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Invited Review Article

Development of *de novo* epithelialization method for treatment of cutaneous ulcers

Masakazu Kurita[a,*], Juan Carlos Izpisua Belmonte[b], Keiichiro Suzuki[c,d], Mutsumi Okazaki[a]

[a] Department of Plastic and Aesthetic Surgery, The University of Tokyo Hospital, Japan
[b] Gene expression Laboratory, Salk Institute for Biological Studies, United States
[c] Institute for Advanced Co-Creation Studies, Osaka University, Japan
[d] Graduate School of Engineering Science, Osaka University, Japan

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ABSTRACT

Cutaneous ulcers are a common cause of morbidity. We have developed a *de novo* epithelialization method for treating cutaneous ulcers by means of reprogramming wound-resident mesenchymal cells *in vivo* into cells able to form a stratified epithelium: induced stratified epithelial progenitors (iSEPs). Administration of 4 transcription factors (*DNP63A*, *GRHL2*, *TFAP2A*, and *cMYC*) expressed *via* adeno-associated viral vectors enabled generation of epithelial cells and tissues, thereby acheiving *de novo* epithelialization from the surfaces of cutaneous ulcers in a mouse model. Generated epithelia, having barrier functions equivalent to the original epidermis, were maintained for more than 6 months. Our findings constitute a proof of concept for future development towards innovative therapies for cutaneous ulcers *via de novo* epithelialization.

1. Introduction

Cutaneous ulcers result from various external and internal causes such as trauma, burns, diabetes, peripheral arterial diseases, and pressure ulcers. Skin grafting and flap plasty are common surgical remedies; however, in cases with absolute epithelial deficiency (*e.g.* broad area burns), current techniques are often insufficient and may not be able to save a patient's life. Early clinical experiences in community medicine suggested that patients with non-healing ulcers have other severe systemic issues, and therefore, are often not candidates for surgical treatment due to the risks these surgeries would pose [1,2].

Regenerative approaches for healing large skin lesions, including cell sheet transplantation and tissue-engineered skin substitutes, have been introduced clinically over the last several decades [3,4]. However, cell culture based approaches require specialized facilities, substantial time for processing and considerable costs. More importantly, it is difficult to attain sufficient survival rates of the graft after transplantation.

Considering these drawbacks of current therapies, there is a need for innovative, non-surgical approaches for epithelial coverage of wounds. This report reviews our recent attempts to establish a method of supplying epithelial cells *via in situ* lineage reprogramming of mesenchymal cells on the surface of cutaneous ulcers in mice [5].

2. *De novo* epithelialization enhances wound healing

Physiologically, epidermal deficits are repaired through migration of epidermal cells supplied from adjacent interfollicular epidermis or appendages, such as hair follicles or sebaceous glands [6]. Namely, the damaged epidermis is only regenerated from the epidermis. Based on this gradual migratory repair process, re-epithelialization of large and deep cutaneous ulcers is inefficient. To overcome this spatial restriction, we sought a novel method of inducing *de novo* epithelialization (epithelial tissue generation) from the surface of the ulcers with the hope of providing a new avenue for prompt wound closures (Fig. 1). The fundamental ideas of other approaches well-known to practitioners (from traditional skin patch grafting [7] to state-of-the-art minced skin grafting techniques [8]) are based on similar principles in terms of wound coverage, but require transplantation of preexisting epidermis.

Abbreviations: iSEPs, induced stratified epithelial progenitors; ES cells, embryonic stem cells; iPS cells, inducedpluripotent stem cells; TF, transcription factor; AAV, adeno-associated virus; MET, mesenchymal to epithelial transition.

* Corresponding author: Department of Plastic and Aesthetic Surgery, The University of Tokyo Hospital, 113-8655, 7-3-1, Hongo, Bunkyo-ku, Tokyo, Japan.
E-mail address: kuritam-pla@h.u-tokyo.ac.jp (M. Kurita).

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3. Introduction of reprogramming technology

Our preliminary investigation studied lineage change using various culture conditions, since many reports have indicated that adult mesenchymal cells harness the greatest potential for differentiating various types of cells, even to ectoderm-derived skin keratinocytes [8–14]. Numerous attempts ended in failure as we could not find any remarkable changes in CDH1 expression, one of the most important markers for epithelial cells, although moderate upregulations of other keratinocyte markers were frequently observed for mesenchymal cells prepared using our protocols [15,16]. Thus, we discovered that the differences between mesenchymal and epithelial cells to be the greatest barrier, when we focused on the mesenchymal-to-epithelial transition (MET). We ultimately were able to discern that MET could be achieved during the reprogramming process of fibroblasts towards pluripotent cells, based on the results from Samavarchi et al. [17] and Li et al [18], and we employed a new reprogramming technology (*i.e.* alter lineage of a cell *via* transduction of a master regulator that specifies cellular lineage) [19].

