Epidermal graft encourages wound healing by down-regulation of gap junctional protein and activation of wound bed without graft integration as opposed to split-thickness skin graft

Muholan Kanapathy1,2 | Nadine Hachach-Haram2 | Nicola Bystrzonowski2 | David L. Becker3,4 | Afshin Mosahebi1,2 | Toby Richards1,2

1Division of Surgery and Interventional Science, University College London, London, UK
2Department of Plastic and Reconstructive Surgery, Royal Free NHS Foundation Trust Hospital, London, UK
3Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore
4Institute of Medical Biology, A*Star, Immunos, Biomedical Grove, Singapore

Abstract

Wound coverage by split-thickness skin graft (SSG) and epidermal graft (EG) shortens healing time, with comparable outcomes. However, the healing mechanism of EG is not as well understood as SSG. The difference in the healing mechanisms of EG and SSG was investigated using gap junctional proteins, proliferative marker, and cytokeratin markers. Paired punch biopsies were taken from the wound edge and wound bed from patients undergoing EG and SSG at weeks 0 and 1 to investigate wound edge keratinocyte migratory activities (connexins 43, 30, and 26), wound bed activation (Ki67), and the presence of graft integration to the wound bed (cytokeratins 14 and 6). Twenty-four paired biopsies were taken at weeks 0 and 1 (EG, n = 12; SSG, n = 12). Wound edge biopsies demonstrated down-regulation of connexins 43 ($P = .023$) and 30 ($P = .027$) after EG, indicating accelerated healing from the wound edge. At week 1, increased expression of Ki67 ($P < .05$) was seen after EG, indicating activation of cells within the wound bed. Keratinocytes expressing cytokeratins 6 and 14 were observed on all wounds treated with SSG but were absent at week 1 after EG, indicating the absence of graft integration following EG. Despite EG and SSG both being autologous skin grafts, they demonstrate different mechanisms of wound healing. EG accelerates wound healing from the wound edges and activates the wound bed despite not integrating into the wound bed at week 1 post-grafting as opposed to SSG, hence demonstrating properties comparable with a bioactive dressing instead of a skin substitute.

KEYWORDS

epidermal graft, gap junctional protein, healing mechanism, skin graft, split-thickness skin graft

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1 | INTRODUCTION

Delayed wound healing is associated with a significant financial and resource burden both to the patient and the health care system.1 Wound coverage by skin grafting, such as split-thickness skin graft (SSG) and epidermal graft (EG), addresses this by shortening healing time; however, the donor site may be problematic where it becomes a second and often painful wound. A recent randomised controlled trial comparing SSG and EG reported that EG demonstrated superior donor site outcomes with faster donor site healing and lower donor site morbidity compared with SSG while having comparable wound-healing outcomes (EPIGRAAFT Trial).2 Patients receiving EG also experienced higher patient satisfaction compared with SSG. Besides that, previous studies by various authors have reported that EG can be performed in an outpatient setting, which may benefit patients who are at high risk for general anaesthesia.3-6

However, because of the difference in the anatomical construct of the two skin grafts, the mechanism of healing of EG may be different to SSG. The healing mechanism of SSG is well understood and is known to involve plasma imbibition, inosculation, revascularisation, and modification.7-10 On the other hand, the healing by EG is poorly understood and has been postulated to be influenced by the interplay of three main mechanisms: keratinocyte activation, growth factor secretion, and reepithelialisation from the wound edge.11 Furthermore, the integration of EG into the wound bed after grafting, or the clinical “take” of the graft, has been debated, with opposing reports in the literature.3,4,6,12 To date, the in vivo wound-healing mechanism of EG has yet to be evaluated in patients. Moreover, the difference in the mechanisms of healing of SSG and EG has not been compared against one another in a standardised patient population and within a controlled trial.

