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Microenvironment-induced myofibroblast-like conversion of engrafted keratinocytes

LI MeiRong, TI DongDong, HAN WeiDong* & FU XiaoBing*

Wound Healing and Cell Biology Laboratory, Institute of Basic Medical Science, Chinese PLA General Hospital, Beijing 100853, China

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Myofibroblasts, recognized classically by α-smooth muscle actin (α-SMA) expression, play a key role in the wound-healing process, promoting wound closure and matrix deposition. Although a body of evidence shows that keratinocytes explanted onto a wound bed promote closure of a skin injury, the underlying mechanisms are not well understood. The basal layer of epidermis is rich in undifferentiated keratinocytes (UKs). We showed that UKs injected into granulation tissue could switch into α-SMA positive cells, and accelerate the rate of skin wound healing. In addition, when the epidermis sheets isolated from foreskin cover up the wound bed or are induced *in vitro*, keratinocytes located at the basal layers or adjacent sites were observed to convert into myofibroblast-like cells. Thus, UKs have a potential for myofibroblastic transition, which provides a novel mechanism by which keratinocyte explants accelerate skin wound healing.

epithelial-mesenchymal transition, epithelial-myofibroblast transition, keratinocyte-myofibroblast transition, myofibroblast, wound healing

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An increasing number of chronic wounds and severe and large burns result in a wide spectrum of socio-economic problems [1]. Healing of these impaired wounds represents a challenge with currently available treatment. Cultured autologous keratinocytes have been used extensively to help treat these patients [2–5]. Autologous epidermal cell suspension transplantation seems to be an effective, simple and timesaving method to treat chronic non-healing wounds [6]. It has been reported that keratinocytes, when placed in a suitable microenvironment, are able to regenerate multi-lineage skin in mice [7]. Moreover, the transplanted keratinocytes facilitate the formation of blood vessels [8]. In addition, skin grafting has been used for the treatment of burns and chronic leg ulcers, which can promote faster re-epithelialization of partial thickness wounds [9–12]. However, the potential mechanism of keratinocytes for accelerated skin wound healing is unclear.

Successful wound healing is a complex process which refills defects through deposition of extracellular matrix in the wound by myofibroblasts. These cells express α-SMA, which are highly active in synthesizing extracellular matrix proteins, such as fibrillar collagens and fibronectin, and the number of myofibroblasts increases markedly in the process of wound repair [13]. Several studies suggest that chronic wounds are often characterized by reduced formation of myofibroblasts [1].

Myofibroblasts that participate in tissue fibrosis, such as the skin, lung, kidney, and liver, can also be derived from epithelial transdifferentiation under suitable microenvironments *in vitro* and *in vivo* [14–16]. Emerging evidence from studies involving wound re-epithelialization in skin suggests that the keratinocytes around the wound edge play a critical role through an epithelial-mesenchymal transition (EMT)

*Corresponding author (email: fuxiaobing@vip.sina.com; hanwdrsw69@yahoo.com)

[17]. Induction of an EMT in the process of wound repair results in the acquisition of mesenchymal traits, promotes closure of the injury, and enhances re-epithelization. In addition, in our earlier study, we showed that UKs exhibit multipotency by acclimatized induction [18]. Therefore, we propose that keratinocyte explants might transit into myofibroblasts under specified conditions. In the current study, we will demonstrate whether or not keratinocytes can be converted into myofibroblastic cells, and investigate the biological function of keratinocyte-derived myofibroblasts.

1 Materials and methods

1.1 Ethics statement

We obtained foreskin samples from healthy donors undergoing routine circumcisions. Keloid samples were selected by experienced plastic surgeons and removed as part of reconstructive procedures. All human skin tissues were collected according to the protocol approved by the Ethics Committee of PLA Hospital and informed consent forms were signed by the donors.

Animal experiments in this study were approved by the Ethics Committee of PLA Hospital. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

1.2 Isolation and preparation of epidermal sheets and cell suspensions

Foreskin samples were washed in phosphate buffer saline (PBS), then fat and membranous materials were removed and the tissue was cut into 1 cm×1 cm pieces and incubated overnight at 4°C in 2.5 mg mL^{-1} of Dispase (Invitrogen/Gibco, USA). The epidermis was mechanically separated from the underlying dermis and washed in PBS several times, and minced with scissors, then placed into 0.25% (w/v) trypsin and 0.25% (w/v) EDTA for 30 min at 37°C. DMEM (Invitrogen/Gibco), supplemented with 15% fetal bovine serum (FBS; Invitrogen/Gibco) was added, and the tube was shaken gently to dissociate the basal layer keratinocytes. The cells were then centrifuged, suspended in Epilife supplemented with HKGS (Invitrogen/Gibco) (fibroblasts cannot survive in Epilife) and plated onto collagen type IV-coated culture dishes (Invitrogen/Gibco). Dissociated keratinocytes were incubated for 10 min at 37°C in a 5% (v/v) CO_2 incubator; after the 10-min incubation, the unattached cells were removed and complete Epilife medium was added to the culture dishes. The medium was replaced every 2–3 days.

