



Original Article

Keratinocyte sheets prepared with temperature-responsive dishes show enhanced survival after *in vivo* grafting on acellular dermal matrices in a rat model of staged bi-layered skin reconstruction

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ABSTRACT

Introduction: Bi-layered skin reconstruction can be achieved by staged grafting of acellular dermal matrices (ADMs) and cultured epithelial keratinocyte sheets (KSs). Both KSs and ADMs have been used for long; yet, their combined use has shown poor effectiveness. This outcome has been related to the enzymatic treatment used in the preparation of KSs, which impairs their adhesion potential to ADMs and the formation of a basement membrane (BM). Temperature-responsive (TR) culture dishes allow for enzyme-free preparation of KSs with preservation of BMs and intercellular adhesion proteins; yet, their use has not been previously applied to staged bi-layered skin reconstruction. Using an *in vivo* rat model, we tested the hypothesis that TR cultures enhance KSs survival and BM preservation after sequential grafting on ADMs.

Methods: In nude rats ($n = 9/\text{group}$), a 9-cm [2] full-thickness dorsal skin defect was repaired with a commercial ADM. At 2 weeks after surgery, we grafted the ADM with KSs (circular, 25 mm diameter), prepared from human cells either by enzymatic Dispase treatment (DT control group) or a TR culture dish (TR experimental group). KSs survival and BMs preservation was assessed one week later by digital imaging, histology (hematoxylin & eosin), immunohistochemistry (collagen IV, pancytokeratins) and immunofluorescence (cytokeratin 1-5-6, laminin).

Results: The TR group showed a significantly higher KSs survival (120 ± 49 vs. 63 ± 42 mm²; $p < 0.05$) and epidermal thickness (165 ± 79 vs. 65 ± 54 μm; $p < 0.01$) compared with the control DT group, as well as higher epidermal maturation (cytokeratin) and a denser laminin and Collagen IV expression in the BMs *in vitro* and *in vivo*.

Conclusion: These findings suggest that KSs prepared with TR culture dishes have significantly enhanced survival when grafted on ADMs; these outcomes could help improve current clinical strategies in wound care by skin reconstruction.

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1. Introduction

A critical, unsolved challenge in reconstructive surgery is the development of bi-layered skin constructs for the repair of cutaneous defects caused by trauma, burns, chronic disease, or surgery [1–3]. Over the past 50 years researchers and surgeons have achieved substantial progress in the *in vitro* manufacturing of epidermal structures, such as cultured keratinocyte sheets (KSs) [4–6], and of dermal substitutes, such as acellular dermal matrices

Abbreviations: KSs, keratinocyte sheet; ADMs, acellular dermal matrices; PBS, phosphate-buffered saline; HE, hematoxylin and eosin; DMEM, Dulbecco's modified Eagle's medium; KCM, keratinocyte culture medium; IHC, immunohistochemistry.

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(ADMs) [7–9]. KSs and ADMs have shown some clinical value in cases where other conventional reconstructive strategies (e.g., grafts, flaps) are either unavailable or not preferred. KSs have been used for over four decades in the treatment of severe burns when no skin donor site is available. Nevertheless, KSs are fragile and do not restore the structural and biomechanical properties of the damaged dermis, often leading to poor functional outcomes (e.g., scar contraction) and unsatisfactory esthetic results [6,11]. In contrast, ADMs are known to facilitate the regeneration of a dermal layer with biomechanical properties similar to native tissues [12,13]. ADMs, however, do not provide wound re-epithelization and require a subsequent overlying skin graft to provide final wound closure.

To date, very limited strategies have successfully replicated a bi-layered (epidermal and dermal) skin construct for clinical use [10]. Attempts to combine KSs and ADMs into a single therapeutic approach have also provided suboptimal outcomes [14–16]. Research and clinical experience have shown that *in vivo* single-stage combined grafting of ADMs and KSs is limited by the inability of KSs to survive uniquely on the diffusion of metabolites from the wound bed through ADMs (usually >200 μm) [17,18]. Although staged grafting of ADMs (first) and KSs (later) theoretically allows blood vessel growth within ADMs, and thus reduces the critical distance for diffusion of metabolites, two-staged combined ADM and KS grafting has also shown surprisingly poor outcomes, with KSs survival rates (“graft take”) as low as 43% [16].

Studies have highlighted how the fragility of KSs grafted on ADMs might derive from the disruption of epidermal basement membranes (BMs) and of intercellular adhesion proteins within KSs [19]. This phenomenon is thought to occur due to the enzymatic techniques used in the *in vitro* manufacturing of KSs, as proteases (e.g., Dispase) are routinely used to detach KSs from culture dishes before *in vivo* grafting [6,20]. Enzyme-free, temperature-responsive (TR) culture dishes have recently been developed. Osada et al. reported that KSs prepared with this method show better-preserved BMs and higher survival when grafted directly on wounds in a rat model [21,22]. Application of this strategy to staged bi-layered skin reconstruction – a more common and relevant clinical scenario – has not been reported yet.

We postulated that enzyme-free, TR manufacturing methods would provide more robust KSs and preserve BMs, leading to increased survival after grafting on ADMs. We test this hypothesis using human-derived KSs in a rat wound model of staged skin reconstruction. Our goal is to provide biological evidence for the improvement of current clinical practice in skin reconstruction using combined ADMs and KSs grafts.

2. Materials and methods

This study was approved by our Institutional Animal and Ethics (human tissue procurement) Committees and the Ethics Committee of Tokyo Women's Medical University (IRB acceptance number: 2555 – R2), and it was performed in accordance with the Declaration of Helsinki (June 1964) and subsequent amendments. All patients provided written informed consent to participate in the study.

