower compared to feeder layer systems.

Overall, this study presents a comparative analysis of the keratinocyte sheet forming capability of various culture systems using animal-derived or animal-free components. The data demonstrate the possibility to reduce the risk profile of large-scale human autologous keratinocyte sheet production by substituting critical components such as bovine serum and a murine feeder layer with animal-free alternatives. Furthermore, it shows that protocols using animal-free alternatives may, in fact, improve the overall quality of the keratinocyte sheets, which may, in turn, improve the graft take rate and wound healing enhancement in vivo. It would be valuable, in future studies, to further characterize keratinocyte sheets by looking at stemness markers and adhesion molecules and understand how they may enhance graft take rate and wound healing capacity.

The need for an efficient xeno-free culture system for clinically compliant applications is currently very topical, not only for the treatment of burn patients but also for all other cell-based applications. In the field of cell therapy and tissue engineering, xeno-free culture conditions are currently being intensively investigated in accordance with official requirements. There are currently major developments, for example, in cell therapies based on the use of mesenchymal stem cells (MSC) to treat Crohn's disease, multiple sclerosis, graft-vs-host disease, type 1 diabetes, bone fractures, cartilage damage, and cardiac disease.[42,43] But also tissue engineering approaches such as the replacement of tissue-engineered heart valves, cartilage, bone, ligaments, etc. are developing xeno-free alternative production protocols to meet the regulatory requirements for clinical use.[44-47]

In conclusion, optimizing autologous keratinocyte sheet cultivation using animal-free components could increase access to this life-saving technology for major burn wound victims.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest. All authors have read and approved the final version of the manuscript. Dr. med. Maurizio Calcagni had full access to all of the data in this study and has taken complete responsibility for the integrity of the data and the accuracy of the data analysis.

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TRANSPARENCY STATEMENT

Dr med. Maurizio Calcagni affirms that this manuscript is an honest, accurate, and transparent account of the study being reported with no important aspects of the study being omitted; and that any discrepancies from the study as planned have been explained.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FULL-LENGTH ARTICLE

Manufacturing

Thermal conditioning improves quality and speed of keratinocyte sheet production for burn wound treatment

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ABSTRACT

Background aims: Cultured patient-specific keratinocyte sheets have been used clinically since the 1970s for the treatment of large severe burns. However, despite significant developments in recent years, successful and sustainable treatment is still a challenge. Reliable, high-quality grafts with faster availability and a flexible time window for transplantation are required to improve clinical outcomes.
Methods: Keratinocytes are usually grown *in vitro* at 37°C. Given the large temperature differences in native skin tissue, the aim of the authors' study was to investigate thermal conditioning of keratinocyte sheet production. Therefore, the influence of 31°C, 33°C and 37°C on cell expansion and differentiation in terms of proliferation and sheet formation efficacy was investigated. In addition, the thermal effect on the biological status and thus the quality of the graft was assessed on the basis of the release of wound healing-related biofactors in various stages of graft development.
Results: The authors demonstrated that temperature is a decisive factor in the production of human keratinocyte sheets. By using specific temperature ranges, the authors have succeeded in optimizing the individual manufacturing steps. During the cell expansion phase, cultivation at 37°C was most effective. After 6 days of culture at 37°C, three times and six times higher numbers of viable cells were obtained compared with 33°C and 31°C. During the cell differentiation and sheet formation phase, however, the cells benefited from a mildly hypothermic temperature of 33°C. Keratinocytes showed increased differentiation potential and formed better epidermal structures, which led to faster biomechanical sheet stability at day 18. In addition, a cultivation temperature of 33°C resulted in a longer lasting and higher secretion of the investigated immunomodulatory, anti-inflammatory, angiogenic and pro-inflammatory biofactors.
Conclusions: These results show that by using specific temperature ranges, it is possible to accelerate the large-scale production of cultivated keratinocyte sheets while at the same time improving quality. Cultivated keratinocyte sheets are available as early as 18 days post-biopsy and at any time for 7 days thereafter, which increases the flexibility of the process for surgeons and patients alike. These findings will help to provide better clinical outcomes, with an increased take rate in severe burn patients.

Introduction

Skin provides an invaluable protective barrier for the human body. If the skin or other tissues are injured by heat, cold, electricity, chemicals, friction or radiation, it is referred to as a burn. Burns are a serious public health problem worldwide. According to the World Health Organization, around 180 000 people die of burns every year [1]. After a severe burn of over 40% of the total body surface,

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patients suffer from dramatic loss of their epidermal and dermal layers, and the natural repair mechanisms are also significantly damaged. These serious injuries lead to a state of immunosuppression, which predisposes burn patients to infectious complications. Patients are at high risk of infection from inhalation injuries and for the development of sepsis secondary to pneumonia, catheter-related infections or suppurative thrombophlebitis [2]. In these patients, the remaining unaffected skin areas are often not sufficient to provide autograft coverage.

To treat this extensive skin damage, large-scale *in vitro* cultivated skin grafts can be made from the patient's own cells (autologous). Keratinocytes, the main cell type present in the epidermis, are isolated from an autologous skin biopsy and cultivated *in vitro* to form a multi-layer cell sheet (Figure 1). These keratinocyte sheets can be transferred to carriers (e.g., Vaseline gauzes) to facilitate subsequent implantation [3]. The original technique was developed by Rheinwald and Green [4] and Green *et al.* [5] in the 1970s. However, despite considerable progress in the large-scale production of keratinocyte sheets in recent years, their successful grafting remains challenging. In fact, graft take rates show enormous variability, especially in deep and infected burn wounds, which necessitates further improvements [6]. For example, a highly inflammatory situation in combination with low neovascularization can be problematic for successful grafting. The use of cultivated skin grafts represents a possibility for the treatment of extensive burn lesions since they allow the resurfacing of a significant area of burn that cannot be achieved with other surgical techniques based on autologous grafts [7]. Compared with allografts, xenografts, dermal substitutes and biosynthetic dressings, however, the production of cultured skin grafts takes a long time, up to 3–4 weeks [8,9]. During this period, the patient's state of health cannot be predicted. To be able to react more flexibly to unforeseen clinical incidents in the patient, an extended and mainly shorter time frame for transplantation would be helpful. For this reason, the authors attempted modifications to the large-scale keratinocyte sheet manufacturing protocol to create an extended flexible transplantation window that allows the surgeon to adjust the time of transplantation according to the patient's state of health.

