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# Keratinocyte Monolayers on Hyaluronic Acid Membranes as "Upside-Down" Grafts Reconstitute Full-Thickness Wounds

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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**Background:** Skin replacement by means of cultured epithelial keratinocytes is a well-accepted method. However, several clinical drawbacks of sheet autografts (CEA – cultured epithelial autografts) have stimulated various efforts to optimize cell culture and cell delivery. Recent developments include use of cell monolayers instead of a fully differentiated epithelium, as well as use of various biomaterials to grow and transport the cultured cells.
To optimize the transfer of human keratinocytes directly to the recipient wound bed, we used an "upside-down" technique, delivering cultured cells directly to the wound with the carrier material on top.

**Material/Methods:** Subconfluent second-passage human keratinocyte monolayers on esterified hyaluronic acid membranes (KHAMC – Keratinocyte-Hyaluronic-Acid-Membrane-Composites) were transplanted either as upside-down grafts or as upside-up grafts onto standardized full-thickness wounds in athymic nude mice versus controls with the cell-free membrane alone.

**Results:** In the upside-down group, 14 days after grafting, a multi-layered, differentiating epidermis was found, whereas the wounds in the upside-up group and in the control group were not completely closed up to day 21. Persistence of human keratinocytes was shown in the upside-down group only, from day 7 until day 35 after grafting.

**Conclusions:** This study confirms that upside-down grafting of subconfluent monolayers of serum-free cultured human keratinocytes on esterified hyaluronic acid membranes is a suitable means to transfer actively proliferative keratinocytes, and reduces wound contraction. Compared to standard grafting protocols of cultured epithelium, such as CEA sheet grafts, it is easier to apply, does not need enzymatic detachment of cells from the culture dish, and limits the number of production steps required.

**MeSH Keywords:** Cell Culture Techniques • Keratinocytes • Re-Epithelialization • Tissue Engineering • Wound Healing

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## Background

Restoration of skin is vital in severe burns. When major parts of the body surface are injured in extensively burned patients, the traditional autograft or allograft is not sufficiently to accomplish a coverage. Transplantation of cultured human epithelium after cell expansion *in vitro* has therefore become an accepted method to treat major burns [1–3]. Nevertheless, several drawbacks have hampered the use of cultured epithelial sheet grafts or engineered skin substitutes (ESS) as such. One reason is the extremely variable overall "take rate", prolonged culture periods, lack of long-term graft adherence, chronic blistering, excessive scarring, and difficulties in handling the fragile grafts. These obstacles have prompted efforts to improve keratinocyte growth and delivery systems. Synthetic, semisynthetic, and biological carriers have been utilized to facilitate keratinocyte delivery to the recipient wound [4–6], and different combinations of cell transfer techniques, as well as composite approaches, have been investigated [7–10].

Among the possible carrier materials used for keratinocyte delivery [9,10], hyaluronic acid (HA) was identified as a promising substance because of its important role in wound healing, by virtue of its abundance in skin. It has been shown to have a stimulatory effect on keratinocyte proliferation both *in vitro* and *in vivo* [11] and to play an important role in scarless fetal wound healing [12].

To overcome the problem of injuring the anchoring filaments of cultured keratinocytes in epithelial sheet grafting when dissolving the cells from the culture dish, we postulated that directly delivering the cultured cells to the wound with the carrier as a backup would facilitate handling and optimize cell attachment to the wound surface. In a previous study we were able to show the feasibility of monolayers of non-confluent cultured human keratinocytes on a bovine collagen membrane to resurface full-thickness wounds when transplanted in an upside-down fashion [13]. While this method showed rapid resurfacing, the problem of wound contraction remained. Since HA has been reported to be associated with scarless fetal wound healing and thus minimize scarring, we investigated the potential use of this substrate as a carrier for the transfer of cultured keratinocytes and as a biomaterial for enhancing tissue repair [12,14].

## Material and Methods

### Cell culture

Skin biopsies for keratinocyte cultures were voluntarily provided by patients undergoing routine operations with skin graft procedures, and informed consent was obtained from all subjects (ethics approval Nr. 264_13 B Friedrich Alexander University Erlangen-Nürnberg). Proliferative human keratinocytes were isolated from fresh human skin with 0.25% Dispase (Boehringer, Mannheim, Germany) after incubation at 40°C for 2 h. Epidermal cells were then isolated into a single-cell suspension by treatment with 0.05% trypsin and 0.02% EDTA (Gibco, Germany) at 37°C for 30 min, resuspended, and expanded in 75 $cm^2$ polystyrene tissue culture flasks in serum-free media containing EGF, BPE (Gibco, Germany), and gentamycin (Merck, Germany) at 5% $CO_2$ and 37°C. At 60–70% subconfluence, the cells were then harvested from the flasks by trypsinization and then re-incubated in a concentration of $1\times10^6$ per 75 $cm^2$ culture flask [4].