1.3 Keratinocyte-myofibroblast transdifferentiation (KMfT) *in vitro* under FBS acclimatized induction

In our previous study, we have showed that Epilife medium allows for the growth of only keratin-positive keratinocytes and prevents their differentiation [18]. Original keratinocytes were cultured in 6-well culture dishes at a density of 1×10^4/well. The original myofibroblastic differentiation medium consisted of nine volumes of complete Epilife medium and one volume of DMEM supplemented with 10% FBS. The induction medium was then changed into seven volumes of complete Epilife medium and three volumes of DMEM supplemented with 10% FBS, and the induction medium was changed into three volumes of complete Epilife medium and seven volumes of DMEM supplemented with 10% FBS. The induction medium was then changed into complete DMEM supplemented with 10% FBS (the induction strategy is shown in Figure 1A), during which the keratinocytes were observed through a microscope for morphologic changes. The medium was renewed every three days. The induction strategy was termed "acclimatized induction" [18]. Cells cultured in Epilife were used as a control. Ten days later, keratinocytes after induced differentiation were examined for E-cadherin, N-cadherin twist-1, slug, vimentin, α-SMA, and collagen I at the RNA level, and E-cadherin (Cell Signaling Technology, USA), vimentin (Sigma, USA), and α-SMA (Sigma) at the protein level. Three parallel induction experiments were performed in this experiment.

1.4 KMfT acclimatized induction of a slice of foreskin and epidermis *in vitro*

Skin samples were cut into 0.2 cm×0.2 cm pieces. A part of the foreskin slice was incubated overnight at 4°C in 2.5 mg mL^{-1} of Dispase (Invitrogen/Gibco). The epidermis was mechanically separated from the underlying dermis and minced with scissors, then washed in PBS several times. The collected foreskin and epidermal slices were incubated in induction medium which was similar to the medium for cell induction. The medium was renewed every day. In addition, before induction, we further detected the specific marker of fibroblast in order to ensure that there is no fibroblast pollution in isolated epidermis (Figures 2, 3A and 4B). Specimens were collected at 1, 2, 3, and 4 d, then embedded in optimum cutting temperature compound (OCT) in liquid nitrogen for immunofluorescence detection and examined for E-cadherin, K14, K10, N-cadherin, twist-1, slug, vimentin, α-SMA, MMP9, and collagen I at the RNA level.

1.5 Repairing full skin loss of nude mice with grafts of human epidermal explants

Full-thickness skin defects (1.5 cm×1.5 cm) were made on the backs of 30 nude male mice, which were randomly divided into two groups. The wounds in the test group were covered with epidermis from human foreskin. An immediate-bonding adhesive (Krazy Glue; Elmer, USA) was used to fix the splint to the skin, followed by interrupted 4–0 ny-