4. Experimental design for identification of reprogramming factors

Protocol design for identification of reprogramming factors linked with functional skin epithelial cells was based on a framework described primarily for human induced pluripotent stem (iPS) cell production [20], and the following steps were employed: prepare culture conditions appropriate for original cell proliferation; prepare culture conditions appropriate for both original cells and reseeded target cells; then prepare culture conditions to facilitate unlimited proliferation and clonal expansion of target cells [21]. We first employed mRNA expression of keratinocyte markers (*CDH1* and *KRT14*) as indicators of conversion. However, during repetitive trials, we found that upregulation of mRNA expression of keratinocyte markers was obviously insufficient to provide function. Therefore, we switched our focus to the form (*i.e.* colony with clearly defined margins from the surrounding fibroblasts, flattened cells in the periphery of the colony, and accumulation of cells in the central part of the colony) and proliferation kinetics of cells on feeders for negative selection, followed by assessment with 3-dimensional organotypic cultures [22] to see whether the cells formed a stratified epithelium (Fig. 2a).

5. Generation of stratified epithelial progenitors (human cells *in vitro*)

Microarray and micro-RNA microarray data comparing primary human fibroblasts and keratinocytes were primarily used for selecting the candidate factors, followed by several types of bioinformatic analyses. Of the assessments, gene expression reversal analysis described by Heinäniemi et al. was the most useful for our study of skin epithelial cells [23]. Gradually increasing the number of candidate factors, over the course of approximately 200 combination trials, we eventually achieved our first successful generation of cells with an ability to form stratified

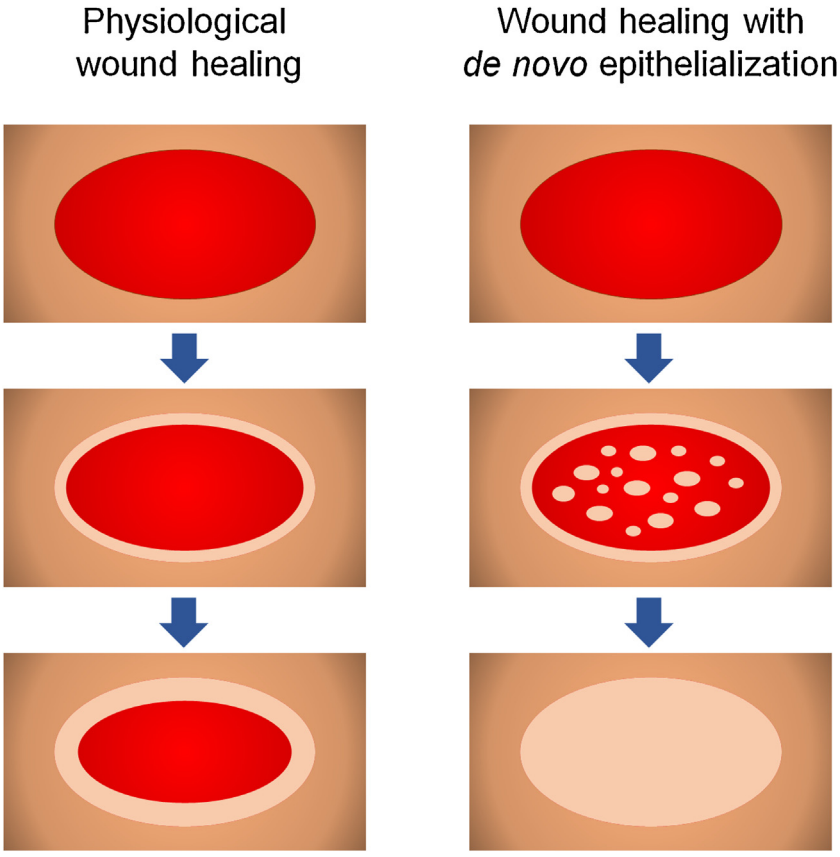

Fig. 1. Comparison between physiological wound healing and *de novo* epithelialization.
During physiological wound healing, epidermal defects are repaired only with gradual epithelialization from the surrounding epithelial tissues (Left), while *de novo* epithelialization enables epithelialization of the wound surface in an all-at-once fashion (Right).