### Hyaluronic acid membranes

Commercially available esterified micro-perforated hyaluronic acid membranes used as a substrate for keratinocyte growth were prepared by phase inversion technique from a 100% esterified benzyl derivative of the hyaluronic acid HYAFF 11 (Laserskin®, Fidia Advanced Biopolymes, Abano Terme, Italy). This product is insoluble in water and soluble in DMSO (>50 mg/ml). Its molecular weight and polydispersity are 145 000 Da. The polymer exhibits good stability to hydrolysis at acid pH and has a high surface energy and hydrophilic behaviour [14]. Membranes were stored at room temperature. For this experiment, membranes with a thickness of 25 µm with perforations were used. The holes (6000/$cm^2$) were made using an UV Laser and were 40 µm in diameter and spaced at intervals of 80 µm, according to the manufacturer's description.

### Preparation of keratinocyte-hyaluronic-acid-membrane-composites (KHAMC)

Human keratinocytes, freshly dissociated from skin samples, were cultured serum-free with defined media according to standard protocols [4,13]. Second-passage keratinocytes were enzymatically detached and isolated when a confluence of 60–70% in the culture flask was achieved (Figure 1). Cells were then inoculated onto a commercially available micro-perforated HA membrane at a density of $4\times10^6$ cells per $cm^2$ and then were submerged in culture medium for 3–5 days until subconfluence was reached on the membranes, as described previously [13] (Figure 2A, 2B).

### Animals

Experimental subjects were athymic nude mice (Balb/c-01aHsd-nu/nu IR; age 6–8 weeks, which were kept in single cages with a Duoflo (Bioclean Lab Procedures, Inc., Maywood, NJ, USA) laminar air-flow at 37°C and 12 h light exposure (government approval Nr. G-98/17 AZ 121.1).





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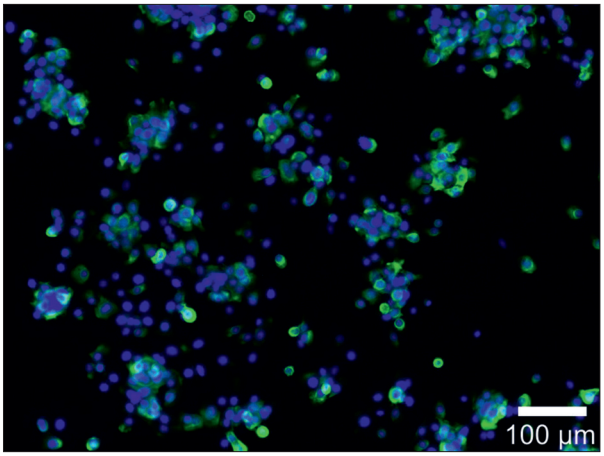


**Figure 1.** Cytokeratin (type 5, 6, 8, 17, and 19) immunofluorescence staining of primary human keratinocytes (green). Counterstaining was performed with DAPI (blue).

### Transplantation procedure

Under aseptic conditions, reproducible standardized 2×2 cm rectangular full-thickness skin wounds were created on the back of anaesthetized nude mice. In group A, 12 freshly created wounds were covered with subconfluent monolayers of cultured human keratinocytes on esterified HA membranes as upside-down grafts with the subconfluent keratinocytes directed towards the wound bed and the HYAFF on top (KHAMC=Keratinocyte-Hyaluronic-Acid-Membrane-Composites) or with the HA membrane underneath (conventional technique=upside-up, as propagated by the manufacturer) (group B, n=12) or with the HA membrane alone (group C, n=12) (Figure 3).

The wounds were dressed with a semipermeable adhesive film (Op-Site, Smith&Nephew, Largo, Fl, USA) and tie-over dressing, consisting of a Vaseline gauze (Adaptic, Johnson & Johnson, New Brunswick, NJ, USA) and a dry cotton gauze, fixed with sutures to the wound margins. Daily inspections were performed to ensure the integrity of the dressings. Biopsies were taken on days 7, 14, 21, and 35 after grafting. Wounds were totally excised, including a small border zone of untreated skin and underlying muscle, and processed for HE staining, as well as immunohistochemical and electron microscopy staining procedures.

### Wound contraction

We performed computer-based image analysis of wound size by photoplanimetric evaluation of the surface area on sequential standardized photographs on days 1, 7, 14, 21, and 35 after transplantation. Data for wound contraction are expressed as a percentage of the original wound area (mean ±SEM). Data from test groups in wound contraction studies were subjected to analysis of variance and Tukey studentized range test, with significance accepted at the 95% confidence level ($p<0.05$).