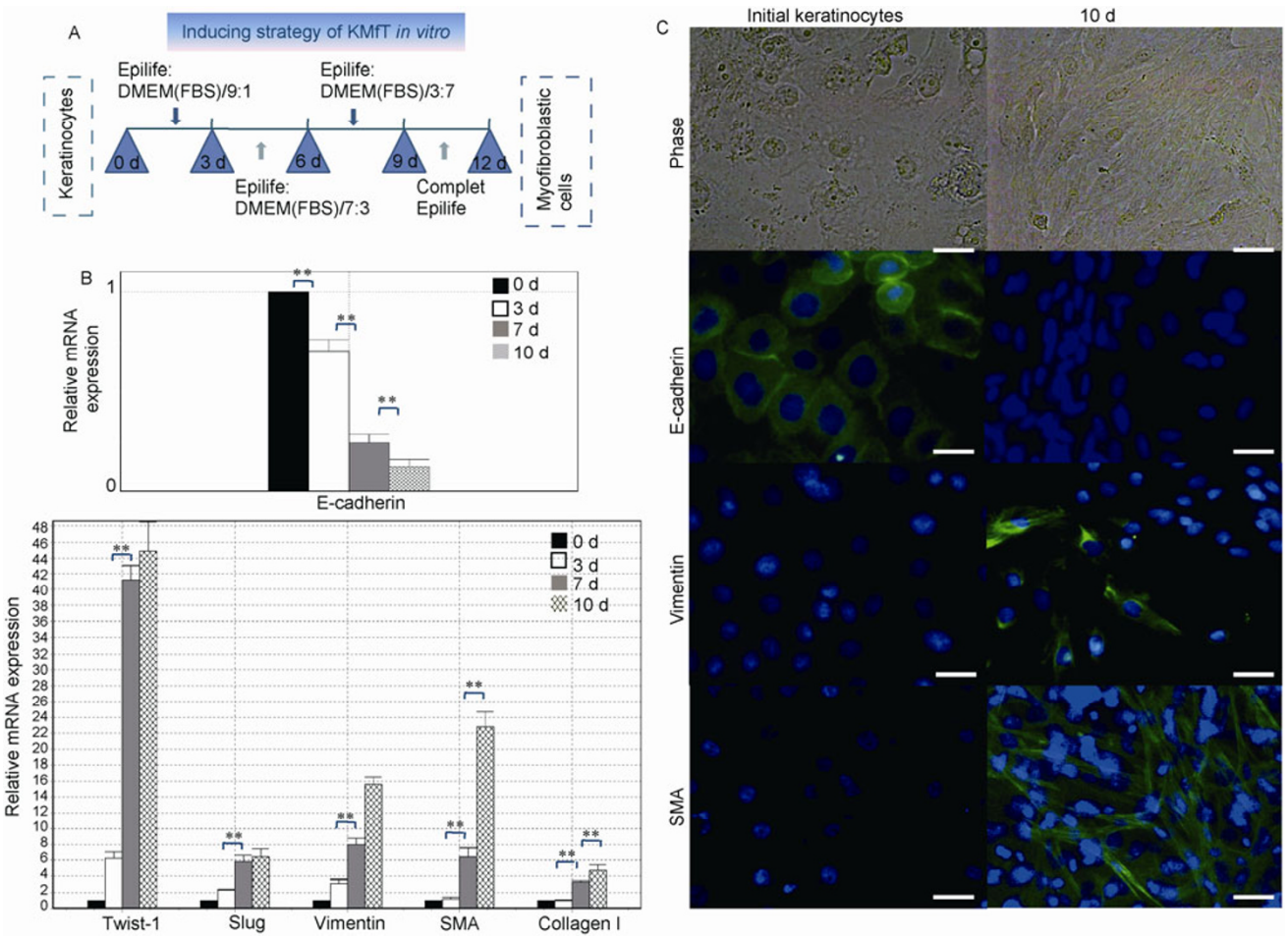

Figure 1 IF and qRT-PCR analysis of KMfT by primary human skin UKs under accumulated induction. Primary keratinocytes were cultured in growth medium to near confluence. A, A schematic representation of the strategy used to observe the KMfT of UKs. B, Gene expressions related to EMT (E-cadherin, twist-1, slug, and vimentin) and fibrosis (α-SMA, MMP-9, and type-I collagen) were measured by real-time qRT-PCR analysis. C, Primary keratinocytes were positive for E-cadherin (IF staining, green), but negative for vimentin and SMA. The mesenchymal induction was demonstrated by expression of vimentin (IF staining, green). The myofibroblastic cells were induced on day 10, as shown by phase contrast microscopy. These cells stain with SMA (IF staining, green). Scale bar, 30 μm.

lon sutures (Ethicon, USA) to ensure position, and Tegaderm (3M, Canada) was placed over the wounds. This *in vivo* technique is shown in Figure 4A. The wounds in the control group were covered with a dressing alone. The wounds were observed every two days. At the same time, we inserted a biological semi-permeable membrane between the transplanted epidermis and wound bed which permitted cytokines and large molecules to pass through without cells shuttling back and forth. Two mice were killed in each group 1, 3, 5, and 7 days post-surgery, and the epidermis was harvested. Each specimen was cut into three pieces as follows: one piece was fixed with 10% formalin and observed with a light microscope (hematoxylin and eosin staining); one piece was embedded in OCT in liquid nitrogen for detecting E-cadherin, vimentin, and α-SMA with a fluorescent microscope; and one piece was examined for E-cadherin, K14, K10, N-cadherin, twist-1, slug, vimentin, α-SMA, MMP9, and collagen I at the RNA level.

1.6 Animal models

1.6.1 Natural rehabilitation murine model

Full-thickness skin defects (1.5 cm×1.5 cm) were made on the backs of 20 C57BL mice. Samples were collected individually at 1, 3, 7, 14, and 30 days, and were embedded in OCT in liquid nitrogen for detection of E-cadherin, vimentin, and α-SMA.