2.1. Preparation of human KSs

Abdominal skin was obtained from surgical discards from deep inferior epigastric artery perforator flap surgeries ($n = 3$; age: 51–67; sex: female). The collected skin was disinfected with povidone-iodine solution, cleared of adipose tissue with surgical scissors, and then minced and treated with Dispase I (400 PU/mL; Godo Shusei, Tokyo, Japan) diluted with phosphate-buffered saline

(PBS) at 4 °C overnight. The epidermis was separated from the dermis using a microscope and a fine cutter, and then treated with 0.25% trypsin in PBS at 37 °C for 30 min. The cell suspension was filtered using a 70- μm cell strainer; Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS was added to stop further trypsinization. The keratinocyte culture medium (KCM) contained DMEM and Ham's F12 medium (3:1), with hydrocortisone (0.4 ng/mL), triiodothyronine (2×10^{-9} M), cholera toxin (1×10^{-9} M), insulin (5 $\mu\text{g}/\text{mL}$), transferrin (5 $\mu\text{g}/\text{mL}$), and epidermal growth factor (10 ng/mL). The cell suspension was centrifuged at $400 \times g$ for 5 min; the pelleted cells were suspended in KCM and seeded in a 35-mm TR cell culture dish (Upsell; CellSeed Inc., Tokyo, Japan) or a 35-mm normal cell culture dish (BD Falcon, Franklin Lakes, NJ) at a density of 2×10^5 cells/cm² with 3T3 feeder cells. Culture dishes were incubated at 37 °C in a humidified atmosphere of 5% CO₂ with alternate-day medium exchange for approximately 10 days until confluence.

We separated KSs from culture dishes either by Temperature-responsive (TR) or enzymatic Dispase treatment (DT). In the TR treatment, keratinocytes were incubated at 20 °C for 30 min, the culture medium was then aspirated, and an artificial support membrane (circular shape, diameter 25 mm) (Millipore SVLP02500 filter; Sansyo, Tokyo, Japan) was placed on the cultured cells. We then harvested KSs with the cell sheet recovery system CellShifter™ (TR sheet). In the enzymatic DT group, KSs were detached after incubation by adding Dispase I (400 PU/mL) at 37 °C for 60 min. KSs were washed twice with culture medium and harvested using the CellShifter™ (DT sheet) (Fig. 1).

2.2. Histological and immunohistochemical analyses of KSs

Cultured KS were fixed with 4% paraformaldehyde, embedded in paraffin and stained with hematoxylin and eosin (HE), using standard protocols. KSs' thickness in each group was measured in HE-stained specimens. Four randomly selected 200 \times magnification images of KSs were divided into five equal segments vertically, and epidermal thickness at the center of each segment was calculated with ImageJ software version 1.47 (National Institutes of Health, Bethesda, MD).

Samples for immunohistochemistry were embedded in optimal cutting temperature freezing compound to prepare 15- μm thick sections, which were positioned on glass slides and then air-dried. Anti-human-collagen IV antibody (ab6586; Abcam, Cambridge, UK) and anti-human-laminin 5 (ab14509, Abcam) were used as primary antibodies (1/500 dilutions). Anti-rabbit IgG goat polyclonal antibody (#424144, Nichirei Biosciences Inc., Tokyo, Japan) was used as secondary antibody, and 3,3'-diaminobenzidine-4HCl for the chromogenic reaction. Mayer's hematoxylin nuclear staining was performed and viewed by an optical microscope (DMi8; Leica Microsystems, Wetzlar, Germany).

2.3. Surgical procedure and experimental design

All animal care and handling procedures were performed in accordance with the Principles of Laboratory Animal Care of the Animal Experimentation Committee of our institution.

F344/NJcl-rnu/rnu athymic nude rats ($n = 18$, 8 weeks old, approximately 200–250 g), purchased from CLEA Inc. (Tokyo, Japan), were anesthetized with 2% isoflurane via a nasal mask attached to a Univentor 400 Anesthesia Unit (Narcobit-E; Natsume Seisakusho, Tokyo, Japan). The back was shaved and washed with 70% ethanol and a full-thickness square-shaped skin defect approximately 30 \times 30 mm² in diameter was made using surgical scissors, sparing the dartos fascia (Fig. 2A). The fascia was covered with a commercially available ADM (Pelnac®, Smith & Nephew