epithelia in 3D organotypic culture with the transduction of 28 transcription factors (TFs) (Fig. 2b). At the same time, several other studies reported that combinations of TFs change cellular phenotypes mostly based on the upregulation of their markers [24,25]. The produced cells were called “induced keratinocytes” without any confirmation of their function. For purposes of clarity, and considering the multiple roles/functions of cells *in vivo*, we will name our artificially-generated cells according to their respective confirmed functions, as is the case for iPS cells (not induced embryonic stem (ES) cells). Thus, hereafter, we define the generated cells able to form stratified epithelial as induced stratified epithelial progenitors (iSEPs).

6. Optimization of the factors

It was obvious that not all 28 TFs were indispensable for generation of iSEPs. Therefore, we attempted to reduce the number of factors with iterative trials with approximately 2000 subset combinations. Consequently, we found that transduction of only 2 TFs, *DNP63A* and *GRHL2*, could induce iSEPs (Fig. 2b). Furthermore, before initiating *in vivo* experiments, we tested 67 combinations composed of *DNP63A*/*GRHL2* plus 1–3 TF s for optimization in terms of cell shape, histological integrity in 3D organotypic cultures, conversion efficiency and cellular proliferation, resulting in identifying *TFAP2A* and *cMYC* as additional factors. Thus, *DNP63A*, *GRHL2*, *TFAP2A*, and *cMYC* (DGTM factors) were employed in the *in vivo* experiments. The rationale behind the combinations of our non-biased selections appears to be supported by recently published reports, which described interactions of TFs between DGTM factors [26,27].

7. Animal experimental model

To prove the occurrence of *de novo* generation of epithelial tissues *in vivo* in a more definitive manner, we developed an animal experimental model which would produce a simple success-failure result. Following several attempts, we eventually established a skin ulcer model in which an ulcer is isolated from the surrounding skin using a chamber surgically fixed to the deep fascia (Fig. 3a). When left in place with no intervention, no epithelial component appeared inside the chamber for least a 1- to 2-month period.

8. Optimization of vector

For *in vivo* gene transduction, primary attempts using lenti- and retro- viral vectors did not work well. We thus chose to use adeno-associated viruses (AAVs) as they are known to facilitate *in vivo* gene transduction and have been used in over 200 clinical trials with promising preliminary results. There are many naturally-occurring AAV serotypes and synthetic variants, each of which has different protein capsids, and thus different gene delivery properties for various cell types (*i.e.* cellular tropism) [28]. Among 18 representative AAV serotypes tested, we noted that AAV-DJ appeared notably superior to the others. The transduction efficiency of AAV-DJ was considered much better than those of the serotypes known for skin cells [29,30].

9. DGTM factors induce generation of epithelial tissue from the surface of ulcers

In the animal model with isolated cutaneous ulcers on the back of mice, we succeeded in generating epithelial tissues histologically showing stratified epithelium structures covered with a cornified envelope, *via* administration of DGTM expressing AAV-DJ vectors (AAV-DGTM) (Fig. 3b). The frequency of epithelial tissue emergence was AAV-DGTM dose-dependent. Chronological observation and clonal tracking (with Confetti mice) suggested the involvement of multiple clones-derived epithelial tissues in *de novo* epithelialization. Further *in vitro* and *in vivo* subset analyses revealed the indispensable role of *DNP63A*, while GTM factors cooperatively worked with *DNP63A* with regards to the incidence and area of epithelized tissues.

10. Optimization of lineage-tracing mouse

To distinguish generated epithelia from the original epidermis after removal of the isolating chamber, we wanted to utilize a lineage-tracing mouse in which mesenchymal origin cells were labelled but not epithelial cells. Potential candidate mice