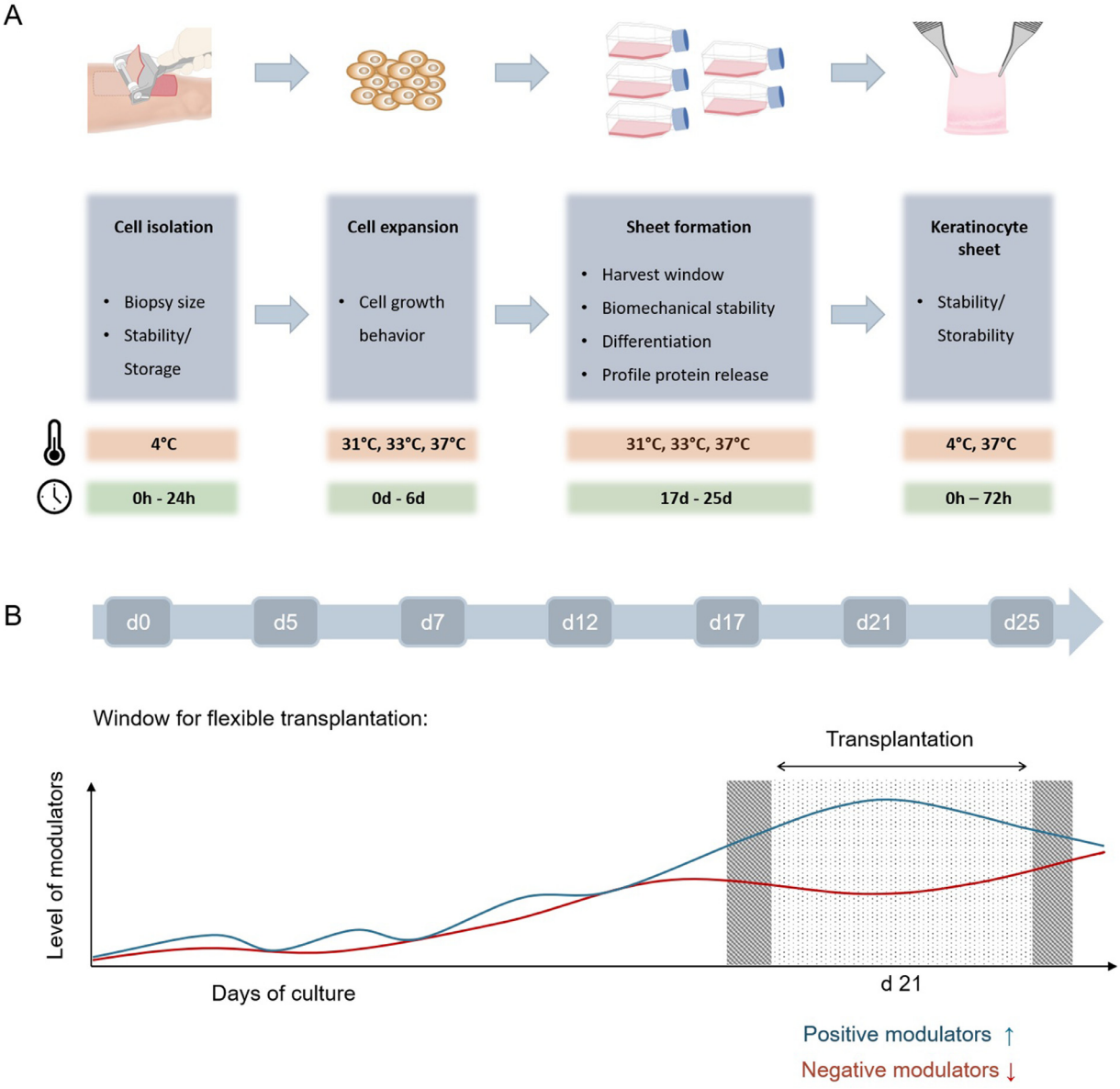


Fig. 1. (A) Schematic representation of autologous keratinocyte sheet production. After skin biopsy, the epidermal cells are isolated and expanded. After reaching the required number of cells, sheet formation is initiated. Thereafter, the keratinocyte sheet can be harvested and transplanted. For production optimization, the effect of temperature and time on the different production steps of cell isolation, cell expansion and sheet formation during keratinocyte sheet production was investigated. (B) To find a possible time window for flexible transplantation, the keratinocyte sheets were examined with regard to their positive (blue) and negative (red) modulators between day 17-25 of total culture. (Color version of figure is available online).

The native skin and its constituent cells, such as keratinocytes, are exposed to large temperature differences. The cells have to go through a temperature gradient from 37°C in the innermost basal layer to 32°C on the skin surface [10]. Since temperature appears to be involved in several pathways that regulate the balance between proliferation and differentiation of human keratinocytes, optimized temperature conditions could be used in a targeted manner to accelerate the process of keratinocyte sheet production and outcome optimization.

The effect of hypothermic culture on large-scale keratinocyte sheet manufacturing and engraftment properties has not yet been described. The authors hypothesized that temperature—specifically hypothermic conditions—may alter and perhaps even enhance the production of wound healing-related biofactors. This would improve overall keratinocyte sheet adhesion to the wound bed and, by extension, provide better clinical results, with an enhanced graft take rate in patients.

To give future burn patients the best possible treatment, the aim of the authors' study was to (i) expand the implantability window and speed up production (time) and (ii) characterize and maximize the activity of implanted keratinocyte sheets (quality). The influence of temperature on the individual *in vitro* phases of keratinocyte sheet manufacturing, such as biopsy storage, cell isolation, cell expansion and sheet formation, was investigated (Figure 1). The main focus was on the reliability and quality of the keratinocyte sheets. In addition to biomechanical stability (sheet thickness), the latter also includes the biological status of the sheets, such as cell differentiation status, biological properties and activity. With regard to a transplantation, the biological properties of cultivated skin grafts have to be ideal for the harsh environment of a debrided burn to successfully reconstitute the skin tissue. Dynamic levels of growth factors, anti- and pro-inflammatory cytokines and other important wound healing modulators secreted by keratinocyte sheets were evaluated at various stages of their *in vitro* development to determine the quality of the sheet cultures.

Methods

Source of human keratinocytes

For the present study, human keratinocytes were isolated from split- or full-thickness skin samples collected from female patients undergoing plastic surgery at the Division of Plastic Surgery and Hand Surgery of the University Hospital Zürich, Switzerland. The informed consent was obtained for experimentation with human material and the privacy rights of the patients participating in the study were always be observed (institutional ethics committee license KEK-ZH-2014-0197). The skin tissue samples were retrieved from remnant operative tissue, which would otherwise have been discarded, excised from the breast or abdominal region. Patient age range and sample number are specified for each assay in the following sections.

Isolation of human keratinocytes

The skin biopsies were placed in a Petri dish and the surface area determined with a ruler. The isolation of keratinocytes from full- or split-thickness samples was performed using the same procedure. Briefly, the skin tissue was micro-dissected in small pieces of approximately 10 × 10 mm and digested with Dispase solution (Corning, New York, USA) 12 U/mL in phosphate-buffered saline (PBS) for 15–18 h at 4°C. A maximum biopsy size of 8 $cm^2$was digested in 15 mL Dispase to maintain enzyme efficiency. Thereafter, the epidermis and dermis were mechanically separated. For isolation of the epidermal cell fraction, the epidermal layer was further digested with trypsin/ethylenediaminetetraacetic acid 5x (Thermo Fisher Scientific, Waltham, MA, USA) for 15–20 min at 37°C. The digestion was halted by adding 3.75 mg/mL soybean trypsin inhibitor (Thermo Fisher Scientific). The cell isolation efficiency was evaluated in 59 biopsy samples (patient age range, 42.2 ± 12.4 years).

To that end, a sample of the isolated cells from each biopsy was collected and stained with Acridine Orange and 4′,6-diamidino-2-phenylindole to determine the number of isolated cells as well as their viability (NucleoCounter NC-200; ChemoMetec, Lillerød, Denmark). The isolation efficiency was reported per biopsy sample as isolated viable cells/cm^2biopsy. In addition, the authors examined whether the age of the donors had a significant impact on the isolation efficiency. The mean ± standard deviation (SD) of the isolation efficiency was calculated. Furthermore, the Pearson correlation was evaluated between isolated viable cells and biopsy surface area as well as between isolated viable cells/cm^2 biopsy and donor age. An independent two-sided *t*-test was performed to compare the number of viable cells/cm^2 biopsy isolated after a 6-h versus 24-h storage period. $P < 0.05$ indicated statistical significance.

Biopsy storability

To test the effect of cold temperature storage on biopsy viability, four biopsies (patient age range, 44.5 ± 1.6 years) were divided into five equal pieces each and placed in a closed and sterile container at 4°C in maintenance medium CnT-XP3 (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland) in a refrigerator. One piece of each biopsy was taken out of storage after 0, 6, 12, 18 and 24 h. Cells were isolated and assessed for the number of isolated viable cells using the NucleoCounter. The number of isolated viable cells/cm^2 biopsy from all four biopsy samples was reported for each time point.

Human keratinocyte proliferation

To analyze the effect of thermal conditioning on cell proliferation kinetics, keratinocytes from three patient biopsies (patient age range, 47.3 ± 24.5 years) were seeded at 10 000 cells/cm^2 in 24-well plates coated with bovine collagen I 6 μg/cm^2 (Symatese, Chaponost, France). Cells were cultivated at 37°C, 33°C and 31°C in triplicate using the chemically defined and xenogeneic-free expansion medium CnT-Prime (CELLnTEC Advanced Cell Systems AG) at 37 °C in a humidified atmosphere and 5% carbon dioxide (CO_2). To minimize the risk of contamination, the medium was supplemented with 100 U/mL penicillin (Sigma-Aldrich, St Louis, MO, USA) and 100 μg/mL streptomycin (Sigma-Aldrich) for the first 3 days of culture. Every 2–3 days, the culture medium was changed with unsupplemented CnT-Prime medium until cells reached 70–90% confluency.

A colorimetric proliferation assay with Crystal Violet (Sigma-Aldrich) was performed to determine the number of viable cells in the monolayer cultures during the expansion phase after 0, 24, 72 and 144 h. A standard curve was established by seeding keratinocytes at 100 000, 50 000, 25 000, 12 500, 6250 and 0 cells/well in a 24-well plate and measuring the Crystal Violet assay signal at day 0. At the individual time points, the cells were fixed with methanol and stained with 0.1% Crystal Violet for 5 min. The cell layer was dried and decolorized with a sodium dodecyl sulfate solution (0.5% with ethanol 50% in 0.5 M tris[hydroxymethyl]aminomethane hydrochloride, pH 7.8) for 60 min at 37°C. The optical density of the supernatant was read at the absorption maximum of 590 nm. Using the internal standard, the mean ± SD of viable cells/cm^2 across all three biopsies was calculated for each time point. A one-way analysis of variance with a Bonferroni post-hoc test was performed for each time point, comparing the three temperatures. $P < 0.05$ was chosen as the statistically significant threshold.