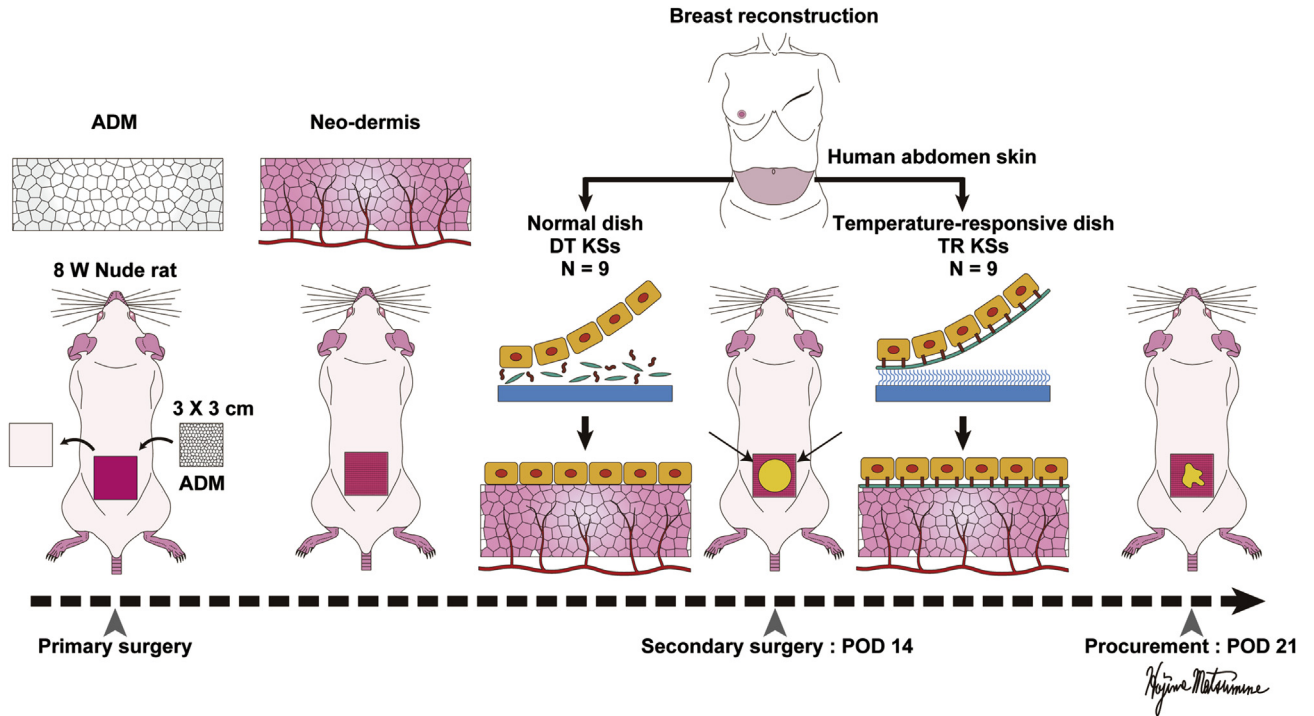


Fig. 1. Schematic overview of experimental procedures. DT, Disperse treatment; TR, temperature-responsive.

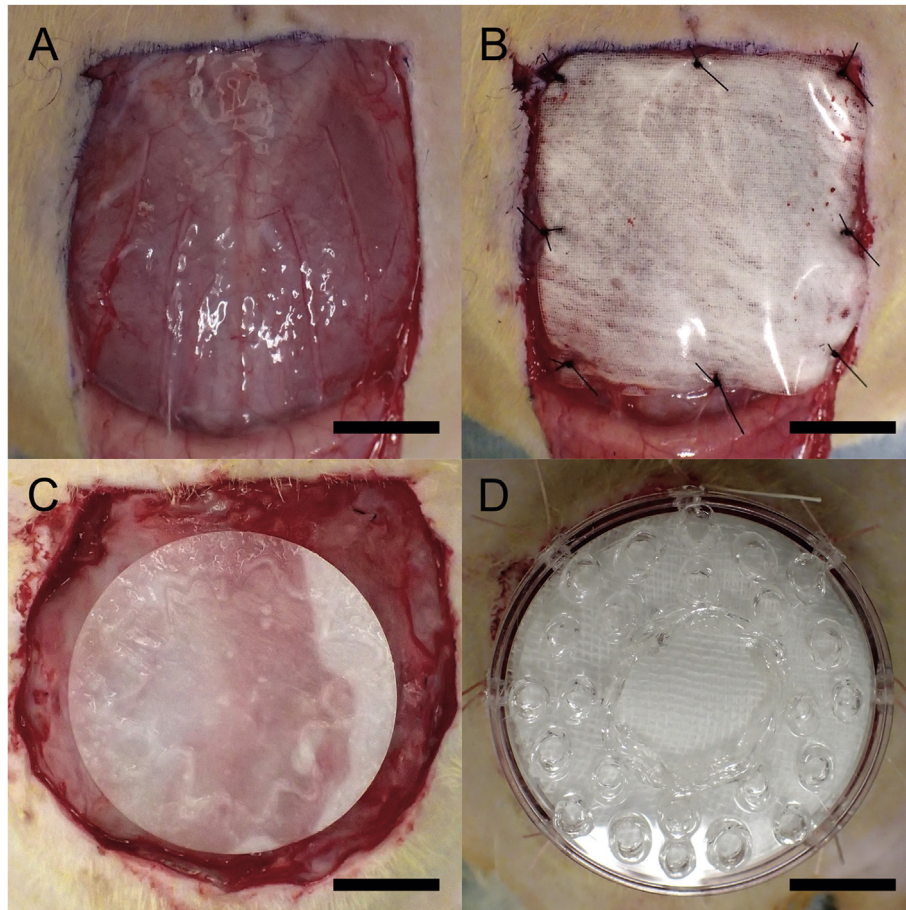


Fig. 2. Human Ks-grafted rat model. (A) Full-thickness square skin defect ($30 \times 30 \text{ mm}^2$) created in the dorsum of experimental rats using surgical scissors. (B) Defect covered by an ADM grafted, placed on the wound. (C) Cultured Ks, collected from temperature-responsive (TR) and normal dishes (DT) transferred with a CellShifter™ cell sheet recovery system (circle, 25 mm diameter) are grafted onto the median of the neodermis. (D) Grafted Ks covered with ointment-impregnated gauze cut to a 35-mm diameter circle in a 35-mm diameter Petri dish. Scale bars represent 10 mm.

Wound Management, London, UK) onto the skin defect wound (Fig. 2B).

Neodermal regeneration within the ADM was visually confirmed 2 weeks after the primary surgery by removing the silicone sheet of the ADM under inhalational anesthesia. We divided the animals into two groups: TR sheet transplant (TR sheet group, $n = 9$) and DT sheet transplant groups (DT sheet group, $n = 9$). Cultured KSs, collected from either TR or normal dishes were transferred with the CellShifter™ (25 mm diameter circle) and grafted onto the central portion of the neo-dermis, leaving the CellShifter™ in place (Fig. 2C). The grafted sheets were then covered with ointment-impregnated gauze, cut to a 35 mm diameter circle and a 35 mm Petri dish (BD Falcon) with the top and side wells drilled with holes, and stitched in 8-way pattern around the skin (Fig. 2D).