### Graft acceptance

Graft acceptance was determined by direct immunofluorescence staining of healed epidermis with a fluorescein-labelled monoclonal antibody against HLA-A, B, and C histocompatibility antigens (Sigma-Aldrich, Munich, Germany).

### Conventional microscopy, Immunohistochemical microscopy, Transmission electron microscopy, Scanning electron microscopy

Biopsies from all groups covering full-thickness wounds excised down to the muscle fascia were obtained between the 7$^{th}$ and 35$^{th}$ days after transplantation. Briefly, histological sections

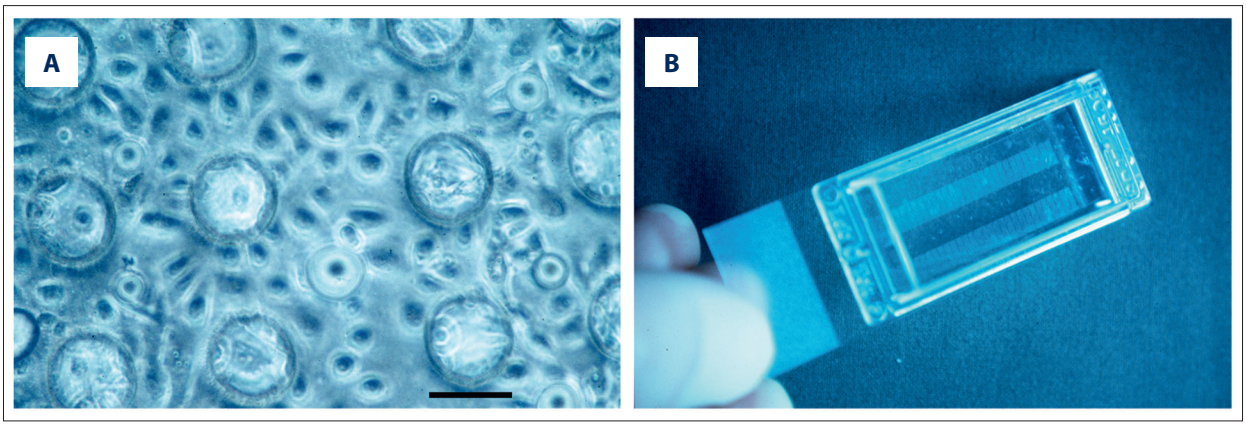


**Figure 2.** (**A**) Subconfluent layer of cultured human keratinocytes on top of a laser-perforated esterified hyaluronic acid (HAYFF) membrane of 25 micron thickness with 40 micron wide regular sized circular laser cut holes/microinterstices; scale bar=40 microns, (**B**) Keratinocyte seeded grafts on laser-perforated HAYFF membrane in a culture dish ready for transplantation size cut to 2×6 cm pieces to be inserted into culture-chamber.





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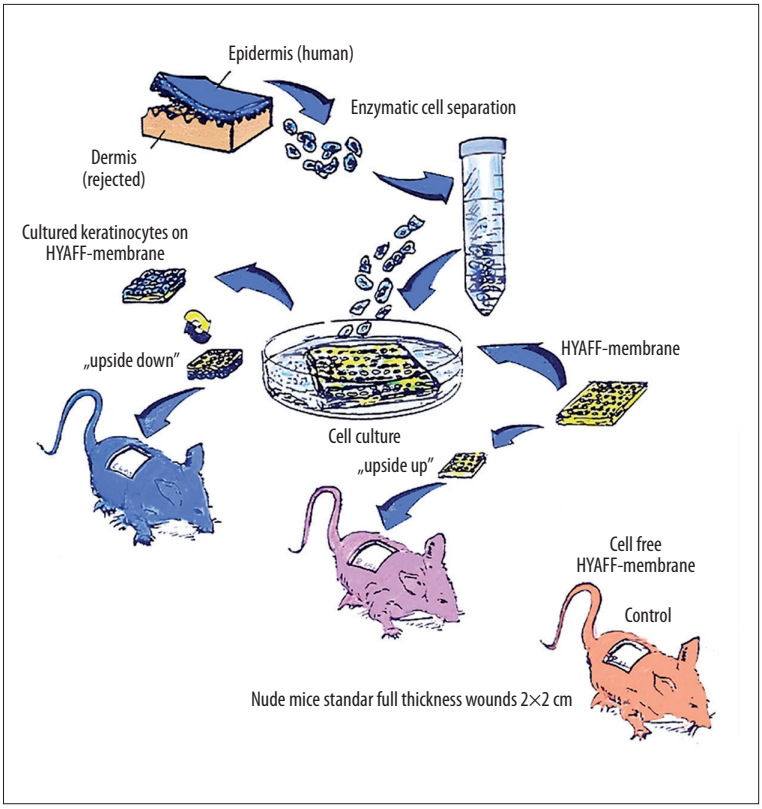


**Figure 3.** Experimental design of upside-down, upside-up, and cell-free control grafting procedures to full-thickness nude mouse wounds.