1.6.2 Repeated excision murine model

A chronic injury model with which to study pathologic physiology does not exist, and chronic repair as demonstrated in diabetic mice cannot reflect pathologic physiology. Therefore, we adopted a "repeat excision model." Full-thickness skin defects (1.5 cm×1.5 cm) were made on the backs of 20 C57BL mice. Due to significant regrowth of central granulation tissue in the 3-day interval between excisions of wound contents, the central granulation tissue was completely excised daily under full anesthesia to

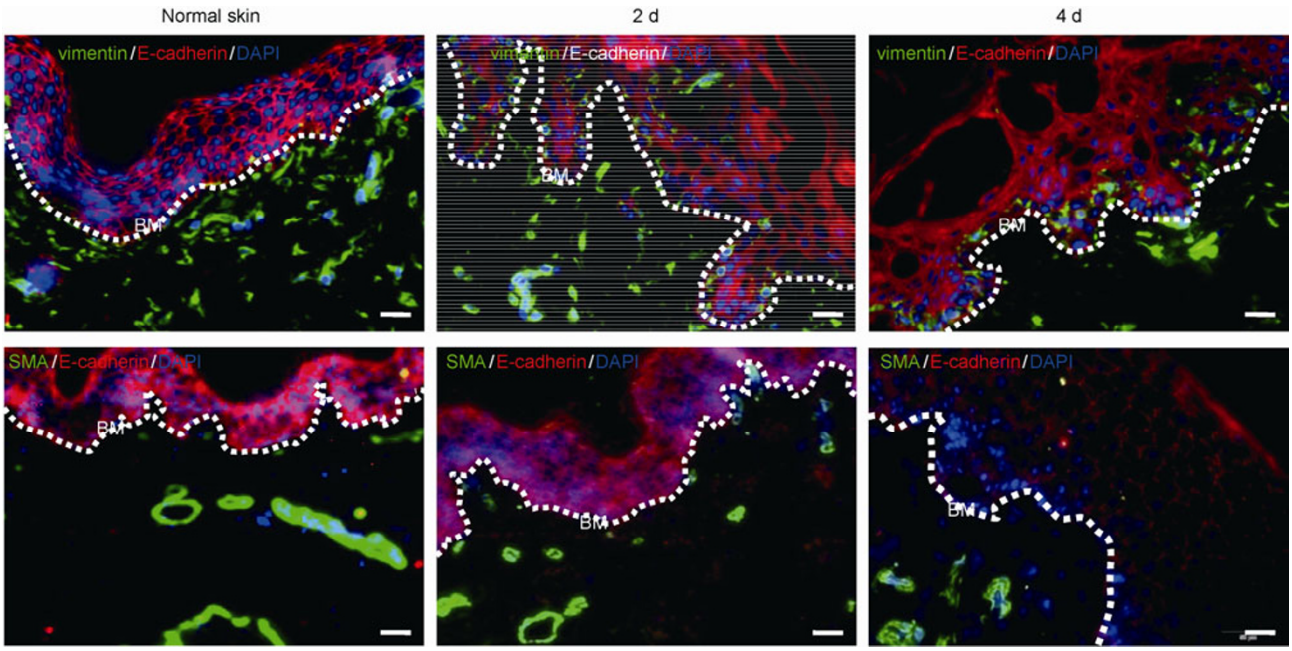

Figure 2 KMT feature detection in normal full-thickness human foreskin under serum induction. A, Adult full thickness foreskin incubates in accumulated induction medium. The mesenchymal transition in epithelial cells was detected by expression of vimentin (IF staining, green). BMZ was designated by dotted white lines. SMA-positive cells is lacking in epidermis in the process of inducing. Scale bar, 30 μm.

<1 mm from the dermal walls of the wound bed down to the deep fascia. We individually collected the samples at 1, 3, 7, and 14 days, and embedded the samples in OCT in liquid nitrogen for detection of E-cadherin, vimentin, and α-SMA.

1.7 Wound healing model and keratinocyte transplantation

Excisional skin wounds were created on the backs of male nude mice (eight weeks old; body weight, 20–22 g) in the midline. The wounds were allowed to heal naturally for 5 d, then each wound received one million cells (Carboxyfluorescein Diacetate Succinimidyl Ester (CSFE)-labeled human UKs), which were injected into granulation tissue at eight injection sites. The animals were placed in individual cages under a warming lamp and allowed to recover fully from anesthesia. The animals were housed individually in the institutional animal facility. A pictorial representation of the wound model can be seen in Figure 5A. We collected the samples at 1, 3, and 6 days and 8 weeks after cell transplantation. Mice were individually anesthetized using an intraperitoneal injection of pentobarbitone sodium. A large diameter excision would be made around the wound, then the samples were embedded in OCT in liquid nitrogen for detecting CSFE-labeled cells and the mesenchymal-related marker, vimentin, and α-SMA.

1.8 Double immunofluorescence

The induced cells cultured on the slides and frozen tissue sections were washed, fixed in neutral-buffered 4% paraformaldehyde (Sigma, USA) for 30 min at room temperature, and incubated at 4°C overnight with primary antibodies (E-cadherin, vimentin, and α-SMA). Next, the induced cells and frozen tissue sections were incubated with a secondary antibody, either DyLight 488-conjugated goat anti-mouse IgG or DyLight 594-conjugated goat anti-rabbit IgG (Invitrogen/Gibco), then subjected to nuclear staining with 4,6-diamino-2-phenyl indole (DAPI) (Invitrogen/Gibco). Appropriate and similarly-titered isotype- matched non-relevant monoclonal antibody (mouse IgG) was used as a negative control.