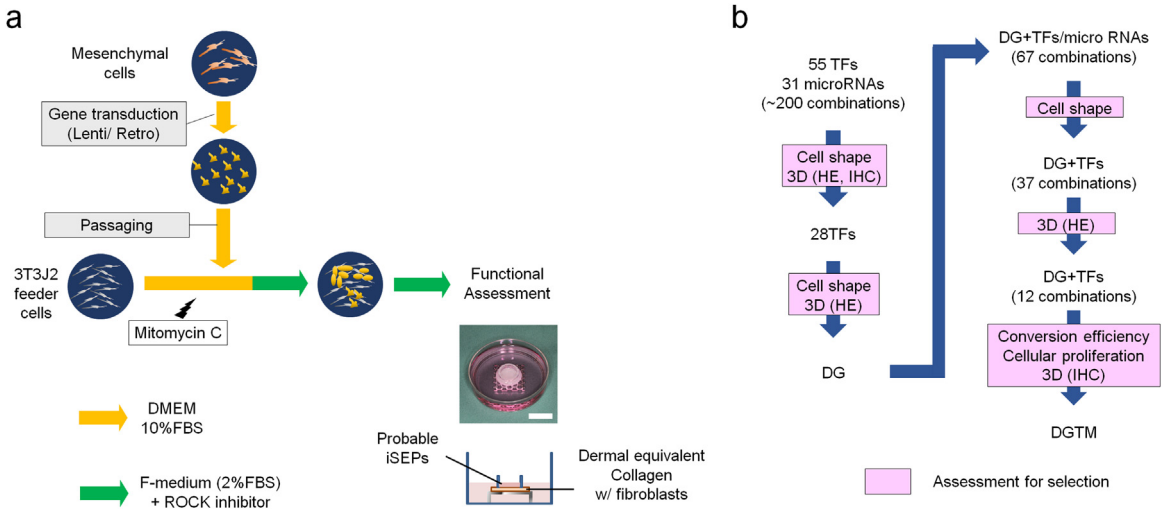

Fig. 2. Identification of reprogramming factors.
a) Schematized experimental design to identify reprogramming factors for cells enabling formation of stratified epithelia in 3D-organotypic culture.
Abbreviations: DMEM = Dulbecco's Modified Eagle Medium, FBS = fetal bovine serum.
b) Flow chart showing our selection and optimization of the reprogramming factors.
Abbreviations: H&E = haematoxylin and eosin, IHC = Immunohistochemistry, TF = transcription factor, DG = DNP63A and GRHL2, TM = TFAP2A and cMYC

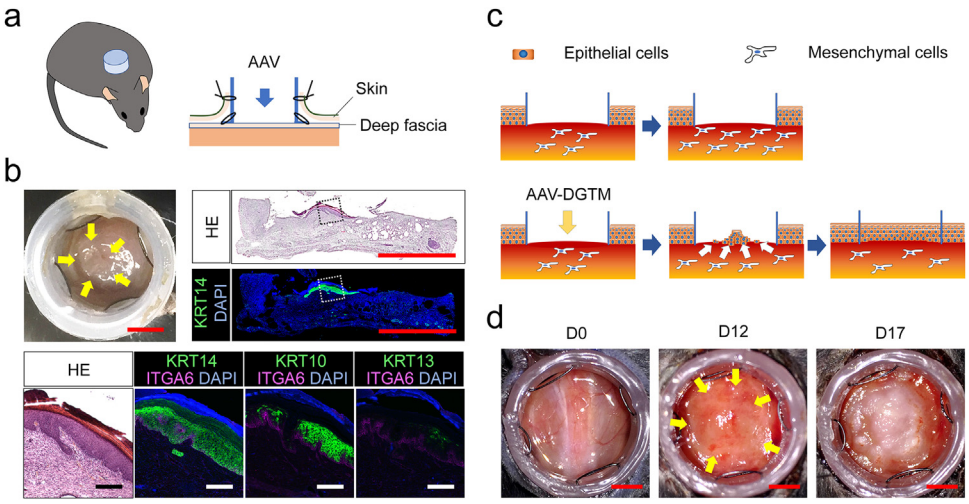

Fig. 3. DGTM factors enable *de novo* epithelialization.
a) Schematized animal model. An artificially-created ulcer was isolated from surrounding skin by a chamber surgically fixed to the deep fascia. b) An ulcer in the chamber 18 days after administration of AAV-DGTM. Haematoxylin and eosin (H&E) and immunofluorescent staining of the sections obtained at the generated epithelium. Yellow arrows indicate the generated epithelium. Red scale bars, 3 mm; black and white scale bars, 200 μm. c) Schematic of ulcer in the chamber. Physiologically, epithelialization could never be achieved within the isolated ulcer (upper row), while AAV-DGTM enabled *de novo* epithelialization *via in vivo* reprogramming. d) An ulcer in the chamber after administration of AAV-DGTM and daily applications of FGF2 and Rock inhibitor. Epithelialization occurred from different regions of the ulcer (yellow arrows) at the same time and enabled entire wound-coverage in all-at-once fashion.