2.4. Graft survival area analysis

One week after KSs grafting, rats were anaesthetized and dressings removed. The area surrounding the graft was collected for histological analysis. Grafted tissues were gently washed with a saline solution-soaked gauze to remove non-grafted (non-adherent) KSs; grafts were then photographed with a stereomicroscope. The graft survival (“take”) area was defined as the epithelialized area present in the center of the neo-dermis in each group. This was digitally measured by three independent double-blinded observers experienced in skin surgery using ImageJ software version 1.47 (National Institutes of Health, Bethesda, MD) and its average value was then calculated and reported.

2.5. Histological analysis of the KSs grafts' epidermal thickness

Cross sections (5 μm thick) obtained from the wound center were stained with HE according to standard protocols. KSs thickness in each group was measured in HE stained specimens. Four 200 \times magnification images of grafted KSs were divided into three equal segments vertically, and epidermal thickness at the center of each segment was calculated with ImageJ software version 1.47 (National Institutes of Health); the mean of the three measurements was then obtained.

2.6. Immunofluorescence staining of the KSs grafts' BMs

To investigate protein expression in the basement membrane of the grafted KSs, double immunofluorescence staining for collagen IV and pan-cytokeratin was performed. Transverse sections (4 μm thick) made with a sliding microtome were mounted on silane-coated glass slides. Sections were incubated successively with blocking solution (Blocking One; Nacalai Tesque, Kyoto, Japan) for 10 min, a cocktail of anti-human-multi-cytokeratin monoclonal antibody (Leica #NCL-L-AE1/AE3) and anti-human-collagen type IV polyclonal antibody (Abcam #ab6586) overnight at 4 °C, and a cocktail of Alexa488- and 594-conjugated species-specific secondary antibodies (Life Technologies Corp., Carlsbad, CA) for 30 min. Sections were counterstained with 4',6-diamidino-2-phenylindole and then cover-slipped with aqueous mounting medium. Images were taken at 200 \times magnification using a confocal laser scanning microscope (LSM710; Carl Zeiss, Oberkochen, Germany).

2.7. Immunohistochemistry of the KSs grafts' keratinocytes

Immunohistochemistry (IHC) was performed to analyze the epidermal maturation of grafted KSs in each group. Transverse sections (4 μm thick) made with a sliding microtome were

mounted on silane-coated glass slides. Sections were successively incubated with blocking solution (Blocking One; Nacalai Tesque, Kyoto, Japan) for 10 min, anti-human cytokeratin 1 monoclonal antibody (Leica #NCL-CK1), anti-human cytokeratin 5 monoclonal antibody (Leica #NCL-CK5), or anti-human cytokeratin 6 monoclonal antibody (novusbio #NBP2-34232) overnight at 4 °C, and secondary antibodies (Nichirei #414191) for 30 min. This was followed by chemiluminescent reactions using 3,3'-diaminobenzidine-4HCl for 1 min at room temperature.

2.8. Statistical analysis

Results are expressed as mean \pm SD, and $p < 0.05$ was considered significant. *In vitro/in vivo* thickness and survival areas of KSs in both groups were analyzed using unpaired Student's t-test in GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA). All histological analysis was performed by three independent double-blinded observers, experienced in skin histology and pathology.

3. Results

3.1. TR KSs have a better-preserved basal membrane *in vitro*

Human KSs were successfully cultured with both TR and standard cell culture dishes; 7–9 days were required from primary culture to reach cell confluence in a sheet-like form. HE staining of cell sheets revealed neater and denser cell arrangement in the basal side of TR-cultured KSs compared to DT-cultured KSs (Fig. 3C and D), but no other macroscopic structural difference between the two groups (Fig. 3A and B).

Immunostaining for collagen IV and laminin revealed widespread expression of collagen IV and laminin on the basal side of TR-cultured KSs. Instead, DT-cultured KSs showed only sparse collagen IV and laminin expression (Fig. 3E–H).

DT-cultured KSs thickness was $15.2 \pm 5.7 \mu\text{m}$, and that of TR-cultured KSs was $17.7 \pm 3.5 \mu\text{m}$, with no significant differences between groups (Fig. 3I).

3.2. TR-cultured KSs grafted on ADMs show higher survival, thickness, and retention of native basement membrane *in vivo*

No surgical site infection was observed in either of the groups one week after grafting onto ADMs (Fig. 4A and B).

At low magnification (50 \times), HE staining revealed thicker stratification of the epidermis in TR-cultured KSs compared with DT-cultured KSs (Fig. 4C and D). At high magnification (200 \times), HE staining revealed sparsely dispersed and disorganized basal cells in DT-cultured KSs as opposed to an homogenous palisade of basal cells between the dermal granulation tissue and TR-cultured KSs (Fig. 4E and F).

Survival area at one week after grafting onto ADMs was 2-fold higher for TR-cultured KSs compared with DT-cultured KSs ($120 \pm 49 \text{ mm}^2$ vs. $63 \pm 42 \text{ mm}^2$; $p < 0.05$) (Fig. 4G). In addition, HE staining showed that epidermal thickness was 2.5-fold higher in the TR-cultured KSs than in the DT-cultured KSs (respectively, $165 \pm 78 \mu\text{m}$ vs. $65 \pm 54 \mu\text{m}$; $p < 0.01$) (Fig. 4H).

Double immunofluorescence staining revealed a qualitatively strong green fluorescence signal of collagen IV in the red fluorescent pan-cytokeratin basal layer in the TR-cultured KSs, which appeared as a straight plane (Fig. 5). In contrast, the green fluorescence signal of collagen IV was only sparsely visible in the red fluorescence of the pan-cytokeratin basal layer and was partially absent in certain areas in the DT-cultured KSs (Fig. 5).