Keratinocyte sheet formation

To analyze the influence of temperature on the production and quality of keratinocyte sheets, differentiation of keratinocytes and sheet

formation at different temperatures were analyzed. Freshly isolated keratinocytes of six patient biopsies (patient age range, 46.0 ± 8.1 years) were seeded at a density of 30 000 viable cells/cm^2 on flasks coated with bovine collagen I and expanded at 37°C in a humidified atmosphere and 5% CO_2. After reaching confluency, production of keratinocyte sheets was initiated by passaging the cells and plating them at a density of 5000 viable cells/cm^2 in CnT-Prime on T115 peel-off flasks (Techno Plastic Products AG, Trasadingen, Switzerland) coated with bovine collagen I. When the cells reached 80–90% confluency after 3–5 days, keratinocyte differentiation was induced using the chemically defined and xenogeneic-free differentiation medium CnT-Prime 2D (CELLnTEC Advanced Cell Systems AG) supplemented with 1.2 mM calcium chloride (Sigma-Aldrich). Henceforth, the culture flasks were cultivated at 31°C, 33°C and 37°C and 5% CO_2 for the remaining days of differentiation culture. A medium change with CnT-Prime 2D and calcium chloride was performed every 2 days in all conditions. For proteomic analysis, medium supernatants from all culture conditions were collected in duplicate before each medium change from day 17 until day 25 of total culture time. Supernatant samples were stored at −80°C until analysis.

Sheet formation assay

From day 17 until day 25 of total culture, the keratinocyte sheets were harvested every second day by enzymatically treating with 0.25% (w/v) Dispase II (Thermo Fisher Scientific) at 37°C for 10 min. After flushing the sheets with PBS, the skin grafts were carefully detached from the cell culture flasks and lifted with two tweezers to see whether the biomechanical stability of the sheets was given at the individual days of culture. For further analysis, representative keratinocyte sheet samples with an inner diameter of 6 mm were taken using biopsy punches.

Histology and immunohistochemistry characterization of keratinocyte sheets

For qualitative evaluation of tissue organization, keratinocyte sheet samples were fixed with 4% formalin for 1 h at room temperature, transferred in a 2% agarose plug (Carl Roth GmbH, Karlsruhe, Germany) and carefully spread using two tweezers. Stepwise dehydration through a series of graded alcohols was performed, followed by embedding in paraffin (Paraplast; Biosystems Switzerland AG, Muttenz, Switzerland) and slicing into 5-μm sections. To assess the tissue composition and architecture of the keratinocyte sheets, hematoxylin and eosin staining was performed. All sections were analyzed by a single operator using an inverted light microscope (Axiovert 40 CFL and Axioplan II; Carl Zeiss AG, Oberkochen, Germany).

In addition, expression profiles of the keratinocyte sheets with regard to early- and late-stage differentiation markers were analyzed using the primary antibodies anti-desmoglein 3 and anti-filaggrin, respectively. Specifically, sections were transferred to target retrieval solution, high pH (K8004; Dako Denmark A/S, Glostrup, Denmark), in a PT Link (PT100/PT101; Dako Denmark A/S) for 20 min at 97°C, performing a three-in-one procedure (i.e., deparaffinization, rehydration and heat-induced epitope retrieval) on the formalin-fixed, paraffin-embedded tissue sections. A standard immunohistochemistry staining protocol was performed on an Autostainer Link 48 instrument (Dako Denmark A/S) for desmoglein 3 (clone 3G133, mouse IgG1, ab14416; Abcam, Cambridge, UK) and filaggrin (mouse IgG, ab17808; Abcam) at a working dilution of 1:100 in antibody diluent (S2022; Dako Denmark A/S) for 20 min at room temperature. The visualization system consisted of the K4001 and K4008 EnVision horseradish peroxidase/3,3′-diaminobenzidine (DAB) system (Dako Denmark A/S), with hematoxylin as counterstain. Tissue formation, including thickness of the keratinocyte sheets and expression intensity of desmoglein 3 and filaggrin, was evaluated using ImageJ 1.52a software (National Institutes of Health, Bethesda, MD, USA). To measure sheet thickness, three measurements were carried out on each histology slide. The expression intensity of desmoglein 3 and filaggrin, indicated by intensity of the DAB stain, was quantified using the DAB color deconvolution algorithm of ImageJ [11]. The mean ± SD sheet thickness as well as DAB intensity of all samples was given for each time point.

Proteomics

To analyze the metabolic state and intercellular signaling of the keratinocytes within the sheets, targeted microarray using multiplex technology (Luminex; R&D Systems, Minneapolis, MN, USA) was used. With this novel technique, complete profiles of cells and cell cultures can be identified [12]. Magnetic beads coated with the specific antibodies were mixed with the sample (supernatant) and incubated. Thereafter, the bonded analytes were detected using the biotinylated streptavidin complex. Using the Bio-Plex Pro human cytokine 27-plex assay (Bio-Rad, Hercules, CA, USA), 27 parameters, including growth factors/proteins, regulating cell growth and division as well as angiogenesis, were analyzed for each sample. The focus during analysis was on pro- and anti-inflammatory cytokines as well as markers regulating proliferation, differentiation and survival of cells within the keratinocyte sheet. Thus, the following eight analytes were chosen for analysis: interferon gamma (IFN-γ), IL-10, IL-4, IL-1b, IL-8, tumor necrosis factor alpha (TNF-α), platelet-derived growth factor type BB (PDGF-BB) and vascular endothelial growth factor (VEGF). Data were acquired using a BioPlex 200 reader (Bio-Rad). The mean concentration (pg/mL) ± SD of the different secretomes was calculated for each time point and presented over time. For statistical significance, a one-way analysis of variance with a Bonferroni post-hoc test was performed. P= 0.05 indicated the statistically significant threshold.

Storability of keratinocyte sheets

To assess the time-dependent usability of the keratinocyte sheets, the storability of the sheets was investigated. Therefore, sheets made of cells from four different patient biopsies (patient age range, 43.7 ± 25.2 years) were cultivated and enzymatically detached as described before. These were then stored either at 4°C in a refrigerator or at 37°C in an incubator (5% CO_2) in CnT-Prime 2D or CnT-XP3 medium. In addition to temperature, two storage conditions were compared: one with the transport dressing Jelonet (Smith & Nephew, Mississauga, Canada) and one without any dressing (n = 4). The viability of the keratinocyte sheets was assessed immediately after harvest (0 h) as well as after 3, 6, 12, 24, 48 and 72 h by a fluorescence-based live-dead assay using fluorescein diacetate (Sigma-Aldrich) and propidium iodide (Sigma-Aldrich). Use of the fluorescent dyes fluorescein diacetate and propidium iodide allowed a simultaneous two-color determination of living cells and the dead cell population, respectively. Keratinocyte sheets of each condition were incubated with 5 mg/mL fluorescein diacetate in acetone and 1 mg/mL propidium iodide in PBS for 5 min. After intensive washing with PBS, the samples were analyzed using an inverted fluorescence microscope as mentioned earlier. The viability of the keratinocyte sheet, indicated by the fluorescence intensity of the fluorescein diacetate stain, was quantified using ImageJ (mean gray value algorithm) and represented as a percentage (mean ± SD) in correlation with the sample without storage of the respective medium and temperature over time.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 25.0.0.1 (IBM, Armonk, NY, USA). Quantitative data are presented as mean ± SD. The individually chosen statistical comparison was specified earlier for each assay. $P < 0.05$ was considered statistically significant.

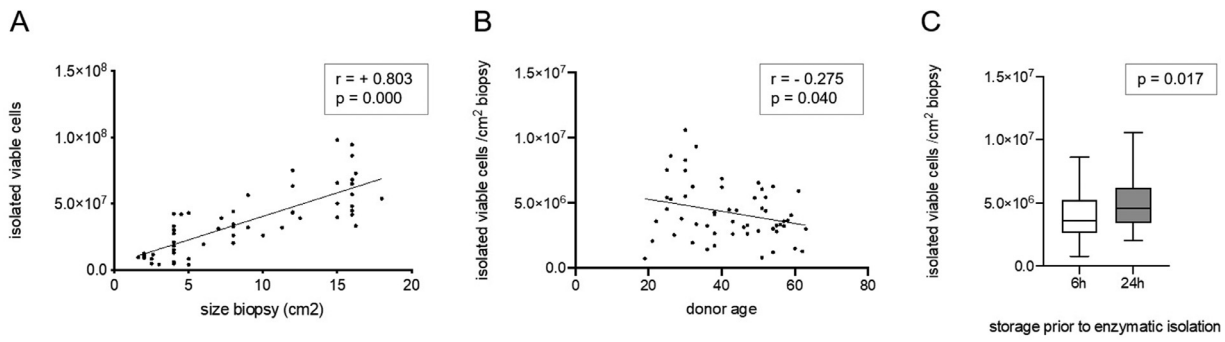


Fig. 2. Cell isolation efficiency. (A,B) Isolating human keratinocytes from 59 skin biopsies, a possible correlation between biopsy size and isolated viable cells as well as between isolated viable cells/cm^2 biopsy and donor age was investigated. (C) The impact of storage before enzymatic isolation was also analyzed. $P < 0.05$ was considered statistically significant.