The first of these mechanisms, which is wound bed activation following keratinocyte activation and growth factor secretion by direct interaction of the graft and the wound, can be evaluated by the pronounced expression of markers of cell proliferation on the wound bed, such as Ki67.7 Wound bed activation is followed by migration of keratinocytes from the wound edge, clinically recognised as reepithelialisation from the wound edge. A factor known to demonstrate migratory activity of keratinocytes at the wound edge that can be used as a marker of reepithelialisation is the gap junctional protein connexin.13-16 Connexin proteins are specialised clusters of plasma membrane channels that have multiprotein interactions, which influence both cellular adhesion and cytoskeletal dynamics and, therefore, cellular migration in wound healing.17 Precise communication via connexin proteins is integral to normal wound healing.16,17 Of the nine different connexins expressed in the human epidermis, connexins 43, 30, and 26 are the most abundant, with connexin 43 being the most ubiquitous.15 Overexpression of connexin proteins in the skin of patients with ulcers has been shown to delay keratinocyte migration, resulting in poor wound healing.13-15 Meanwhile, the down-regulation of connexin 43 accelerates wound healing.15,17 Finally, the integration of a graft into the wound bed can be confirmed by the presence of keratinocyte markers, such as cytokeratins 6 and 14, on the wound bed.18 The presence of these markers on the wound bed after a duration following grafting would indicate successful integration of the grafts into the wound bed.

The aim of this study was to explore the difference between the healing mechanisms of EG and SSG. Here, the mechanism of healing of EG was compared with SSG using a gap junctional protein to evaluate the migratory activity of wound edge keratinocytes, a proliferative marker to evaluate wound bed activity, and a cytokeratin marker to evaluate the integration of EG into the wound bed after grafting.

2 | METHODS

2.1 | Patient selection

Tissue biopsy was obtained from patients enrolled in a randomised controlled trial with two parallel groups: EG and SSG (EPIGRAAFT Trial).19 All consenting patients were randomly assigned to one of the treatment groups (allocation ratio of 1:1). By nature of the interventions, the surgical team, clinical staff, and patients were not
blinded to the procedure. The trial protocol and the final clinical results have been published.\textsuperscript{2,19} Tissue biopsies were collected from the first 12 patients in each study arm of the trial to be included in this study. Participants were recruited at the Royal Free London NHS Foundation Trust Hospital as per published protocols.\textsuperscript{19} This trial and tissue sample analysis were approved by the National Research Ethics Service Committee London-Fulham (15/LO/0556) and registered with ClinicalTrials.gov (NCT02535481). The inclusion and exclusion criteria have been summarised in Table 1.

### 2.2 Procedures and biopsy acquisition

All wounds were prepared as per normal clinical practice to achieve a healthy granulating bed, and a wound swab was taken to exclude infection. The wound bed was deemed “ready for grafting” following agreement between two senior clinicians.

EG was performed using a semi-automated EG system, the CelluTome Epidermal Harvesting System (Acelity, San Antonio, Texas),\textsuperscript{5,20} that enables multiple small EG to be harvested (totalling 1% of body surface area) with ease in an outpatient setting without the use of anaesthesia. The harvested EG was then transferred onto the wound using a non-adhering silicone dressing (Adaptic Touch, Systagenix, Gatwick, UK). The graft was then secured with a secondary dressing, based on wound type, for exudate control and secured with a crepe bandage or a Mefix dressing (Mölnlycke Health). The donor site was dressed with an occlusive dressing (Tegaderm Film, 3M).

SSG was performed in the operating theatre under general or local anaesthesia. Skin was harvested from the thigh using an air dermatome, at a thickness of 8/1000 in., and meshed to a 1:1.5 ratio. The wound was grafted and dressed using Adaptic Touch, gauze, and a Mefix or wool and crepe bandage depending on the site of the graft. The donor site was dressed with Kaltostat and secured with Mefix. The wound and donor site for SSG and EG were reviewed on day 7 ± 2 post-grafting and then at a weekly interval.

Skin punch biopsies (4 mm) were taken from two locations from all patients, at the centre of the wound (wound bed) and at the wound edge. This procedure was performed prior to grafting (week 0) and repeated 1-week post-grafting at the two similar locations.