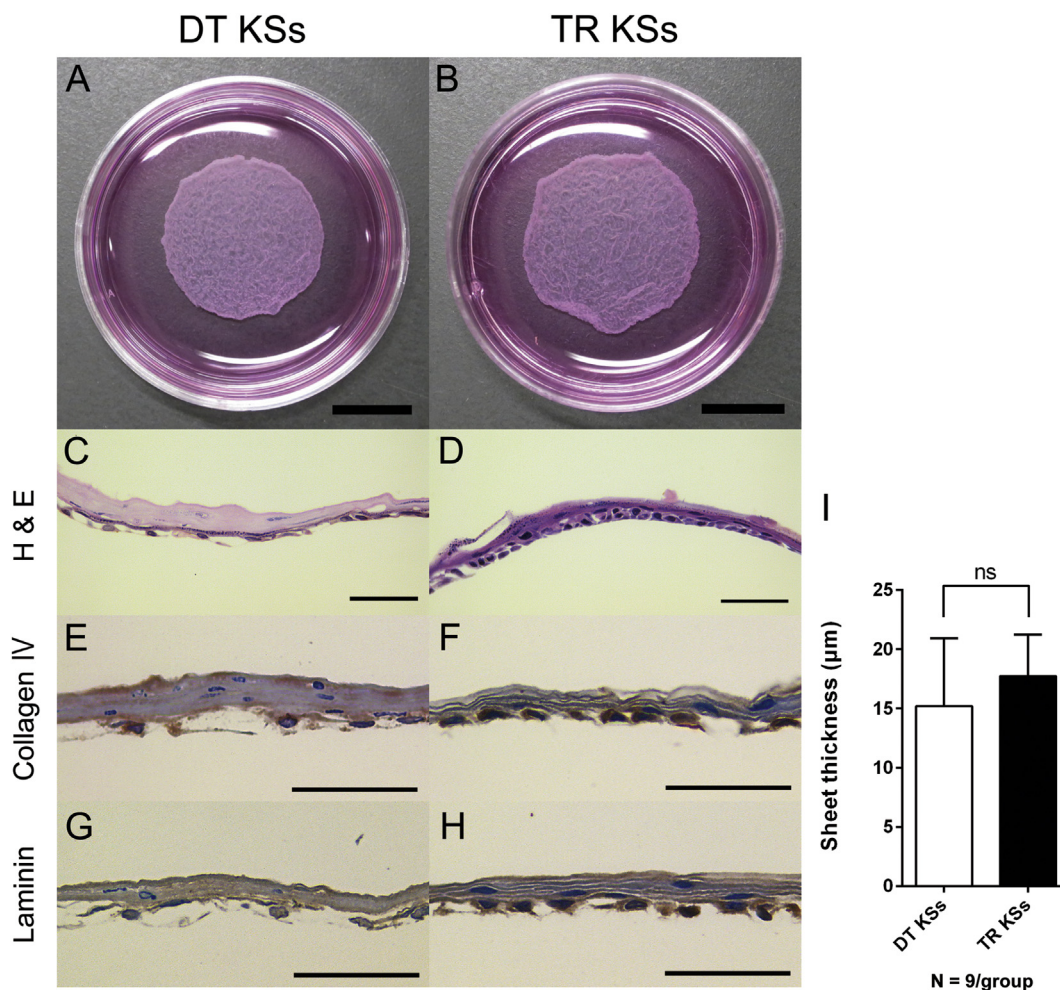


Fig. 3. Human KSs: Comparison of external appearance, tissue structure, and sheet thickness between the groups. External appearance of (A) DT-cultured KSs and (B) TR-cultured KSs does not differ greatly after cell sheet harvesting. Scale bars represent 1 mm. HE staining shows a more orderly and dense basal side cell structure for (D) TR-cultured KSs compared to (C) DT-cultured KSs. Scale bars represent 50 μm. Immunostaining for Collagen IV and laminin showing widespread expression of Collagen IV and laminin in TR-cultured KSs on the basal side (F and H); in contrast, DT-cultured KSs show only sparse expression of Collagen IV and laminin (E and G). Scale bars indicate 50 μm. (I) Average thickness of DT-cultured KSs was 15.2 ± 5.7 μm and that of TR-cultured KSs was 17.7 ± 3.5 μm; there was no significant difference in the *in vitro* thickness of the KSs between groups.

3.3. TR-cultured KSs grafted on ADMs show higher epidermal maturation

In both DT-cultured KSs and TR-cultured KSs, stacked cells showed positive staining for Cytokeratin 5 (an epidermal cell marker expressed also in the epidermal basal cells), although the two groups showed a different epidermal thickness. Cytokeratin 1 (a marker of cell layers above the basal one) was positive only in an extremely thin cell layer in DT-cultured KSs, and in a thicker and densely stacked spur cell layer and granular cell layer in TR-cultured KSs. Cytokeratin 6 (a marker of hyperproliferative squamous cells) was sparsely positive in DT-cultured KSs, but was diffusely positive in cells of all epidermal layers in TR-cultured KSs; the proliferative ability of grafted keratinocytes was found to be extremely high (Fig. 6).

4. Discussion

In this study, we show that KSs obtained from TR cultures provide superior outcomes when grafted onto ADMs in two-stages wound repair and skin reconstruction, as compared to KSs obtained through standard enzymatic DT culture. Specifically, our rat model showed that the survival area (“take”) of TR-cultured KSs grafts was

twice that of DT-cultured KSs grafts, that TR-cultured KSs promoted significantly higher cell proliferation *in vivo* (epidermal thickness), and—most importantly—that TR-cultured KSs showed histological structural characteristics (laminin/cytokeratin deposition and alignment) most closely resembling the normal skin architecture, with highest preservation of the BM *in vitro* and *in vivo*.