Results

Cell isolation efficiency

The cell isolation procedure was reliable. Human keratinocytes were successfully isolated from all 59 biopsies, with biopsy surface sizes between 1.6 and 18 cm^2. The mean number of isolated viable cells was 4.29 ± 2.12 million cells/cm^2 biopsy (Figure 2A). A linear positive correlation between isolated viable cells and biopsy surface area was found (r = +0.803, P = 0.000) (Figure 2A). A weak negative correlation was found between isolated viable cells/cm^2 biopsy and donor age (r = −0.275, P = 0.040) (Figure 2B). However, a relation between isolation efficiency per cm^2 biopsy and storage prior to enzymatic isolation was demonstrated (Figure 2C). When comparing cell isolation after 6 h and 24 h of storage, it was shown that the efficiency after 24 h of storage at 4°C was significantly better. The number of viable cells/cm^2 isolated after 6 h of storage was 3.9 ± 1.8 million cells/cm^2, and this increased after 24 h of storage, reaching 5.2 ± 2.1 million cells/cm^2 (P= 0.017). The number of viable cells/cm^2 isolated from split-thickness (4.1 ± 2.8 × 10^6 cells) versus full-thickness biopsies (4.7 ± 2.0 × 10^6 cells) was not significantly different.

Biopsy stability

To investigate variability within the samples, biopsies were divided into five equal parts and stored at 4°C in CnT-XP3 maintenance medium until cell isolation. After 0, 6, 12, 18 and 24 h, cells were isolated as previously described.

As can be seen in Figure 3, the number of isolated viable cells/cm^2 biopsy was not significantly adversely affected during the first 12 h of storage. However, after 12 h, some patients showed higher isolation efficiency (see biopsy one), whereas others showed an opposite trend (see biopsy three).

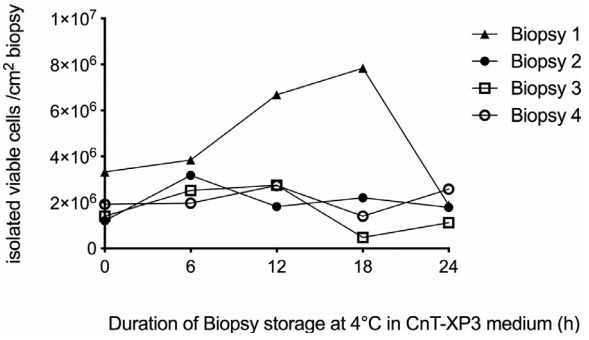


Fig. 3. Biopsy storability. Four human skin biopsies were stored at 4°C in CnT-XP3 medium. Over a period of 24 h, cells were isolated every 6 h and the number of isolated viable cells/cm^2 biopsy assessed.

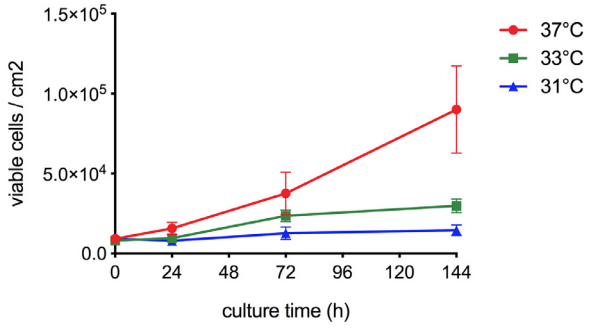


Fig. 4. Impact of thermal conditioning on keratinocyte proliferation. Cell expansion was investigated after 0, 24, 72 and 144 h at low and subphysiological temperature of 31°C (blue), intermediate temperature of 33°C (green) and high temperature of 37°C (red). (Color version of figure is available online).

Thermal conditioning cell expansion

After successful cell isolation, the effect of hypothermic conditioning on the proliferation of human keratinocytes was examined at cultivation temperatures of 31°C, 33°C and 37°C. Thermal conditioning had a significant influence on the expansion behavior of human keratinocytes (Figure 4). Lower temperature conditions of 31°C and 33°C impaired cell expansion compared with 37°C. Population doubling after 144 h at 37°C reached 3.25 ± 0.29 doublings, or 1.7 times that seen at 33°C, with 1.88 ± 0.23 doublings, and five times that seen at 31°C, with 0.64 ± 0.25 doublings. After 144 h, the number of viable cells was highest when cultivated at 37°C (9 ± 2.7 × 10^4 cells/cm^2). This was three times (P= 0.011) and six times (P= 0.004) higher than that seen in cultures cultivated at 33°C (3 ± 0.4 × 10^4 cells/cm^2) and 31°C (1.5 ± 0.3 × 10^4 cells/cm^2), respectively.

Keratinocyte sheet formation

The keratinocyte sheets cultivated at all three temperature conditions showed successful sheet formation (Figure 5A). However, thermal conditioning demonstrated a significant influence on the formation of a multi-layer structure. At 17 days of culture, the keratinocyte sheets of the individual patients featured biomechanical stability (i.e., were liftable) when cultivated at 33°C or 37°C. However, the sheets cultivated at 31°C obtained a biomechanical stability at a later time point and were not liftable before day 19. All liftable sheets showed a homogeneous cell multi-layer, and no macroscopic difference between harvesting at day 17 or day 25 was observed. The tissue structure was more compact with cultivation at 37°C compared with that seen at lower temperatures. The thickness of the keratinocyte sheets showed a higher homogeneity during the day 17 to day

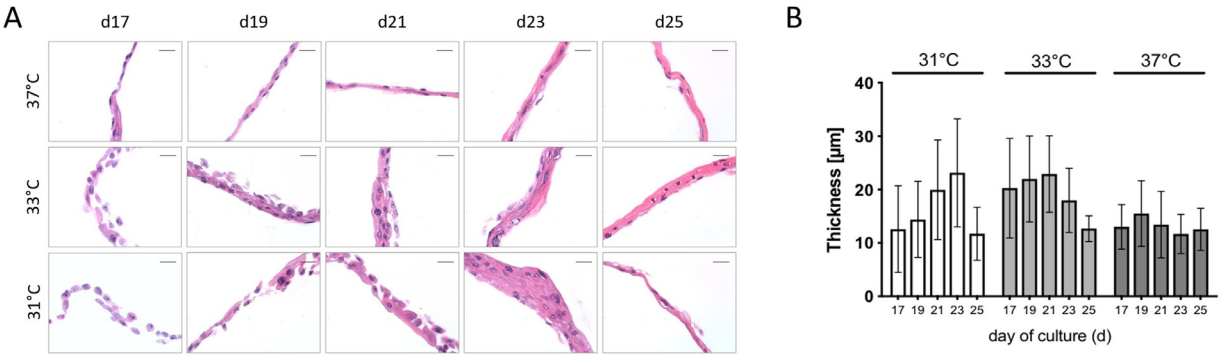


Fig. 5. Time- and temperature-dependent keratinocyte sheet formation. Keratinocyte sheets cultivated at 31°C, 33°C and 37°C were harvested every second day from day 17 to day 25. (A) Paraffin-embedded and sectioned keratinocyte sheets were analyzed for tissue architecture and sheet formation using hematoxylin and eosin histology staining. (B) Thickness of the keratinocyte sheets was evaluated using ImageJ software (mean ± SD, n = 6). Scale bar for all panels = 20 μm. (Color version of figure is available online).

25 window when cultivated at 37°C (13.3 ± 5.0 μm) (Figure 5B). By contrast, sheets cultivated at 33°C were 18.9 ± 7.5 μm in thickness, and those cultivated at 31°C were even thinner, at 17.1 ± 9.1 μm. Nevertheless, no statistically significant difference in thickness was detected between the three groups (31°C, 33°C and 37°C).

The early differentiation status of keratinocytes, as indicated by the expression profile of desmoglein 3, did not vary regardless of culture temperature or time (Figure 6A). From the 17th day (earliest time of observation), keratinocytes showed constant expression over time. By contrast, the late-stage differentiation marker filaggrin was increasingly expressed from day 19 onward when cultivated at 37°C and 33°C (Figure 6B). A notably delayed and heterogeneous expression of filaggrin was found at low temperature (31°C).