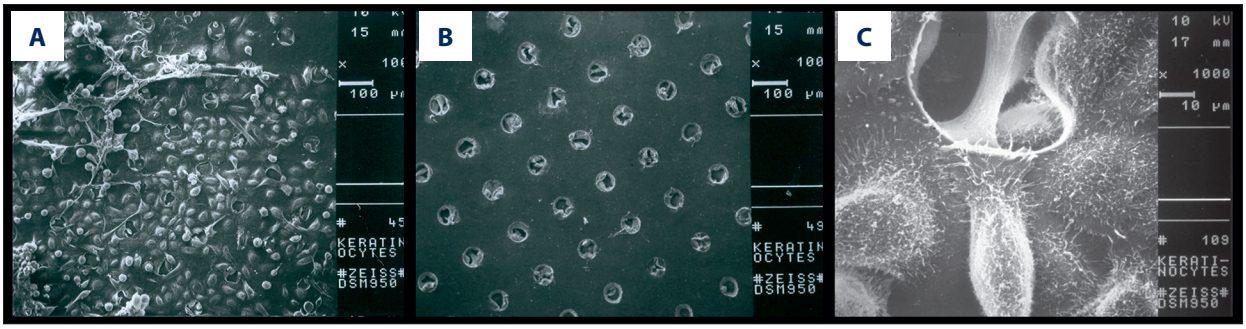


**Figure 4.** (**A**) Scanning electron microscopy of subconfluent keratinocyte monolayer on laser-perforated HYAFF membrane (scale bars=100 ym) and (**B**) undersurface of membrane without cells (scale bars=100 ym, (**C**) Scanning electron microscopy of cultured human keratinocytes on perforated HYAFF membrane migrating through the holes (scale bars=10 ym) (all scale bars=visible on right side of images **A–C**).

were fixed in formalin and embedded in paraffin and HE stained for routine histological examination. Immunohistochemistry, immunofluorescence, and ultrastructural electron microscopy were performed in all experimental groups between the 7th and 35th days after transplantation. For further details of the staining protocols, see previous publications [13].

## Results

Human cultured keratinocytes, cultivated in serum-free medium and without feeder layers of fibroblasts, seeded onto non-woven HYAFF-11 membranes were able to attach to the biomaterials, grow, and expand in number. Expansion occurred within 3 days to a 60–70% subconfluent monolayer and the composites were then ready for grafting to experimental animal wounds (Figures 2, 4).

### Results of upside-down grafting KHAMC to nude mice full-thickness wounds

*Wound healing*

KHAMC with 60–70% subconfluent monolayers of human keratinocytes transplanted upside-down with the cells in direct