1.9 Quantitative real-time RT-PCR

Total RNA was extracted from tissues using TRIzol® reagent and reversely transcribed using oligo $(dT)_{15}$ primers and M-MLV reverse transcriptase. 1 μL of cDNA was added to SYBR Green PCR Master Mix (Qiagen, USA). PCR was performed on ABI 7500 Real-Time PCR System (Applied Biosystems, USA) using the following thermal settings: one cycle of 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 1 min at 60°C using the primers in Table S1 in Supporting Information. Relative mRNA expression was calculated with the $2^{-\Delta\Delta C_t}$ method. β-actin was used as the internal control.

1.10 Statistical analysis

SPSS PC software (release 13.0) was used for statistical

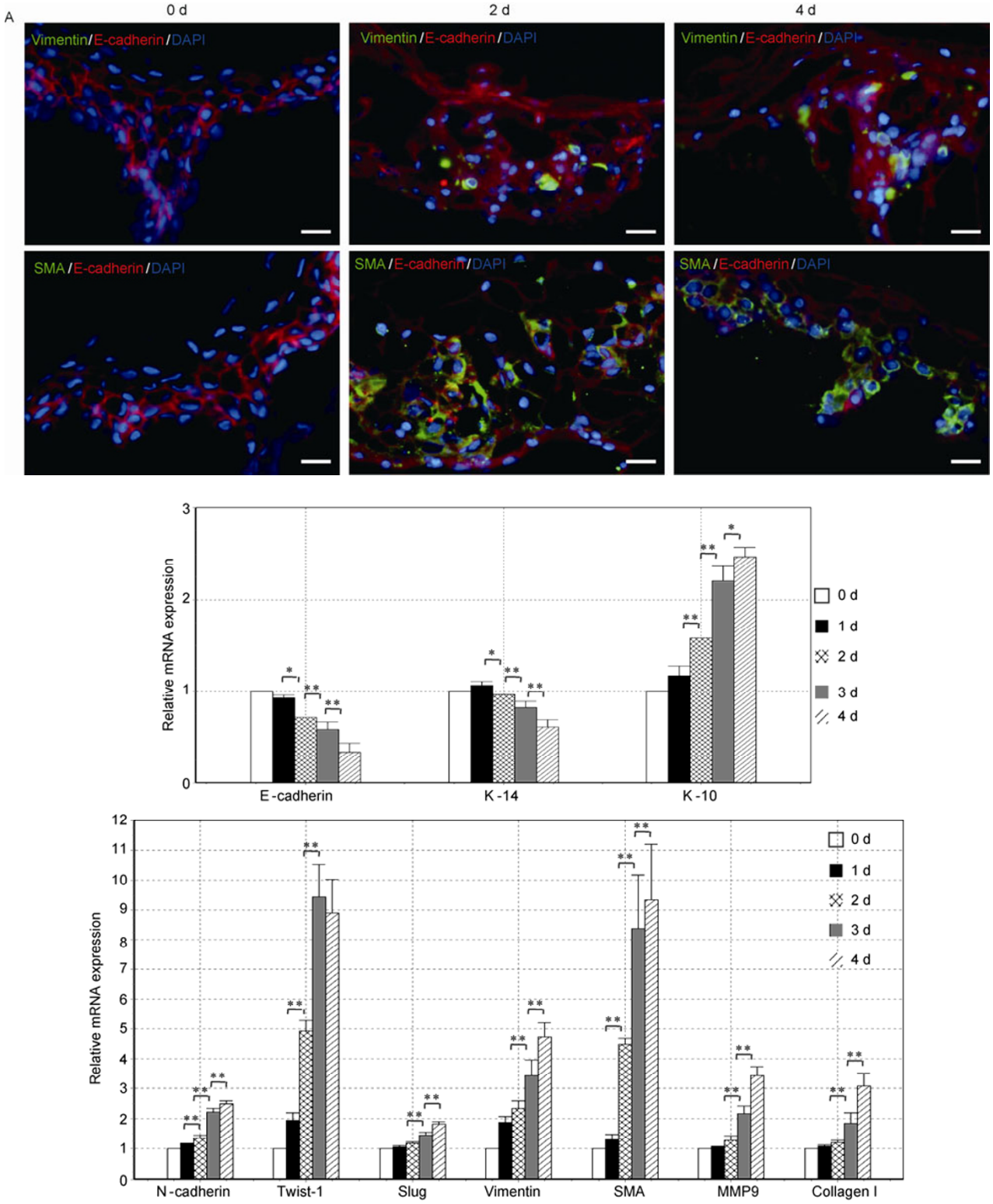

Figure 3 Induction of EMT features in epidermis derived from human foreskin *in vitro*. Epidermis derived from foreskin was primed by accumulated Induction medium, followed by culture at 37°C for 1, 2, 3, and 4 d. A, Before inducing, the isolated epidermis had full-thickness stain with E-cadherin, but rarely any vimentin- and SMA-positive cells. After serum induction, the gain of mesenchymal characteristics was examined by expression of vimentin (IF staining, green) and SMA (IF staining, green). B, The gene expression, related to EMT (E-cadherin, K14, K10, N-cadherin, twist-1, slug, and vimentin) and fibrosis (type-I collagen, α-SMA, and MMP-9) in the transplanted epidermis at different time points (*, $P<0.05$ as significance by ANOVA; **, $P<0.01$ as significance by ANOVA). Scale bar, 30 μm.