KSs have been a valuable adjuvant treatment in wound care and reconstructive surgery for 50 years, first as stand-alone therapy for epidermal replacement and more recently in combination with dermal scaffolds for bi-layered skin constructs [23,24]. The significance of this strategy is particularly evident in severe burns and other reconstructive procedures burdened by the lack of large-enough donor sites for autologous skin grafting. The lack of donor site morbidity could have boosted the adoption of KSs in a broader range of surgical scenarios. Yet, the fragility of KSs and their low survival after *in vivo* grafting (among other manufacturing, financial, and regulatory challenges) have substantially limited interest, use, and outcomes of the procedure, and relegated it to a restricted number of cases and clinical indications.

The inability to recreate/retain a biomechanically functional basement membrane has been shown to be a major contributor and determinant of the fragility of KSs. Commonly used culture methods employ proteolytic enzymes (e.g., Dispase) to detach

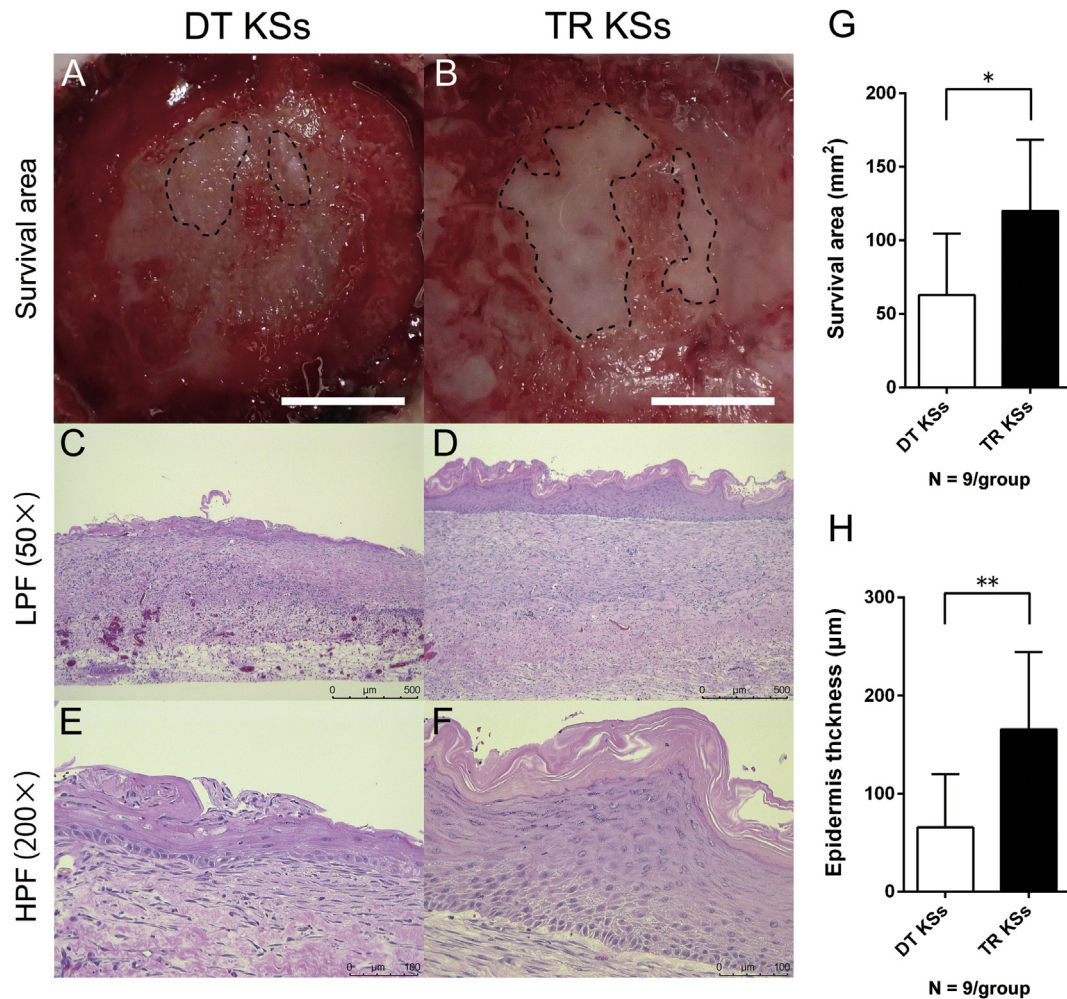


Fig. 4. External appearance, pathological features, graft survival area, and epidermal thickness of KSs grafts one week after grafting onto an ADM. (A) DT-cultured KSs grafts and (B) TR-cultured KSs grafts, one week after grafting onto ADMs. Scale bars represent 1 mm. At $\times 50$ magnification, HE staining shows thicker stratification of the epidermis for (D) TR-cultured KSs grafts, compared to (C) DT-cultured KSs grafts; Scale bars represent 500 μm . At $\times 200$ magnification, HE staining shows sparse unaligned basal cells for (E) DT-cultured KSs grafts; in contrast, (F) TR-cultured KSs grafts show a palisade of basal cells seen between the dermal granulation tissue and the KSs. (G) KSs graft survival area one week after transplant onto ADMs was $120 \pm 49 \text{ mm}^2$ for TR-cultured KSs grafts, 2-fold higher than that of the DT-cultured KSs grafts ($63 \pm 42 \text{ mm}^2$) ($p < 0.05$). (H) On HE staining, epidermal thickness was $165 \pm 78 \mu\text{m}$ for TR-cultured KSs grafts, 2.5-fold higher than that of DT-cultured KSs grafts ($65 \pm 54 \mu\text{m}$) ($p < 0.01$).