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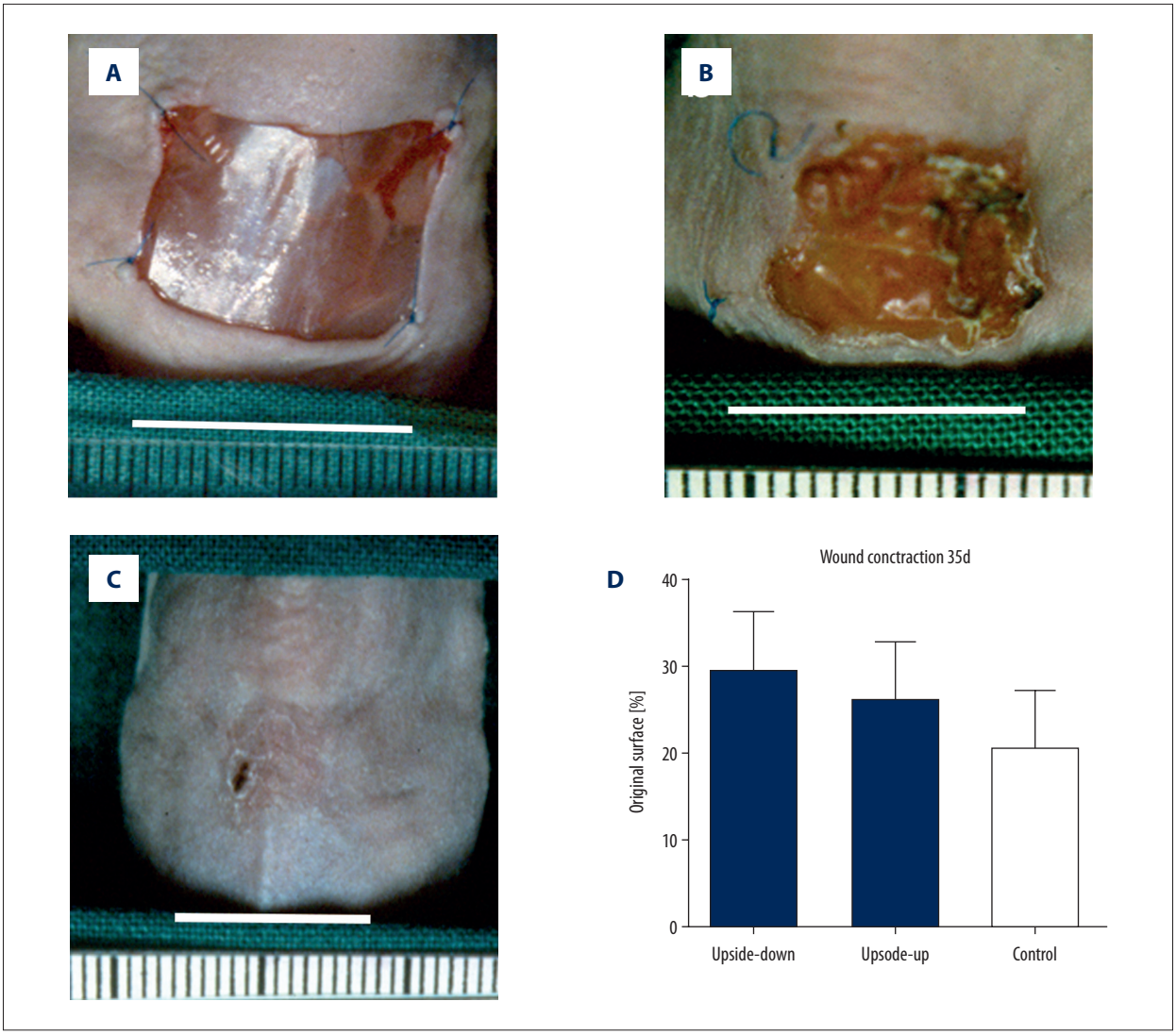


**Figure 5.** (**A**) Full-thickness wound on the back of a nude mouse with sutures to fix the 4 edges of the wound to the undersurface, (**B**) upside-down grafted human keratinocyte grafts of HYAFF membranes on the back of a nude mouse at day 7 after grafting, (**C**) Wound at day 35 after human keratinocyte grafts of HYAFF membranes on the back of a nude mouse, scale bars=20 mm (**D**) Wound contraction in% of the original wound size for KHAMC upside-down grafting procedure, upside-up grafting procedure and control with cell-free HYAFF membrane at day 35

contact to the wound and the biomaterial on top of the cells adhered to the full-thickness wounds within a few hours after transplantation, as did the upside-up transplants and the membranes alone. By gross inspection, there was no visible difference in grafted material adherence to the wound surfaces within the first week between the groups A, B, and C.

*Wound contraction*

During the observation period of 5 weeks, the size of all wounds decreased in the original wound area, with variations among the groups. In Group A (upside-down group) on day 35 after transplantation, the wound size was 29.48% of the original wound size (p=0.05), in Group B (upside-up group) the wound size was 26.04% of the original wound size (p=0.05), and in the control group (Group C) it was 20.43% (n. s.) of the original wound size (Figure 5). Due to the decreasing number of animals towards the end of the study, these differences were not statistically significant on day 35 at the end of the study.

*Wound morphology*

Histological findings

Examination of biopsies from mouse wounds receiving KHAMC grafts by using the upside-down technique revealed a thin