Keratinocyte sheet proteomics

The eight analyzed wound healing-related biofactors (IFN-γ, VEGF, PDGF-bb, TNF-α, IL-1b, IL-8, IL-4, IL-10) were detectable in the supernatant of the keratinocyte sheet cultures at all time points and temperatures tested. Thermal conditioning as well as duration of culture affected the levels of biofactors in both the detected peak and kinetics (Figure 7). Within the sheet harvest window of 17–25 days, those cultured at 37°C exhibited an earlier increase in biofactor release, a narrower peak and a trend toward an earlier decrease in secretion within the time window compared with other temperatures. Sheets cultured at 31°C systematically exhibited lower levels of biofactors and slower release kinetics. With regard to sheets cultured at 33°C, biofactor levels were the highest, and the most sustainable release kinetics occurred in this time window. Specifically, IFN-γ release kinetics exhibited a 2-day delay, with levels at 37°C peaking on day 21 (190 ± 26 pg/mL) and levels at 33°C rising to approximately the same level (197 ± 33 pg/mL) and plateauing at day 23. Levels at 31°C eventually rose starting at day 23. The peak (detected at 37°C and 33°C, extrapolated at 31°C) did not seem to be affected by thermal conditioning, only the kinetics of release. The same profile was observed for TNF-α and IL-4, with levels at 37°C peaking on day 21 (TNF-α, 30 ± 4 pg/mL, IL-4, 2 ± 0.5 pg/mL) and levels at 33°C rising to approximately the same level (TNF-α, 27 ± 3 pg/mL, IL-4, 1.8 ± 0.4 pg/mL) but continuing to increase. VEGF also showed a temperature-related delay in

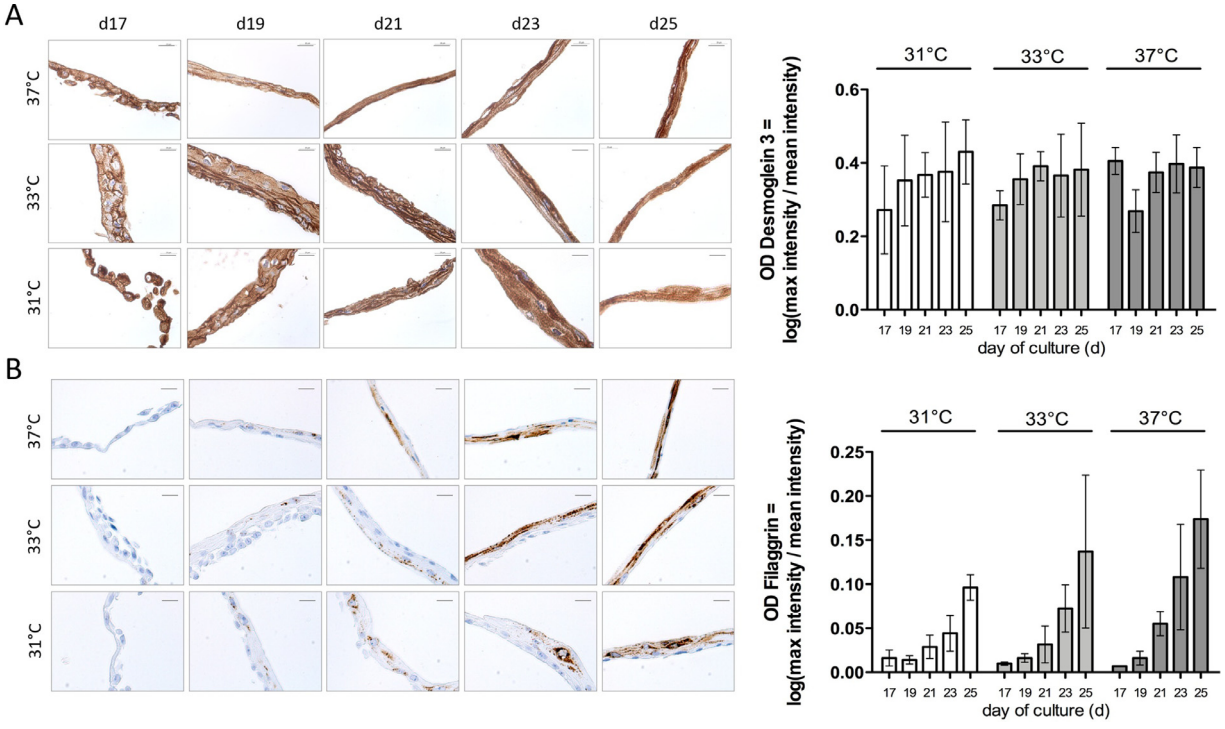
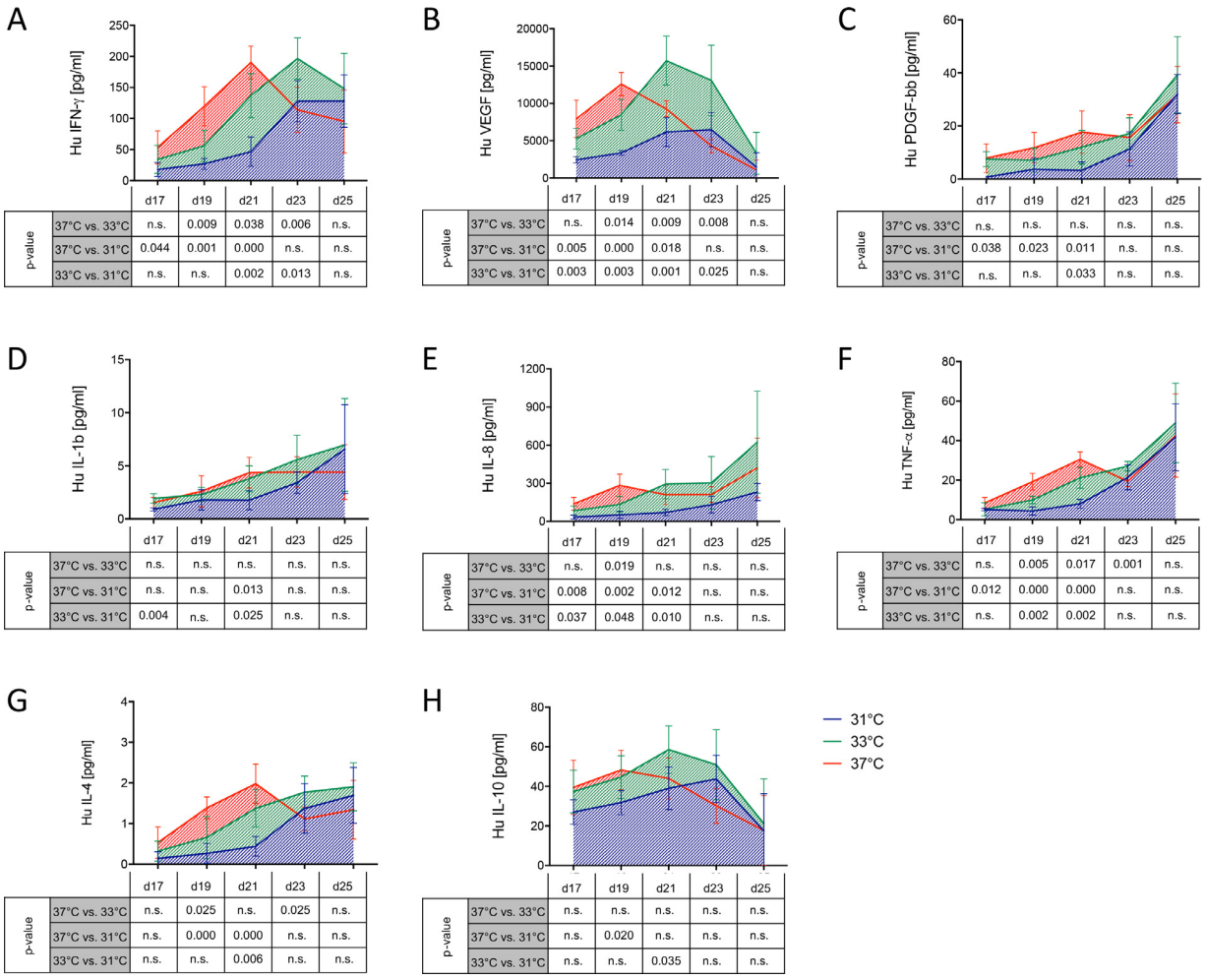


Fig. 6. Influence of time and temperature on keratinocyte sheet formation. From day 17 to day 25, keratinocyte sheets were immunohistochemically analyzed for graft maturation. (A) Anti-desmoglein 3 indicates the early differentiation phase and (B) anti-filaggrin shows the late-stage differentiation phase of the keratinocytes within the sheets. For quantification of the two stains, ImageJ software was used (mean ± SD, n = 6). Scale bar for all panels = 20 μm. (Color version of figure is available online).