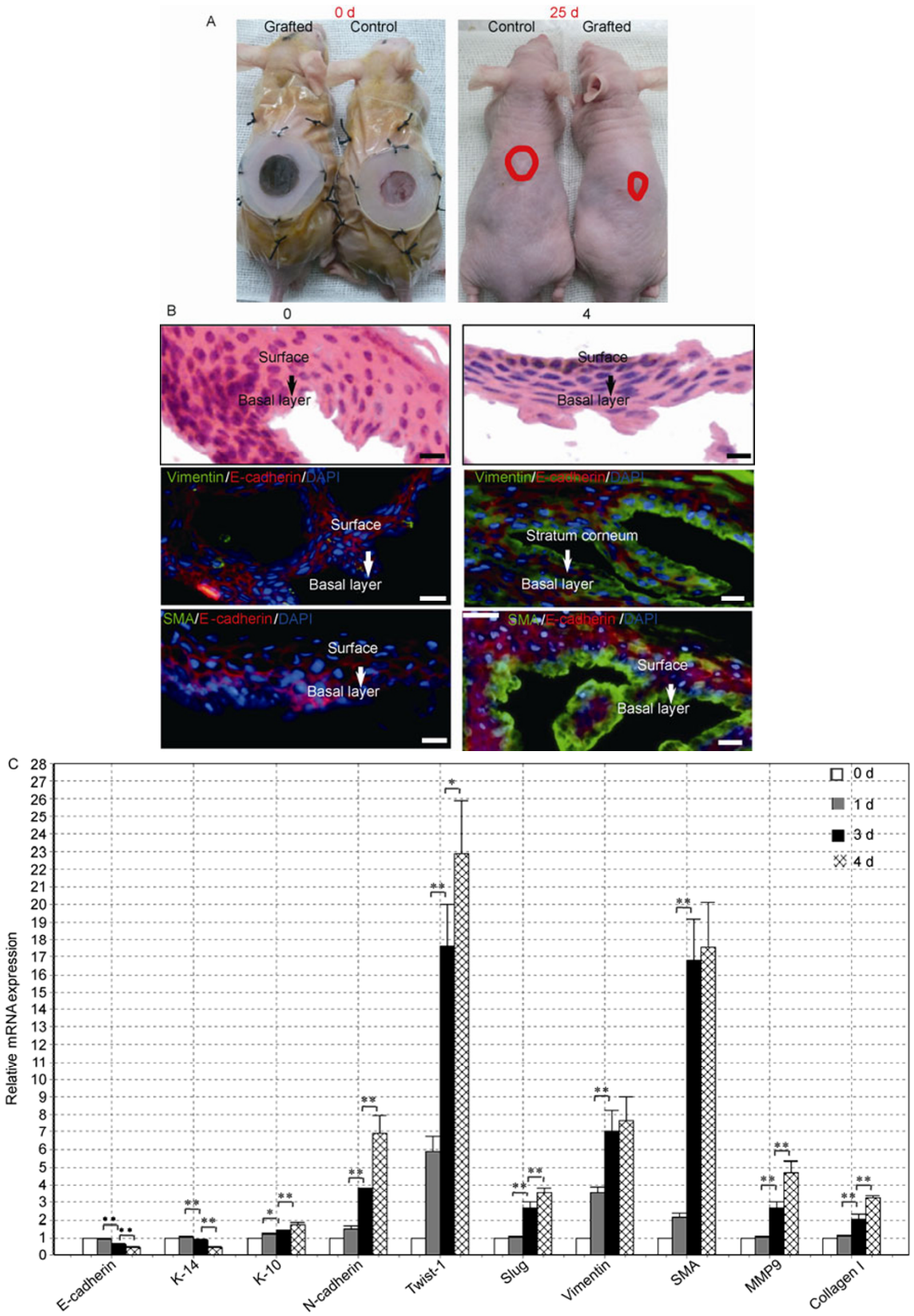

Figure 4 Induction of myofibroblastic transition in epidermis under attachment to the wound bed. A, A schematic representation of the strategy used to observe the KMfT of human epidermis. The transplanted group has a smaller scar. B, Gain of myofibroblastic features by the keratinocytes in epidermis is demonstrated by vimentin (IF staining, green) and α-SMA expression (IF staining, green). Scale bar, 30 μm. C, The gene expressions, related to EMT (E-cadherin, K14, K10, N-cadherin, twist-1, slug, and vimentin), and fibrosis (type-I collagen, α-SMA, and MMP-9) in the transplanted epidermis at different time points (*, $P<0.05$ as significance by ANOVA; **, $P<0.01$ as significance by ANOVA).

analysis. Statistical significance was evaluated using Student's *t*-test or ANOVA. Values of $P<0.05$ were considered significant, unless otherwise stated.

2 Results

2.1 Keratinocytes at the tongue of the wound margin acquire mesenchymal features in normal and abnormal wound healing

Previous research has shown that keratinocytes at the margin of the wound gain a reversible phenotype of mesenchymal-like cells in normal wound healing [17]. We further tested the biological change of keratinocytes at the margin of the wound. In our natural rehabilitation model, the keratinocytes at the margin of the wound showed vimentin staining approximately on day 3. As shown in Figure S1 in Supporting Information, several vimentin-positive cells were located in the basal or superbasal layer of the epidermis on day 9; however, we did not find α-SMA-positive cells in the epidermis at any time point. Thus, we speculate that KMfT may occur during abnormal wound healing, especially in keloids. We examined additional biopsies from keloids to elaborate the features of keratinocyte-mesenchymal transition (KMT). The keloid in the analysis varied from 12 to 24 months after injury and was in the growth phase, as judged by the raised erythematous appearance and stiffer consistency than surrounding skin. Keratinocytes were characterized by E-cadherin expression, and mesenchymal features were defined by vimentin and α-SMA. Vimentin-positive cells were frequently found in the thickened epidermis of keloids (Figure S1 in Supporting Information); however, α-SMA-positive cells were rarely found in the epidermis of keloids (Figure S1 in Supporting Information). Together, these results suggest that acute normal wound healing and chronic fibrotic scaring may share common KMT characteristics, but no KMfT occurs in these processes.

2.2 Keratinocytes at the tongue of the wound margin acquire mesenchymal features in a chronic injury model