### 2.3 Laboratory studies methodology

#### 2.3.1 Biopsy preservation and cryosectioning

All samples were blinded prior to analysis using anonymous codes. All biopsies were fixed overnight in 4% paraformaldehyde, then transferred to 20% sucrose in phosphate-buffered saline (PBS), and stored at 4°C until processing. Tissues were embedded in an optimal cutting temperature (OCT) medium (BDH-Poole, UK) and cryosectioned at a thickness of 10 μm using a Leica CM1900 UV cryostat (Leica, Wetzlar, Germany).

#### 2.3.2 H&E staining and analysis

All sections were stained with hematoxylin and eosin (H&E) using standard methods. Imaging was performed using the Zeiss AxioScan Z1 slide scanner at ×40 magnification and analysed using Zen 3.2 software (Carl Zeiss, Germany, 2019). The number of polymorphonuclear leukocytes on the wound bed was quantified based on the average number of cells in three separate areas, each measuring 500 μm by 500 μm, that best represent the skin section. Cell count was performed using Image J (http://imagej.nih.gov/ij/).

#### 2.3.3 Immunohistochemical staining for wound edge biopsies

Tissue sections were permeabilised for 15 minutes in 0.2% Triton X-100 and blocked using 0.1 M lysine-PBS for 30 minutes. Primary antibodies were prepared in PBS: anti-connexin 43 (1:4000; C6219, Sigma), anti-connexin

### Table 1 Inclusion and exclusion criteria

| Inclusion criteria | Exclusion criteria |
|--------------------|-------------------|
| 1. Male or female  | 1. Infected wound  |
| 2. Age ≥ 18, who after clinical review by a consultant Plastic Surgeon had been referred for skin grafting | 2. Wounds on the plantar aspect of the foot |
| 3. Wounds measured ≥1 cm × 1 cm and ≤6 cm × 6 cm with a healthy granulating wound bed | 3. Wounds unsuitable for SSG |
| 4. Able to comply with weekly visits and follow-up regime | 4. Uncontrolled diabetes mellitus (HbA1c ≥ 10%) |
| 5. Presence of one or more medical conditions, including renal, hepatic, hematologic, or autoimmune diseases; use of systemic steroid or immunosuppressant | 5. Presence of one or more medical conditions, including renal, hepatic, hematologic, or autoimmune diseases; use of systemic steroid or immunosuppressant |
| 6. Patient not fit for surgery (ASA classification ≥ 4) | 6. Patient not fit for surgery (ASA classification ≥ 4) |

Abbreviations: ASA, American Society of Anesthesiologists; SSG, split-thickness skin graft.
26 (1:200; 10202093, Fisher Scientific), and anti-connexin 30 (1:200; 10795723, Fisher Scientific). The tissues stained for connexin 43 were incubated with the primary antibody for 1 hour at room temperature, while tissues stained for connexins 30 and 26 were incubated overnight at 4°C. The tissues were then stained with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:400; 10729174, Fisher Scientific) at room temperature for 1 hour. Nuclei were stained using Hoechst (1:10 000; 10150888, Fisher Scientific) for 5 minutes. Coverslips were mounted using Citifluor (Citifluor Ltd, London, UK) and sealed with nail varnish.

2.3.4 | Immunohistochemical staining for wound bed biopsies

Tissue sections were immersed in Citrate Buffer solution (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) and placed in a water bath at 90°C for 15 minutes to break protein cross-linking to enhance staining intensity. Tissues were then rinsed with 0.1% PBS Tween 20 and permeabilised in 0.2% Triton X-100. Sections were then blocked with 0.25% Gelatin from cold water fish and 10% foetal bovine serum for 60 minutes. Primary antibodies were prepared in PBS: anti-Ki67 (1:400; rabbit polyclonal, ab15580, abcam), anti-cytokeratin 6 (1:100; mouse monoclonal, ab18586, abcam), and anti-cytokeratin 14 (1:100; mouse monoclonal, ab7800, abcam). Sections were incubated with the primary antibody overnight at 4°C followed by secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG [1:400; 10729174, Fisher Scientific] or DyLight 488 goat anti-mouse IgG [1:400; ab96879, abcam]) at room temperature for 1 hours. Nuclei were stained with Hoechst (1:10 000) and mounted using Citifluor (Glycerol/PBS solution, Citifluor Ltd) and sealed with nail varnish.