A	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	0.009	0.038	0.006	n.s.
37°C vs. 31°C	0.044	0.001	0.000	n.s.	n.s.
33°C vs. 31°C	n.s.	n.s.	0.002	0.013	n.s.

B	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	0.014	0.009	0.008	n.s.
37°C vs. 31°C	0.005	0.000	0.018	n.s.	n.s.
33°C vs. 31°C	0.003	0.003	0.001	0.025	n.s.

C	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	n.s.	n.s.	n.s.	n.s.
37°C vs. 31°C	0.038	0.023	0.011	n.s.	n.s.
33°C vs. 31°C	n.s.	n.s.	0.033	n.s.	n.s.

D	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	n.s.	n.s.	n.s.	n.s.
37°C vs. 31°C	n.s.	n.s.	0.013	n.s.	n.s.
33°C vs. 31°C	0.004	n.s.	0.025	n.s.	n.s.

E	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	0.019	n.s.	n.s.	n.s.
37°C vs. 31°C	0.008	0.002	0.012	n.s.	n.s.
33°C vs. 31°C	0.037	0.048	0.010	n.s.	n.s.

F	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	0.005	0.017	0.001	n.s.
37°C vs. 31°C	0.012	0.000	0.000	n.s.	n.s.
33°C vs. 31°C	n.s.	0.002	0.002	n.s.	n.s.

G	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	0.025	n.s.	0.025	n.s.
37°C vs. 31°C	n.s.	0.000	0.000	n.s.	n.s.
33°C vs. 31°C	n.s.	n.s.	0.006	n.s.	n.s.

H	d17	d19	d21	d23	d25
37°C vs. 33°C	n.s.	n.s.	n.s.	n.s.	n.s.
37°C vs. 31°C	n.s.	0.020	n.s.	n.s.	n.s.
33°C vs. 31°C	n.s.	n.s.	0.035	n.s.	n.s.

Fig. 7. Secretome release of eight analyzed biofactors—the immunomodulatory factor (A) IFN-γ; the angiogenic factors (B) VEGF and (C) PDGF-bb; the pro-inflammatory factors (D) IL-1b, (E) IL-8 and (F) TNF-α; and the anti-inflammatory factors (G) IL-4 and (H) IL-10—at the three different temperatures, 31°C (blue), 33°C (green) and 37°C (red). Data are shown as mean ± SD, n = 6. Results from the ANOVA post-hoc Bonferroni statistical analysis comparing biofactor levels for each time point are shown in tables under each graph. $P < 0.05$ was considered statistically significant. ANOVA, analysis of variance; n.s., not significant ($P \geq 0.05$). (Color version of figure is available online).

release, first peaking at 37°C, followed by 33°C and then 31°C. However, the peak levels of VEGF were lowest at 31°C (6479 ± 2337 pg/mL), followed by 37°C (12 599 ± 1570 pg/mL), and highest at 33°C (15 730 ± 3295 pg/mL), which peaked at day 21 and maintained the highest levels afterward compared with other temperatures. IL-10 showed a similar profile, with less notable temperature-related differences. With regard to PDGF and IL-8, the levels at all temperatures rose progressively over time, with levels at 31°C remaining systematically lower. A progressive increase in IL-1b levels over time was also observed, but the difference between temperatures was less notable.

Keratinocyte sheet storability

Overall, keratinocyte sheets harvested at day 21 exhibited a progressive loss in viability when stored at 4°C or 37°C in CnT-2D (growth) or CnT-XP3 (maintenance) medium (Figure 8A,B), maintaining a viability above 50% of the 0 h signal (mean gray value, 22 ± 4) for varying amounts of time (Figure 8C–F). A rapid loss in viability with a similar shelf half-life (mean gray value, 22 ± 9) was seen in all conditions within the first 12 h of storage regardless of medium or temperature (Figure 8C,E). After 24 h of storage, the viability of the keratinocyte sheets fell below the threshold of 50% of the initial viability signal (0 h) in all conditions. However, after this time point, the loss of cell viability within the keratinocyte sheets slowed down, as indicated by the flattening of the curves (Figure 8C–F). Overall, storage at a colder temperature was beneficial regardless of medium or presence of carrier gauze, but this difference was mostly notable from 24 h of storage onward.

In the presence of the transfer gauze Jelonet, cell viability further decreased compared with the control sheet without carrier. A smaller drop in viability due to the carrier was observed when using CnT-2D medium, especially at 4°C. A notable decrease in viability was observed after 48 h of storage in CnT-XP3 medium. Storage on Jelonet at 4°C reduced viability by 25% compared with storage in CnT-XP3 medium only (P= 0.015). The same was observed at 37°C, with a 22% decrease compared with the control without gauze carrier (P= 0.037). Starting at 6 h, Jelonet reduced viability by 12%. Nevertheless, sheets stored in CnT-2D medium at 4°C on Jelonet maintained viability at 34 ± 20% (mean gray value, 14 ± 8) for longer storage, up to 72 h (Figure 8C). In the absence of the transfer gauze, a stabilization of viability was detected (Figure 8D,F), with the use of CnT-XP3 medium showing an obvious beneficial effect compared with CnT-2D medium, maintaining the 50% level up to 48 h.

Discussion

In this study, the authors demonstrated that temperature has a clear influence on *in vitro* keratinocyte sheet production. Thermal conditioning could be tailored to benefit each manufacturing step. Biopsy cold storage and keratinocyte sheet cold storage were