We reasoned that during the re-epithelization phase, the epidermal cells in the migrating tongue may be mobilized through gain-of-mesenchymal features, and we further speculated such partial EMT may be transitioned into KMfT under chronic injuries. Therefore, we established a repeat incision animal model to confirm this presumption. The biopsies were harvested at different stages of repair after repeat excision. In these specimens, we identified epithelial cells that had undergone EMT and gained mesenchymal markers, such as vimentin. The keratinocytes around the wound edge were shown to be mesenchymal marker-positive (Figure 6). In contrast, keratinocytes distant from the wound edges in the spinous and granular layers clearly lacked the mesenchymal markers (Figure 6). Taken together, these results further confirmed that keratinocytes around the wound edge gain EMT features during chronic wound healing, but could not transdifferentiate into myofibroblastic cells.

2.3 Gain of myofibroblastic features by transplanted UKs in the wound bed

Cultured autologous keratinocytes have been used extensively to help treat non-healing wounds; however, the potential mechanism of the positive effects for wound closure is still unclear. We adopted two animal models to track the fates of the transplanted keratinocytes. Our basic assay for transition *in vivo* involves isolated keratinocytes labeled with CSFE implanted into granulation tissue (Figure S2 in Supporting Information). After 5 days, we collected samples at the wound sites. Compared with untreated wounds, treated defects exhibit a higher rate for healing and leave smaller scars, but have more obvious contractions around the wound (Figure 5A and B). We detected the genes related to wound repair and showed that these gene expression was significantly increased when compared with the control group (Figure 5C). Myofibroblasts are responsible for wound contraction. Thus, we propose that transplanted keratinocytes might transdifferentiate into myofibroblasts directly, or accelerate wound closure by enhancing another cell-myofibroblast transition. To monitor the fate of transplanted keratinocytes, we made frozen sections to track the changes in CSFE-labeled cells. We observed that CSFE-labeled cells were diffusely distributed in granulation tissue, and a significant fraction of the inoculated cells switched into α-SMA-positive and E-cadherin-negative cells (Figure 5D).

Several earlier studies have demonstrated the incorporation of donor cells in host tissues; transdifferentiation has been explained by fusion of donor and host cells and the formation of heterokaryons [19–21]. In contrast, in the current study almost all of the injected keratinocytes expressing α-SMA contained only one normal-appearing nucleus (Figure 5D). This suggested that the expression of α-SMA in donor-derived cells was not the result of fusion with host cells.