newly formed KSs from culture dishes. These enzymes, however, concurrently damage the intrinsic structure of the KSs, especially the basal membrane. Very recently, advances in tissue engineering and biochemistry have yielded TR culture techniques that exploit temperature changes to detach cell sheets from culture dishes, without the need for enzymatic reactions. This approach has provided more robust tissue constructs and better-preserved histological architecture closely resembling native tissue. A thermo-responsive polymer [poly(*N*-isopropylacrylamide) (PIPAAm)] is applied to the surface of TR culture dishes by electron beam irradiation. This coating makes the dish surface slightly hydrophobic at 37 °C and hydrophilic below the lower critical solution temperature (LCST) of about 32 °C. During standard culture at 37 °C, keratinocytes adhere to the hydrophobic surface, spread, and proliferate. Once culture temperature is reduced below the LCST, keratinocytes detach from the hydrophilic surfaces, yet maintain intact cell-to-cell junctions and BMs. As rapid hydration of PIPAAm occurs, a separating layer forms and cells can be collected as a single contiguous cell sheet [22]. TR techniques have been successfully adopted to develop cell sheets for *in vivo* repair of corneal defects [25–27], esophageal mucosa [28], oral and nasal mucosal lining

[29], ear canal mucosa [30,31], cartilage [32], and endometrium [33,34], among others. Besides positive outcomes observed in animal models, initial studies in patients are also promising [35].

Application of these innovations in cell sheet technology to the field of wound care has been limited and recent. Hamada et al. described the use of TR culture to create cell sheets from adipose-derived mesenchymal stem cells and use these constructs for cutaneous wound healing in diabetic rats [36,37]. To our knowledge, Osada et al. are the only group to create epithelial KSs using TR culture, and to demonstrate that this approach improves KSs survival *in vivo* in a rodent model [21]. Here, we leveraged the experiences of Osada et al. and others and applied these to a model of combined, staged KS and ADM grafting, which we believe more adequately addresses current clinical needs and best practice in wound care and reconstructive surgery. Our results are consistent with previous reports and confirm the efficacy of this approach (TR-cultured KSs) in combined KS and ADM grafting for wound repair.

The translational relevance and implication of our research is substantial, especially given other recent advances in cell engineering, which have paved the way for more effective cell therapies, lower manufacturing challenges, decreased costs, and a more

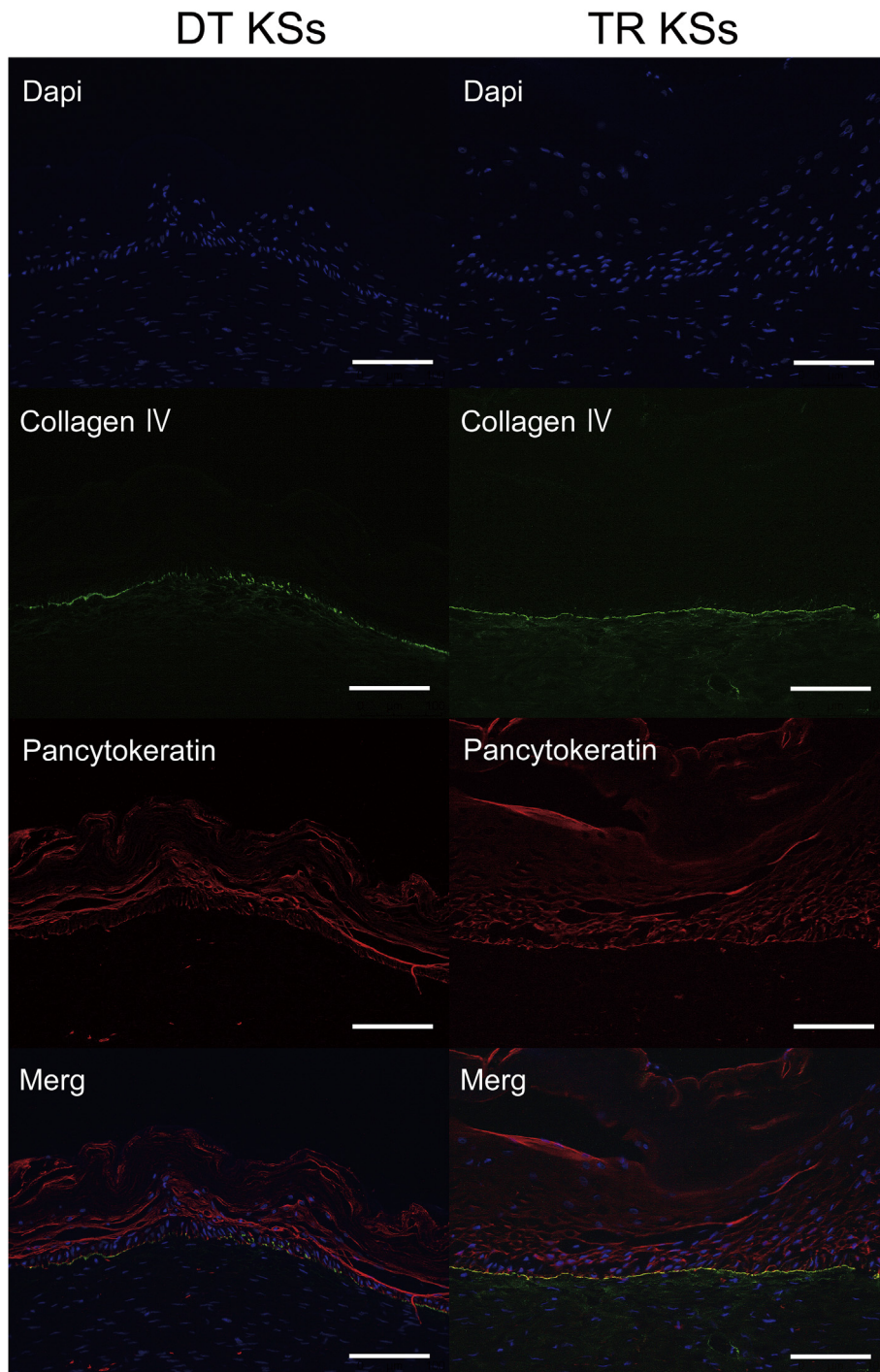


Fig. 5. KSs prepared with a disperse treatment (DT) or TR culture dish and grafted onto an ADM at one week after grafting. Double immunostaining with anti-pan-Cytokeratin (red) and anti-Collagen IV (green) antibodies. Scale bars represent 100 μ m.