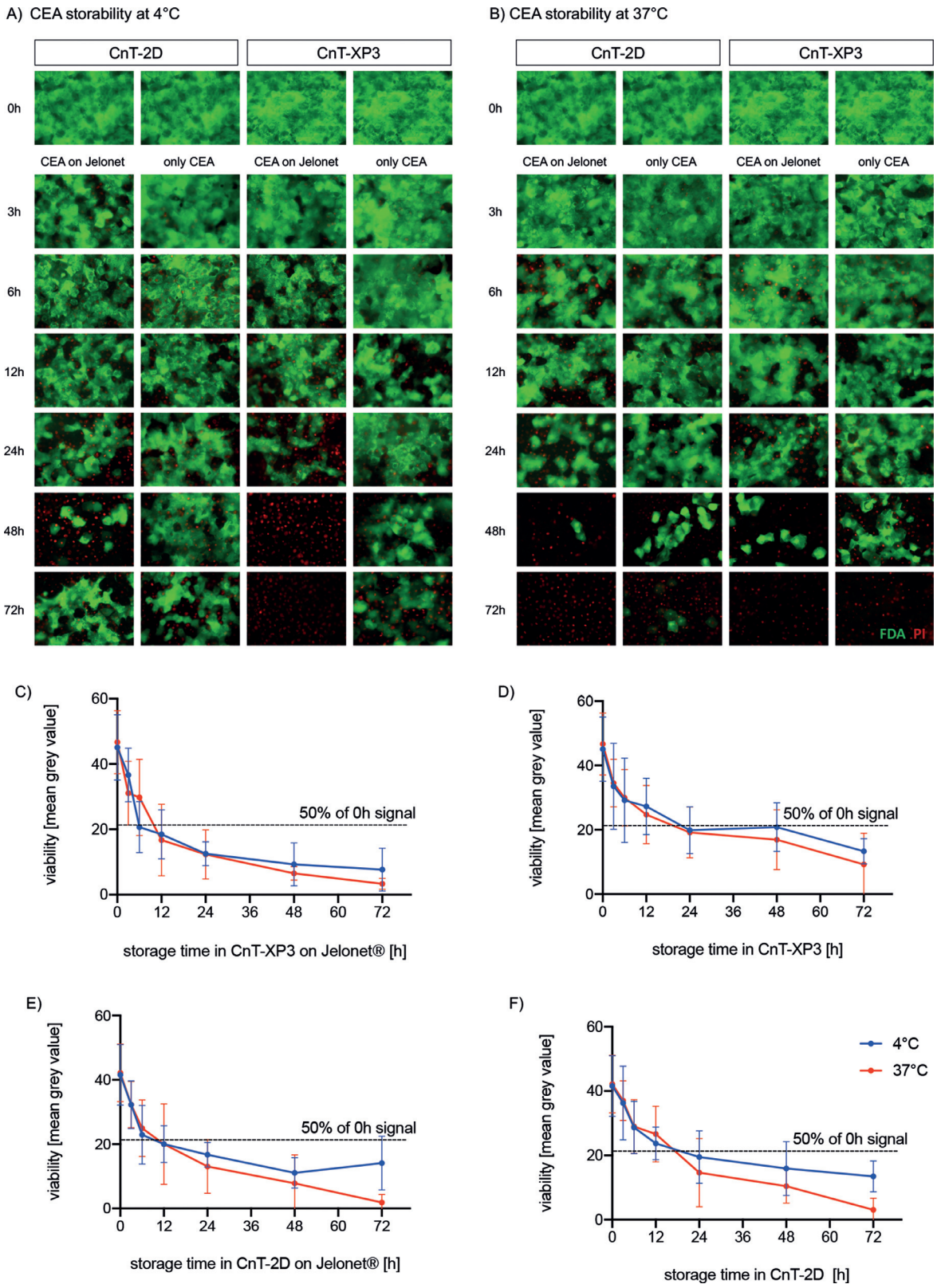


Fig. 8. Stability of cultivated keratinocyte sheets. Sheets were harvested and subsequently stored either in Cnt-2D or CnT-XP3 medium at 4°C (A) respectively at 37°C (B) on the wound dressing Jelonet® and without wound dressing. After 0, 3, 6, 12, 24, 48 and 72 h, viability was assessed using the live-dead stain fluorescein diacetate/ propidium iodide. To quantify the fluorescent signal of the fluorescein diacetate stain, ImageJ software was used. (C–F) The course of viability over time is represented as the mean gray value of the respective temperature and medium conditions. Mean ± SD, n = 4. (Color version of figure is available online).

beneficial overall. Human keratinocytes showed their maximum proliferation rate at 37°C compared with lower temperatures. During differentiation and sheet formation, keratinocytes benefited from cultivation at 33°C, particularly with regard to improved epidermal structure, biomechanical stability and biological activity (secretome release). Finally, tailored thermal conditioning of the various sheet production steps allowed large-scale sheet production to be ready for implantation by day 18 following biopsy harvest and also allowed for a wider implantability window, from day 18 to day 25, which could then be tailored to the patient's needs.

Production time and hypothermic conditioning of keratinocyte sheets

Initially, the technique of growing keratinocytes in confluent sheets, with the expansion coefficient needed for large-scale production (i.e., 600 times the surface expansion from biopsy to sheet area), required 5 weeks [13]. Because of intensive process optimization in the last decades, this manufacturing period was subsequently reduced to 3 weeks [14]. Nevertheless, the characterization and harnessing of a transplantation efficiency window would enable surgeons to make an informed decision regarding the timing of a grafting procedure by allowing them to choose the time frame when the patient is most stable and graft maturation is ideal. Determining the duration of the efficiency window would also allow surgeons to work around delays caused by the sudden onset of complications in a patient. This would undoubtedly translate into better clinical outcomes in burn patients in the future.

In this study, the maximal window of transplant efficiency was examined. The aim was to find the earliest time point when keratinocyte sheets can be transplanted despite patient-to-patient variability. To achieve the greatest possible clinical flexibility with regard to transplantation, the extension of the window for a possible transplantation was also examined. The current literature describes degenerative features of cultivated skin transplants in cases of prolonged cultivation (up to 28 days) [8]. After a mature phase of 14–21 days, a senile phase is observable. Therefore, keratinocyte sheet quality was analyzed in the current study between day 17 and day 25 of total production time. With the help of optimized temperature conditions, the individual manufacturing steps should be accelerated and the safety and quality of the sheets optimized.

For this purpose, keratinocytes were isolated from 4 cm^2 human skin biopsies, expanded and cultivated to form multi-layer keratinocyte sheets. Skin tissues were obtained by full- or split-thickness collection from female patients aged 48.5 ± 10.5 years. On average, $3.2 \times 10^6 \pm 1.35 \times 10^6$ viable epidermal cells/cm^2 tissue were isolated. In accordance with the current literature [15], no differences in cultured keratinocyte doublings were found among cells isolated from the skin of different body areas (breast, abdomen) or from donors of different ages.

To keep the cellular stress level as low as possible when isolating primary cells and thus increase the number of isolated viable cells, the authors examined storage of the skin biopsies in the maintenance medium CnT-XP3. CnT-XP3 regulates tissue homeostasis, thereby reducing cellular stress after biopsy. This observation was confirmed in the present study, showing that storage in CnT-XP3 for 24 h significantly increased the number of viable isolated cells (Figure 2C) compared with isolation without storage. However, a closer look revealed that there were enormous differences in isolation efficiency between individual patients after 12 h of storage. Although in some cases one can benefit from a 4-fold increase in cell isolation efficiency after overnight storage, this is not the case systematically; in fact, the opposite may happen (Figure 3). This patient-to-patient variability, inherent to autologous therapies, means that to reduce overall time until transplantation, the sheet preparation process should be started as soon as possible.

To accelerate keratinocyte proliferation as well as keratinocyte sheet formation, cells were cultivated at temperatures of 37°C and 33°C, which keratinocytes also experience in their native *in vivo* development [10], as well as at subphysiological temperature conditions of 31°C. The comparison confirmed that cultivation temperature *in vitro* has a significant influence on the proliferation of the cells. During the *in vitro* proliferation phase, the keratinocytes benefited from an internal body temperature of 37°C. The two lower temperature conditions showed an adverse effect, with a significantly lower proliferation rate. In other mammalian cell cultures, it has already been demonstrated that a cultivation temperature of 31°C places cells into cold shock and arrests cells in the G1 phase of the cell cycle, which leads to reduced expansion of the cells, decreased biomass and less cells [16]. Since the aim of this study was to accelerate cell proliferation, a keratinocyte expansion temperature of 37°C would be recommended based on these results.

The influence of the three temperatures was also examined with regard to keratinocyte sheet formation between day 17 and day 25 of harvest to assess biomechanical stability, sheet thickness and expression profile of the differentiation markers. Temperature had a profound impact on the differentiation phase of keratinocytes. At 31°C, sheet formation was significantly delayed (day 19), which was reflected in the mechanical stability as well as the expression of the late-stage differentiation marker filaggrin. Overall, the composition of the sheets after cultivation at 31°C was more heterogeneous, which was confirmed by the thickness measurement of the sheets, and biomechanical stability was not reached before day 19 of culture. The subphysiological temperature of 31°C had an obviously negative influence on keratinocyte metabolism with regard to the differentiation capacity of the cells. Keratinocyte sheets cultivated at a mildly hypothermic temperature of 33°C generated better epidermal structures and an increased differentiation potential, which might be associated with increased mitochondrial adenosine triphosphate synthesis [17–19]. Therefore, from a macroscopic and differentiation point of view, forming human keratinocyte sheets at 37°C, and even 33°C, would be preferable to forming them at 31°C. In future studies, it would be valuable to further characterize keratinocyte sheets by looking at stemness markers and adhesion molecules to understand how these may be influenced by thermal conditioning and how they may enhance graft take rate and wound healing capacity.

Proteomics and hypothermic conditioning of keratinocyte sheets

Upon injury, a sequence of events starts in the epidermis to repair the wound. This healing process is divided into three phases: inflammation, proliferation and maturation. The biochemical mechanisms underlying the wound healing process involve a number of cytokines and growth factors [20]. The generation of a proteolytic environment by inflammatory cells infiltrating the wound site as well as prolonged upregulation of pro-inflammatory cytokines and chemokines inhibits, for example, the normal progression of wound healing. One parameter that influences the productivity and duration of the biofactor production phase is cultivation temperature. The temperature-dependent release of biofactors has been demonstrated using the kinetics of individual factors [16,21]. With Chinese hamster ovary cells, it has already been shown that the yield of recombinant proteins and their quality can be improved at temperatures below 37°C [22].

As observed with Chinese hamster ovary cells, the experiments carried out in the present study showed that a standard temperature of 37°C led to an early and rapid increase in biomarker secretion in human keratinocytes as well. However, the secretion reached a peak value for only a short time and quickly dropped within the analyzed time window. By contrast, lower temperatures led to longer-lasting secretion of biomarkers, with higher overall yields at 33°C and lower yields at 31°C within the day 17 to day 25 observation window,

which was the graft harvest window. Cultivation conditions of 33°C for keratinocyte sheets resulted in optimal secretion of the angiogenic factors VEGF and PDGF-bb within the observed time window between day 17 and day 25 of culture. The increased level of released angiogenic factors in the keratinocyte sheets could favor the formation of blood vessels after transplantation *in vivo*. Furthermore, dermal wound repair could be improved by the enhanced release of the biomarker PDGF-bb, which is known to be a chemoattractant for fibroblasts, monocytes and neutrophils [23]. Patients have already been treated in the United States with PDGF-bb in completed randomized clinical trials to improve wound healing [24].