(*Pdgfra*^{creER};*LSL*^{tdTomato}, *Pdgfra*^{creER};*Rosa*$^{mT/mG}$, *Pdgfra*^{cre};*LSL*^{tdTomato}, *Pdgfra*^{cre};*Rosa*$^{mT/mG}$, and *Fsp1*^{cre};*LSL*^{tdTomato}) were tested for the labelling of a broad spectrum of mesenchymal cells to screen for the presence of signal leakage. Though all strains showed aberrant epidermal labelling, we noted that a portion of the *Pdgfra*^{creER};*LSL*^{tdTomato} mice was free from epidermal leakage. For a more stringent lineage-tracing system and to eliminate animals with epidermal leakage, we examined *Pdgfra*^{creER};*LSL*^{tdTomato} mice *via* a two-step selection process: histological investigation of ear biopsy specimens and investigation with primary keratinocyte cultures of the skin removed to generate the wound. One mouse of every 5–10 pups satisfied the inclusion criteria and was used for the lineage tracing experiments.

11. Generated epithelial tissue serves as outermost layer of skin

Using the lineage-traced animal, we confirmed that generated epithelial tissues definitely originated from mesenchymal cells (Fig. 3c), which served as an outermost layer of skin in continuity with the surrounding original epidermis for more than 6 months after chamber removal. The generated epithelial tissue showed equivalent barrier function to that of the original epidermis based on two types of dye penetration assays (for outside-in barrier function) and transepidermal water loss assessments (for inside-out barrier function).

12. Characteristics of *in vivo* iSEPs

In vivo iSEPs were separated from primary cultured epithelial cells obtained from skin tissue, including the generated epithelial layer in lineage-traced mice. Clonogenicity (clonal expansion potential) of *in vivo* iSEPs, as well as *in vitro* iSEPs, was higher than that of primary keratinocytes. However, no pathological tumorigenic potential was detected in the soft agar colony formation assay (*in vitro* testing for anchorage independent growth ability of cells) and tumorigenicity/teratogenicity assay (*in vivo* testing for cell behavior in NOD/SKID mouse). Transcription profiling *via* RNA-seq analyses supported the finding that *in vivo* iSEPs are similar to primary keratinocytes. Continuous expression of AAV-derived transcripts in *in vivo* iSEPs implied genomic integration of vector-originated sequences.

13. Potential in the clinic

To examine this methodology as a potential, viable future therapy, we confirmed that *de novo* epithelialization can be induced, even in non-fresh ulcer wounds (1 week after creation). Additionally, use of FGF2 and Rock-inhibitor (reagents which promote re-epithelialization) enabled concomitant *de novo* epithelialization in different regions within the ulcer to achieve rapid coverage in approximately 2.5 weeks (Fig. 3d).

With the experiments designed carefully to avoid false conclusions, we confirmed: 1) forced expression of DGTM factors confer the ability to form stratified epithelia in human cells *in vitro* and mouse mesenchymal cells *in vivo*; 2) *in vivo* reprogramming with AAV-DGTM enables generation of epithelial cells and tissues, thus *de novo* epithelialization from the surface of cutaneous ulcers; and 3) generated epithelia have sufficient function to close the wound. Undefined issues, such as long-term influences associated with potential mutagenic impact of DGTM transduction and influences of local/distant off-target effects associated with viral gene transduction, should be carefully assessed. The true, detailed origin of the reprogrammed cells (*i.e.* specific fraction of tissue-resident cells or circulating myeloblastic cells) are also of interest.

Our observations constitute an initial proof of principle for future development of innovative therapies for treatment of cutaneous ulcers based on *de novo* epithelialization. Also, we consider the current approach paves the way for the realization of true regeneration of skin (such as with skin appendages) *in vivo*.

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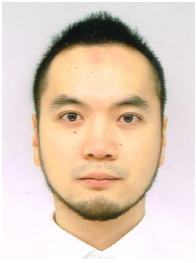

Masakazu KURITA (M.D., PH.D) received his M.D. at Faculty of Medicine, The University of Tokyo (2003) and PH.D. at Faculty of Medicine, Kyorin University (2010). He was assigned as an Assistant Professor at Department of Plastic surgery, Kyorin University hospital (2007–2015), chaired by prof. Kiyonori HARII, and a Research Associate at Gene expression laboratory, Salk Institute for Biological Studies (2015–2018) under supervision of Prof. Juan Carlos IZPISUA BELMONTE. He is currently Assisstant professor of Department of Plastic Surgery, the University of Tokyo Hospital, chaired by Prof. Mutsumi OKAZAKI.