2.3.5 | Confocal microscopy

A Leica TCS SP8 confocal microscope (Leica, Mannheim, Germany) was used to obtain ×40 images: Hoescht was excited by a 405-nm laser, while Alexa Fluor and DyLight were excited by a 488-nm laser. All parameters were kept constant between weeks 0 and 1 to allow direct comparison.

2.4 | Connexin quantification

Confocal images for connexin proteins were captured at six locations along the epidermis per biopsy to ensure true representation of distribution, and the mean expression was calculated. ImageJ was used for connexin quantification. Images were converted to binary images, and the epidermal threshold was kept constant, set at 80, with a recognised pixel threshold size of 2 to infinity for all images. Regions of interest were manually marked to include the epidermis only. Data were presented based on wound type; acute (<6 weeks in duration) and chronic (≥6 weeks in duration).

2.5 | Assessment for Ki67

Confocal images for Ki67 at the wound bed were captured at three locations: 500 μm from each edge and one at the centre of the biopsy, with a zoom factor of 2.0 to magnify the nuclei. The number of nuclei expressing Ki67 was manually calculated. Because of damaged samples during processing, only samples from five patients in the EG group and four in the SSG group were included in the final analysis of Ki67.

2.6 | Assessment for cytokeratins 6 and 14

Cytokeratins 6 and 14 were imaged at three random locations along the wound bed. Qualitative assessment was performed to evaluate their expression on the wound bed.

2.7 | Outcomes

The cellular mechanism of healing was assessed from the paired punch biopsies taken at weeks 0 and 1 to investigate histological changes, wound edge keratinocyte migratory activities (connexin 43, 30, and 26), wound bed activation (Ki67), and the presence of the grafts on the wound bed after transplantation (cytokeratins 6 and 14).

2.8 | Statistical analysis

Data on the expression of connexin proteins and Ki67 were presented as mean ± standard deviation and compared using paired t test (weeks 0 vs 1). Normality testing was performed using the Shapiro-Wilk test. A P value of less than .05 was considered significant, and all tests were two-sided. All statistical analyses were performed using SPSS Statistics 24 software (IBM, Armonk, New York).
3 | RESULTS

3.1 | Patient demographics

Samples from the 24 patients were analysed (EG, n = 12; SSG, n = 12). There were 17 men (EG, n = 8; SSG, n = 9) and seven women (EG, n = 4; SSG, n = 3). The mean age of patients in the EG group was 62.92 ± 20.06 years compared with 67.00 ± 12.88 years in the SSG group (P = .06). There were 12 acute wounds (<6 weeks old) (EG, n = 6; SSG, n = 11) and 12 chronic wounds (≥6 weeks old) (EG, n = 6; SSG, n = 1). The most common wound aetiology was because of trauma (EG, n = 6; SSG, n = 4), followed by wound dehiscence (EG, n = 5; SSG, n = 1), skin cancer excision (EG, n = 1; SSG, n = 5), amputation (SSG, n = 1), and wound debridement (SSG, n = 1).

All patients had skin punch biopsies taken at the wound bed and the wound edge at weeks 0 and 1. A total of 96 biopsies were analysed.

3.2 | Analysis of wound edge biopsy

3.2.1 | Histological feature of the wound edge

There was no obvious difference in the thickness of the epidermis or the distribution of inflammatory cells seen between weeks 0 and 1 in either of the groups (Figure 1A).

3.2.2 | Connexin expression at the wound edge

A general down-regulation of the connexin proteins was seen at the wound edge after treatment, which was more prominent in the EG group. A different pattern of down-regulation was seen between acute and chronic wounds in all three connexin proteins.

A significant down-regulation of connexin 43 was seen in the chronic wounds after EG (P = .023) (Figure 2). Connexin 30 demonstrated a similar pattern of down-regulation, with significant down-regulation in chronic wounds after EG (P = .027) (Figure 2). Connexin 26, on the other hand, demonstrated down-regulation after treatment in both treatment arms; however, this was not statistically significant in either group or wound type (Figure 2).