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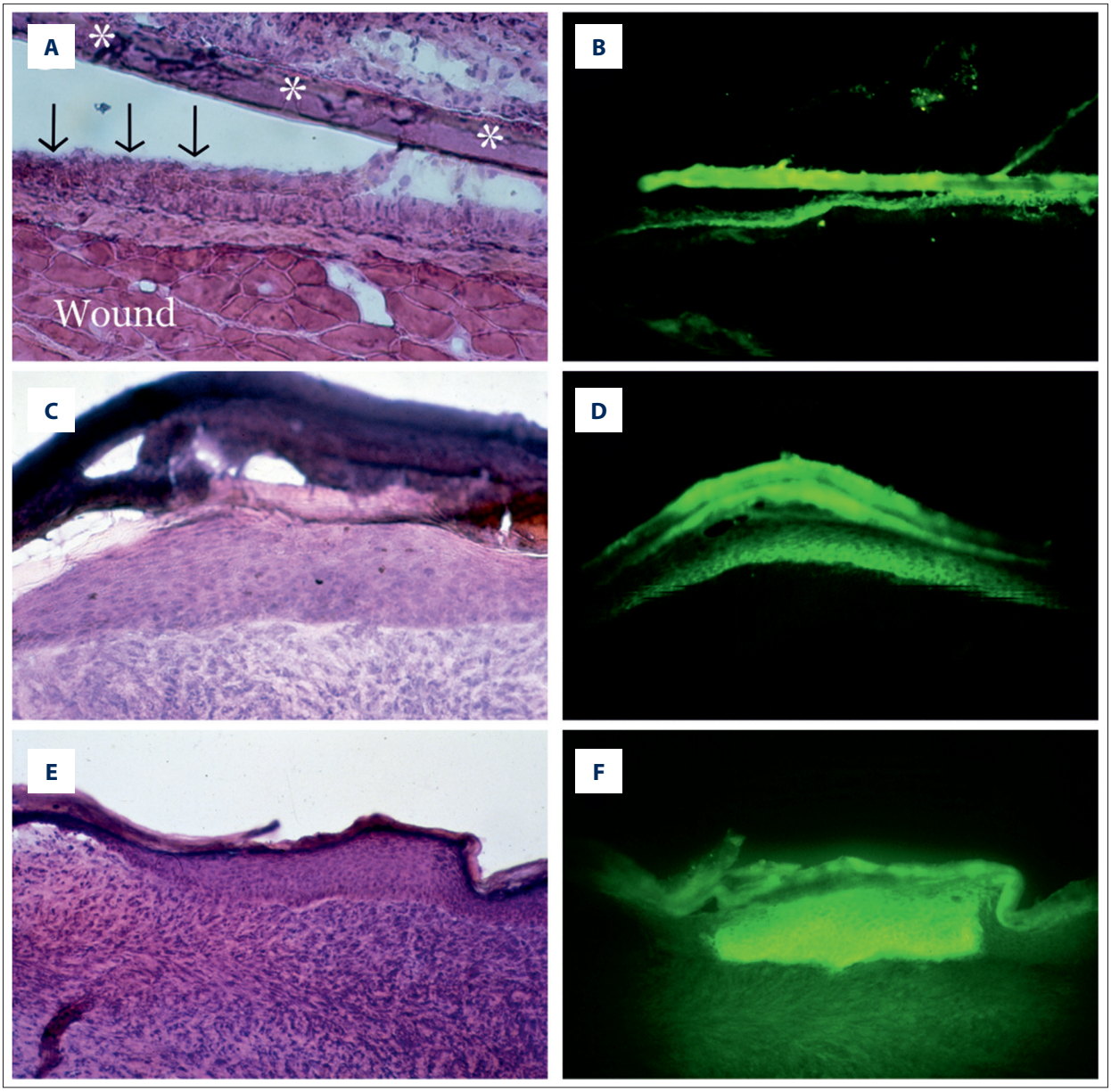


**Figure 6.** Cross-section of a healing wound after upside-down grafting of KHAMC at day 7 [HE staining (**A**), Anti-human HLA (**B**)], and on day 14 [HE staining (**C**), Anti-human HLA (**D**)]. Typical appearance of a healing wound after upside-down grafting of KHAMC at day 21, [HE staining (**E**), Anti-human HLA (**F**)]. * Membrane.

sheet-like epithelial structure with obvious attachment to the wound ground as early as day 7 after transplantation (Figure 6A, 6B). Until day 21, a multi-layered, cornifying epidermis was formed (Figure 6C–6F). The subepidermal tissue was well vascularized and revealed many collagenous fibers. The human origin of the neoepithelium was confirmed in all biopsies by staining with an anti-human HLA-A, B, C antibody. Positive anti-human HLA- A, B, C staining was detected up to day 35 after grafting. Wounds of the upside-up group and the control group consisted mainly of granulation tissue, and an organized epidermal layer could be detected only from day 21 after the operation (Figure 6E, 6F). The anti-human HLA- A, B, C staining in those groups remained negative throughout the complete observation period of 35 days, apart from a local anti-HLA- A, B, C positive area in 1 animal of the upside-up group on day 7 after transplantation.