The pro-inflammatory biomarkers IL-1 and IL-8 further support the healing process *in vivo* [25]. In addition to the regulation of angiogenesis [26], IL-1 is a highly active and pleiotropic pro-inflammatory cytokine that stimulates keratinocyte growth and collagen synthesis by fibroblasts and regulates hematopoiesis [27] by inducing the production of different hematopoietic growth factors [28]. The release of the biofactor IL-1b seems to be less temperature-sensitive during keratinocyte sheet formation compared with the other biomarkers tested. At all three temperature conditions, a comparable IL-1b release over time was observed. During the inflammatory phase of wound healing *in vivo*, TNF-α is also an important pro-inflammatory mediator. As with pro-inflammatory cytokines, high levels of matrix metalloproteinases have been found in inflammatory states such as chronic wounds. In full-thickness human skin organ cultures, TNF-α stimulates the release of active matrix metalloproteinase 2, which is a type IV collagenase. This suggests a mechanism whereby TNF-α can promote wound healing by indirectly stimulating inflammation and increasing macrophage-produced growth factors [29].

The anti-inflammatory properties of the cytokines IL-10 and IL-4 demonstrate a negative feedback on keratinocytes when cultured at lower temperatures. The ability of IL-10 to facilitate regenerative healing is likely the result of pleiotropic effects through regulation of the inflammatory response [30] as well as upregulation of endothelial progenitor cells. Epidermal keratinocytes are a major source of IL-10 in skin. The production by keratinocytes *in vitro* was most notably upregulated at 33°C compared with the conventional culture at 37°C. The induction of IL-10 may contribute to the anti-inflammatory effect leading to local and systemic immunosuppression after autologous keratinocyte sheet transplantation.

The second investigated anti-inflammatory cytokine, IL-4, is able to activate connective tissue cells and stimulate the accumulation of extracellular matrix macromolecules such as collagen I and III. It regulates extracellular matrix biogenesis via pre-translational mechanisms [31] and might be involved in the regulatory role of epithelial differentiation [32]. In experimental studies, the topical administration of IL-4 to wounds has shown significant acceleration of the healing rate [33]. In the current study, overall higher levels of secreted IFN-γ could be observed when sheets were cultivated at 33°C or 37°C. As a multi-functional immunomodulatory cytokine, IL-4 initiates in keratinocytes the host's anti-viral defenses [34]. Furthermore, it is involved in angiogenesis and collagen deposition [35] and induces cytokines specific to mononuclear cells. The IFN-γ levels when sheets were cultivated at 31°C seemed to only increase from day 23 onward—a typical delay in secretion shown for many of the investigated biomarkers.

Table 1
Influence of thermal conditioning at 31°C, 33°C and 37°C on secretome release of selected biofactors during keratinocyte sheet formation.

Effect	Analyzed biofactors	31°C	33°C	37°C
Immunomodulatory	IFN-γ	–	++	++
Anti-inflammatory	IL-10, IL-4	–	++	+
Pro-inflammatory	IL-1b, IL-8, TNF-α	–	++	+
Angiogenic	VEGF, PDGF-bb	–	++	+

++ high, + medium, – low release.

These results demonstrate that a cultivation temperature of 33°C beneficially affected the production of immunomodulatory (IFN-γ), anti-inflammatory (IL-10, IL-4) and angiogenic (VEGF, PDGF-bb) biofactors by keratinocytes during sheet formation (Table 1). Pro-inflammatory factors (IL-1b, IL-8, TNF-α) were also more expressed at 33°C and may play a role in wound healing through a positive feedback loop. Overall, the *in vivo* effect of various biofactors secreted by the cultivated sheets could not be accurately predicted *in vitro*. However, cultivating sheets at 33°C showed the highest and most sustained levels of biofactor release during the investigational graft harvest window 17–25 days after biopsy harvest. This would indicate that such active grafts may exhibit a better take rate and stimulate the wound bed to accelerate wound closure and healing once implanted in patients.

Although this study analyzed the keratinocyte sheet secretome in the supernatant of sheet cultures, it may also be valuable in future studies to determine protein levels in the graft itself and how these may be influenced by thermal conditioning.

Storability

The regeneration of skin after skin graft transplantation depends mainly on the viability of the graft. Viable grafts facilitate further remodeling of the wound bed *in vivo* compared with non-viable transplants [36]. In case of unforeseen events, the clinical process may be delayed. It is questionable to what extent such short-term storage would affect the viability of enzymatically harvested keratinocyte sheets. It has been shown that cultured epidermal allografts can be stored long-term through cryopreservation [37]. However, to the authors' knowledge, a shelf life between sheet harvest and transplantation has not yet been described. The authors' experiments showed that the first 12 h of storage effected the greatest loss of viability regardless of medium, temperature and presence of transfer gauze. After 24 h of storage, it could be clearly seen that the effect diminished. Compared with the sheets kept in storage without gauze, those placed on Jelonet showed reduced viability. Nevertheless, transfer gauze is necessary for handleability, and therefore investigating various carriers may be valuable for maximizing graft stability prior to implantation.

Although viability drops significantly after 12 h and may reach levels below 50% of initial viability (0 h), the 50% threshold reference is not indicative of clinical efficacy potential. Indeed, sheets may recover after transplantation, and their efficacy in improving wound healing may not require high viability at the time of transplantation. Although there may be indications that the *in vivo* milieu may cause cell death in transplanted cell product [38], the required dosage of viable cells at transplantation has not yet been linked to clinical efficacy. The findings in this study simply show that the tested media had no significant influence on cell viability, but temperature appeared to have an effect, with a lower storage temperature systematically improving viability in all groups at all time points from 24 h onward.

The sheets maintained a similar viability of 34 ± 20% (mean gray value, 14 ± 5) (Figure 8E) on the transport gauze Jelonet compared with the control without transport gauze (viability, 32 ± 11%, mean gray value, 13 ± 5) (Figure 8F) when kept at 4°C in CnT-2D medium. When using Jelonet as a carrier gauze, it would therefore be preferable to store harvested sheets at 4°C in CnT-2D medium until transplantation is possible. That being said, investigating alternate carrier materials side by side may be beneficial in improving not only storage viability but also keratinocyte sheet bioactivity and take rate.

Influence of temperature and pH buffer systems

To maintain the physiological pH value during *in vitro* cultivation, suitable buffer systems have to be used. Thermal conditioning experiments can have the limitation of coupling temperature changes to pH changes simply as a result of CO_2 solubility. The most common buffer

systems include physiological bicarbonate buffer (CO_2/bicarbonate) and non-volatile buffers such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Compared with buffering with bicarbonate, HEPES buffer maintains the physiological pH value despite temperature-mediated changes in CO_2 solubility [39]. At cultivation temperatures below 37°C, HEPES shows more effective buffering for maintaining enzyme structures and functions since its dissociation decreases with decreasing temperatures [40]. In this study, temperature and pH changes could be decoupled using the chosen CELLnTEC medium, which contains both a bicarbonate and a HEPES buffering system. Bicarbonate and HEPES would be resistant to temperature-mediated changes in CO_2 solubility and therefore the culture medium pH in the temperature ranges used in this study.

Conclusions

The authors' study may have important clinical implications, in that the expanded window of grafting keratinocyte sheets (21 ± 4 days after biopsy harvest) will give surgeons the flexibility to decide when a grafting procedure would be most successful based on the patient's health status. Primary keratinocytes display high proteomic consistency related to constitutional factors such as sex, age and anatomic site [41]. However, hypothermic conditioning clearly affects the secretion of biofactors during keratinocyte sheet culture. The optimized temperature conditions of 37°C during the cell proliferation phase and 33°C within the differentiation phase of human keratinocytes reflect the native temperature gradient in normal healthy skin tissue [17]. By using 37°C during the proliferation phase and 33°C during the sheet formation phase of human keratinocytes, the production of cultivated skin grafts can be accelerated and the quality of the grafts, as well as their bioactivity, can be improved, particularly at 33°C. Furthermore, using 4°C during biopsy storage and sheet storage maximizes cell yield and viability. With this protocol optimization, a flexible time window of day 18 to day 25 was created in which a transplantation could take place. These results could be of great benefit with regard to future clinical approaches to the production of high-quality keratinocyte sheets and could significantly improve the treatment of burn patients.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: L. Frese, S.E. Darwiche, M. Calcagni. Acquisition of data: L. Frese. Analysis and interpretation of data: L. Frese, S.E. Darwiche, M. Calcagni. Drafting or revising the manuscript: L. Frese, S.E. Darwiche, B. von Rechenberg, S.P. Hoerstrup, P. Giovanoli, M. Calcagni. All authors have approved the final article.

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