In both groups, a similar pattern of change in the distribution of the three connexin proteins was observed after treatment, whereby a reduction in the expression of the connexin proteins was seen in the basal layer of the epidermis, and this was especially evident in connexin 43.

3.3 | Analysis of wound bed biopsy

3.3.1 | Histological feature of wound bed

At week 1, increased polymorphonuclear leukocytes, mainly neutrophils, were seen in both groups, although they were more pronounced in the EG group (Figure 1B). The increase appeared to be higher in the EG group (week 0 = 1805.22 ± 582.69 vs week 1 = 2141.44 ± 503.27, P = .109) compared with the SSG group (week 0 = 1648.78 ± 431.55 vs week 1 = 1841.69 ± 381.02, P = .260), although these were not statistically significant.

3.3.2 | Ki67 expression in wound bed biopsy

A general increase in the nuclear expression of Ki67 antigen was observed, which was significant in both acute (P = .017) and chronic (P = .048) wounds after EG (Figure 3). Despite an increased expression of Ki67 after treatment in the SSG group, statistical significance was not achieved (P = .124).

3.3.3 | Cytokeratins 6 and 14 expressions in wound bed biopsy

Keratinocytes expressing cytokeratins 6 and 14 expressions were observed on all wounds treated with SSG, but they were absent on all wounds treated with EG at week 1, signifying the absence of graft integration following EG (Figure 4).

4 | DISCUSSION

The healing mechanism of EG was previously postulated to occur via keratinocyte activation, growth factor secretion, and reepithelialisation from the wound edge, while there were inconsistent observations on the integration of the graft to the wound bed. Furthermore, the differences between the healing mechanisms of SSG and EG have not been compared against one another in a standardised patient population and within a controlled prospective study. Here, the healing mechanism of EG was explored and compared against SSG within the same clinical
The study revealed that both autologous skin grafts have different healing mechanisms. EG demonstrated down-regulation of epidermal connexin gap junctional proteins at the wound edge and activation of the wound bed while not achieving graft integration (clinical “take”) at week 1 post-grafting as opposed to SSG. These findings suggest that EG accelerates wound healing from the wound edges while activating the wound bed following interaction with the wound despite not integrating into the wound bed. Meanwhile, as previously known, SSG integrates into the wound bed and provides a skin coverage.

The connexin gap junctional proteins were used as a marker of migratory activity of the wound-edge keratinocytes in this study. The connexin proteins are specialised clusters of plasma membrane channels that facilitate communication between adjacent cells and also act as a nexus, interacting with adhesion molecules, tight junctions, and cytoskeletal components, either directly or via adaptors. Up-regulation of connexins 43, 30, and 26 in chronic wounds is known to impair keratinocyte migration and results in poor wound healing. On the other hand, down-regulation of connexin in murine and human have been shown to significantly accelerate wound healing. The attenuation of connexin 43 expression leads to reduced cell adhesion via a reduction in adhesion molecule (N-cadherin) expression and activation of regulators of cytoskeletal dynamics.
GTPases Rac1 and RhoA), enabling increased cell motility.\textsuperscript{15} Besides increasing the migratory activity, connexin 43 down-regulation at a wound site also promotes angiogenesis and keratinocyte proliferation and decreases the number of infiltrating neutrophils and macrophages.\textsuperscript{23} In this study, significant down-regulation of connexins 43 and 30 was observed at the wound edges after EG, especially in chronic wounds. These down-regulations suggest increased migratory activity of keratinocytes from the wound edge towards the centre of the wound after EG. Similar down-regulation was not seen in the SSG group, likely because of the integration of the SSG into the wound bed, thus not encouraging wound healing from the wound edges.