A reconstituted dermal-epidermal junction zone was detected in the upside-down group by anti-laminin staining on day 14 and in the other groups on day 21 after grafting (Figure 7). Immunohistochemical staining with a human-specific collagen type VII antibody was positive in the upside-down group





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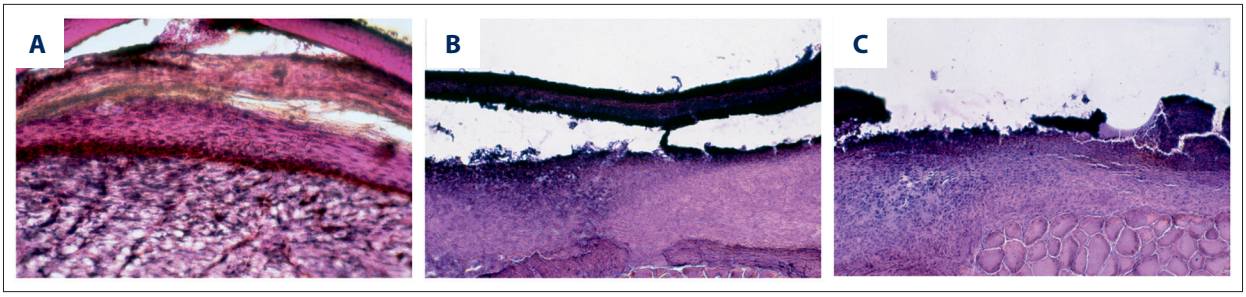


**Figure 7.** Anti-laminin staining of a healing wound after upside-down grafting of KHAMC at day 14 (**A**), after upside-up grafting of KHAMC at day 14 (**B**), and cell-free HYAFF membrane (**C**).

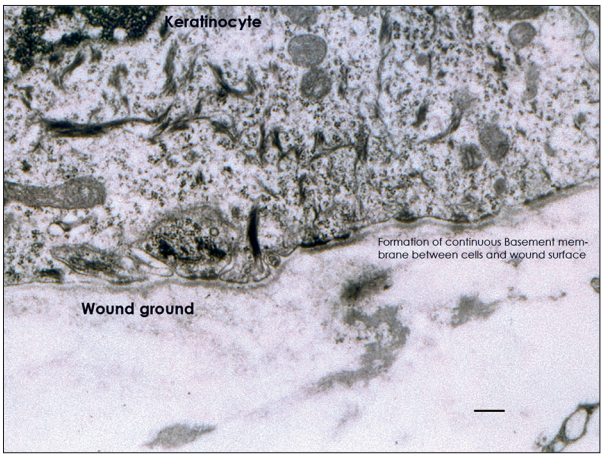


**Figure 8.** Transmission electron micrograph at day 35 after KHAMG grafting with development of a continuous double-structured basement membrane between keratinocyte and wound ground (scale bar=1 µm).

by day 14 after transplantation of the human keratinocytes, whereas in the upside-up group and in the control group, no human-specific structures were found.

Ultrastructural findings

Representative biopsies drawn from re-epithelialized nude mice wounds at day 35 after engraftment of the different composite grafts were analysed by transmission electron microscopy (Figure 8). Upside-down grafted areas revealed a fully differentiated, cornifying, intact epidermal structure, as well as a continuous basement membrane with numerous hemidesmosomes and tonofilaments at day 21, whereas the upside-up group and the control group showed fewer epidermal layers and an immature basement membrane.

## Discussion

This process of repair and the subsequent reorganization of the dermal matrix following an injury with skin loss is known as scar formation and maturation. The restoration of lost dermis is achieved by scarring, with the exception of fetal repair at specific gestational ages of specific species [15]. Hyaluronan has been repeatedly reported to be involved in scarless fetal wound healing, and the repair of fetal wounds shows a variety of responses in regard to wound healing and scarless repair [16]. Open wounds made in fetal sheep at mid-gestation close and show scarless repair, and suture-closed wounds heal in a scarless manner depending upon the species of animal and its gestational age when the fetus was wounded [17,18]. Therefore, the utilization of hyaluronan to optimize scarring and to promote healing of full-thickness wounds seems worthwhile. It has been applied in combination with cultured human keratinocytes to treat wounds [18] or as a method to retain wound fluid with a reduction in wound contraction [19]. Hyaluronic acid membranes with 40–60 µm holes and a distance between the centers of the holes of 120 µm with vertically oriented pores have been shown to be potential carriers of cultured human keratinocytes [20]. However, other researchers used laser-perforated HYAFF membranes with stratified multilayered cultured epithelial cells on top of the carrier and used a feeder layer of irradiated fibroblasts to promote keratinocyte growth *in vitro* [21]. Such sheets have been proposed to be superior in terms of their physiological appearance in the laboratory when compared to standard sheet grafts in culture flasks [21]. In superficial burns, this approach has been found to lead to earlier re-epithelialization than in control patients [21].

However, in such systems, the keratinocytes meant to attach to the wound bed have to migrate through the laser-drilled holes until they can reach their proper destination. It seems logical that differentiated cells located in the upper cell layers on top of the membrane might not contribute to the wound healing capacity of cell transplantation clinically until cells are connected with the recipient microcirculation. Models to enhance performance of tissue engineered grafts via arterio-venous microvascular loops are promising, but are not suitable for skin replacement yet [22,23]. Many of these keratinocytes might be lost due to lack of nourishment as a result of inadequate diffusion hindered by the carrier *in vivo*. This is one of the well-known problems clinically encountered with composite grafts [24]. Under *in vitro* culture conditions, epithelial





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reformation can occur beneath the hyaluronic membrane undersurface when an appropriate attachment site is provided [21]).