favorable regulatory landscape. Furthermore, with research focused on improving ADMs performance having reached a plateau, combination of these constructs with cellular components has gained popularity. Diverse strategies have been attempted over the years to tackle this (cell spraying, micro-grafting), often with suboptimal outcomes [38–40]. Insights on the efficacy of TR culture-derived KSs might make this a competitive approach in this scenario. We believe that research in this field can further expand the clinical application of the combined KSs and ADMs beyond

cases of severe burn or critical wounds, to more conventional “daily” wound care.

The outcomes we present are limited by the nature of our study, which was performed in a preclinical rodent model and with a surgical skin injury, using immune-deficient animals and xenologous cells to treat a relatively small cutaneous defect. Despite this being an established model with demonstrated robust translational value, future studies using larger animal models and clinical trials in patients are needed to validate our approach,

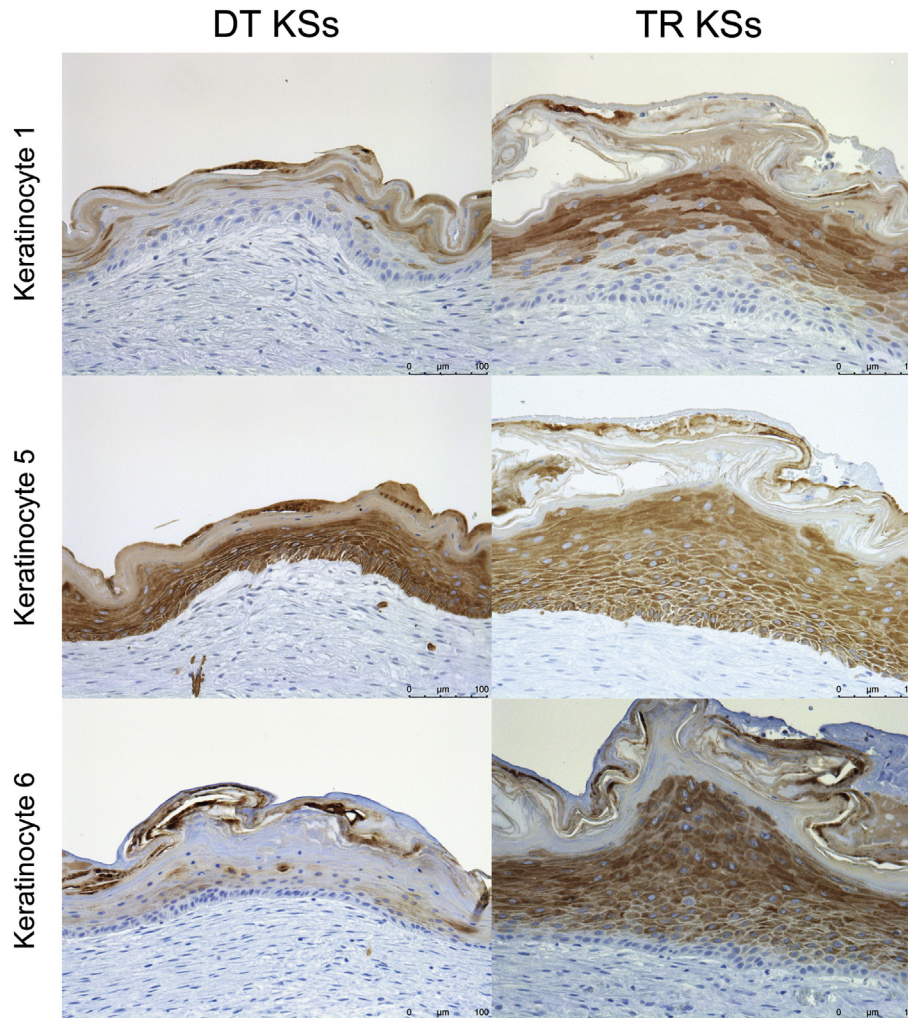


Fig. 6. Immunohistochemistry of keratinocytes. In both DT-cultured KSs grafts and TR-cultured KSs grafts, stacked cells showed positive staining for Cytokeratin 5 (pan-epidermal cell marker), despite differences in their thicknesses. Cytokeratin 1 (epidermal marker expressed above the basal cell layer) was positive only in an extremely thin layer in DT-cultured KSs grafts, but was positive in a thicker and densely stacked spur cell layer and granular cell layer in TR-cultured KSs. Cytokeratin 6 (hyperproliferative squamous cells marker) was sparsely positive among the cells in DT-cultured KSs grafts, but was positive in all epidermal layers in TR-cultured KSs grafts. Scale bars represent 100 μm .

especially in pathological conditions such as diabetic wound healing and burns.

5. Conclusions

This study showed that the KS prepared with TR culture dishes have a remarkably improved survival rate when grafted on ADMs in two-stage skin reconstructive procedures, as compared to conventional KSs. These preclinical outcomes offer evidence-based translational insights that, if confirmed in clinical trials, could help improve current therapeutic strategies and standards of wound care.

Conflicts of interest

The authors declare no conflict of interest.

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