The wound bed, on the other hand, demonstrated increased proliferative marker (Ki67) and inflammatory cell expression after EG, suggesting the activation of the wound bed after grafting. Similar activation was not seen

\textbf{FIGURE 2} Expression of connexin proteins at the wound edge. A, Confocal images of connexin 43 expression in chronic wounds in each group at weeks 0 and 1. Scale bar = 50 μm. Magnification ×40. B and C, Mean connexin 43 expression in acute and chronic wounds (epidermal graft [EG], n = 12; split-thickness skin graft [SSG], n = 12). D and E, Mean connexin 30 expression in acute and chronic wounds (EG, n = 12; SSG, n = 12). F and G, Mean connexin 26 expression in acute and chronic wounds (EG, n = 12; SSG, n = 12). Values expressed as mean ± standard deviation. *P < .05 (paired t test)
after SSG as the grafted skin forms an epithelial coverage for the wound, thus progressing towards the phenotype of a healed wound. In chronic wounds, wound bed activation after EG was also observed to be accompanied by reorganisation of the extracellular matrix to become denser because of increased production of granulation tissue. In normal wound healing, the first step of the healing cascade is inflammation, which involves recruitment of neutrophils and macrophages that secrete cytokines, chemokines, and growth factors, which then initiates the production of collagen, seen clinically as granulation tissue. The increased collagen content in the extracellular matrix is a change that is normally observed in the proliferative phase of wound healing, whereby the growth and deposition of granulation tissue is critical for an inductive and supportive role for reepithelialisation from the wound edge. Therefore, the transplanted keratinocytes in the form of EG stimulate cell proliferation and create a conducive micro-environment for healing.

Wound bed activation was seen despite the absence of keratinocytes on the wound bed a week after EG, as demonstrated by the absence of cytokeratins 6 and 14. There have been mixed reports on the visibility and viability of EG on the wound bed after grafting in several observational clinical studies. The absence of keratinocytes or the graft on the wound bed at week 1 in this study provides robust evidence that EG does not integrate into the wound bed as seen with SSG. The difference in the graft integration between EG and SSG is likely because of the lack of dermis in the EG, which prevents capillary ingrowth into the graft as the epidermis is an avascular layer. The absence of dermis, however, enables direct interaction between the actively proliferating basal keratinocytes and the wound bed. This interaction enables wound bed activation as the basal keratinocytes carry keratinocyte stem cells and, at the same time, expresses a cocktail of cytokines and growth factors onto the wound bed. Hence, it may be postulated that the EG behaves like a bioactive dressing instead of a skin substitute. However, to confirm this and to fully elucidate the mechanism of action of the grafted epidermis, further research on the kinetics and integration of the EG, which analyses biopsies at week 2 or 3 post-grafting, is required.

There were several limitations to this study. First, there were various wound aetiologies and durations...
included in the study. This is because patients were enrolled as part of a larger randomised controlled trial despite adhering to strict exclusion criteria to eliminate factors that could severely influence wound healing. To limit this variation, subgroup analysis based on the chronicity of the wound (acute vs chronic) was performed. On the other hand, despite highlighting the difference in healing mechanisms between EG and SSG, this study does not include a negative control by obtaining tissue biopsy from patients managed with standard dressing therapy. This is because previous evidence in the literature suggests that EG has a healing mechanism that is comparable with other autologous skin grafts. However, the findings from this study confirm EG to have a mechanism that is comparable with a bioactive dressing instead of a skin substitute. As our study did not evaluate the secretion or expression of growth factors by the grafts, this cannot be confirmed with the current available evidence. Moving forward, a prospective study comparing the mechanism of healing and clinical outcome of EG against standard dressing therapy is required to further understand the healing mechanism and outline the clinical outcome.

5 CONCLUSION

Despite EG and SSG both being autologous skin grafts, they demonstrate different mechanisms of wound healing. EG accelerates wound healing from the wound edges and activates the wound bed despite not integrating into the wound bed at week 1 post-grafting as opposed to SSG. This difference is likely because of the difference in the anatomical construct of the grafts, resulting in EG having properties comparable with a bioactive dressing instead of a skin substitute. A future study comparing EG with standard dressing therapy is required to further delineate the healing mechanism and the clinical outcome.

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CONFLICT OF INTEREST

The authors have no competing interests to declare.
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