In our transplantation system, monolayers of keratinocytes (instead of multi-layered cell sheets) are directly transplanted towards the wound bed with the carrier on top. This guarantees an optimal delivery of the cultured cells to the wound bed and an optimal cell yield. Using non-confluent monolayers makes use of the proliferative capacity of undifferentiated epithelial cells and delivers the keratinocytes at a very early time after seeding them onto the biomaterial. This is of clinical relevance in the treatment of major burns, where the time until reepithelialisation can be achieved is critical for patient survival. In an earlier trial, we found that dense membranes of collagen type I are able to carry monolayers of cultured human keratinocytes to wounds when transplanted with the cells in an upside-down manner [13]. However, the handling of such membranes is more difficult than with HYAFF membranes, which have different physical properties. In addition, the wound contraction was reduced more efficiently than with the use of collagen membranes [15]. It has been speculated that so-called activated keratinocytes, unlike "sedentary" keratinocytes (those of the well-organized and well-differentiated epithelial layers) are the key elements to reconstruct an epithelium on a wound bed [25].

The lack of adherence of standard cultured multi-layered sheet grafts as a potential source of blistering has been described earlier. It has been associated with the enzymatic detachment of cultured cells from the culture dishes [26]. Using biocompatible carriers like hyaluronan membranes that can be simply peeled off the petri dish without enzymatic digestion provides an obvious simplification in handling the grafts.

No potentially unfavorable enzymatic treatment of the grafts is necessary prior to the application, which might enhance the proper function of epithelial cells after their delivery to the wound.

Data suggest that the hyaluronic acid-rich wound matrix may play a pivotal role in fetal scarless wound healing and may be involved in cell migration and in stimulating cell function [27], although the underlying mechanisms remain unknown. Esterification of HA has made it possible to create HA membranes of nearly any design suitable for *in vitro* and *in vivo* studies and as a biomaterial suitable for the transplantation of epithelial cells [21,28,29]. A diminished wound contraction rate has been found compared to pure epithelial grafts in similar series with other transplantation techniques and different biomaterials [4,13]. In our study, the HA was placed on top of the keratinocyte monolayers without the use of additional fibroblast feeder layers, as previously published by others. In our model, a possible influence of HA would suggest that degradation products of this biological matrix may be incorporated into the healing wound and interact with the intercellular matrix. Others have shown the presence of HA in extracellular matrix modulated collagen synthesis in the process of wound repair in experimental animal wounds, with a higher content of type III collagen. Also, a possible interaction of HA with fibroblasts on tissue regeneration, and especially mesenchymal stem cells delivered conventionally or with 3D printing [30], could play a role in the reduced contraction

However, it must be stated that in contrast to classical cultured composite skin graft techniques, the dermal regeneration is not addressed with this special epithelial transplantation technique. Nevertheless, this animal model serves as a proof of principle of epithelial regeneration in a standardized and reproducible full-thickness wound model. Further research on the potential mechanisms by which HA influences wound healing and tissue regeneration in this context is necessary.

The use of biologically active and tissue-friendly membranes as a cell carrier that offer possible additional advantages on wound healing is therefore of great interest [31]. HYAFF lends itself in this context as a biodegradable scaffold, having a hyaluronic acid-rich environment that has embryonic-like concentrations, and is suitable for *in vitro* cell culture. It can be cryopreserved and has good handling properties and thus an increased ease in handling cultures with a reduced risk of rupture of cultivated tissue. So far, no relevant immunologic response to this material has been described. Due to the (in principle) adjustable or tailorable degradation rates and tailorable hydrophilicity and processability, further modifications according to the needs of the specific tissue regeneration guidance seem to be possible and should be evaluated according to the specific application and needs.

Our data confirm the possibility of this new modality of cell transplantation in the form of cultured monolayers of non-confluent keratinocytes to resurface full-thickness experimental wounds oriented directly towards the wound surface with the carrier on top (upside-down technique). This approach ensures that the cultured cells are delivered directly onto the wound surface and no cells are lost after their propagation on the biomaterial. The biomaterial can also serve as a dressing on top of the cultured cells.

## Conclusions

Overall, our study shows the efficacy of this method, which combines the *in vitro* expansion of graftable cells with the advantage of transplanting actively proliferating cells on an appropriate biological carrier. The reduction in time required for grafting compared to standard sheet grafts and the low





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wound shrinkage rate are attractive effects of this transplantation system, which can be of great value in the treatment of acute and severe wounds such as in burns.

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