2.4 Gain of myofibroblastic characteristics by keratinocytes derived from transplanted epidermis in the wound bed

Skin grafting has been commonly used for the treatment of chronic wounds. To explore the possible mechanism for accelerating wound healing, we used epidermis sheets which were isolated from foreskins, and transplanted onto full-thickness skin wounds in immunodeficient BALB/c nude mice (Figure 4A). Before grafting, the epidermis was negative for vimentin or α-SMA, but was strongly positive

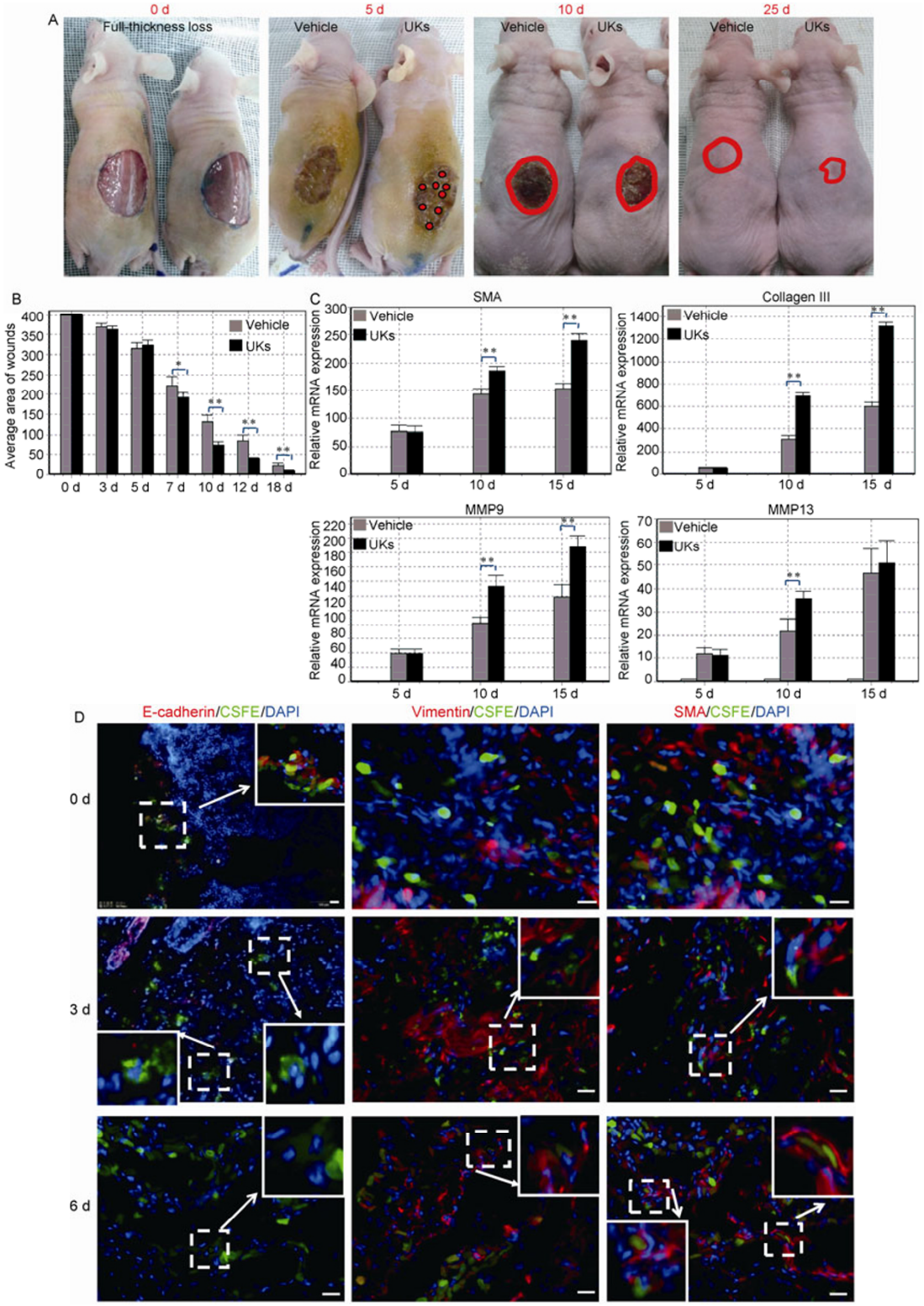

Figure 5 Induction of myofibroblastic transition in UKs under granulation tissue transplantation. A, A pictorial representation of the strategy used to observe the KMfT *in vivo*. After natural wound rehabilitation for 5 d, CSFE-labeled cells were injected into newly formed granulation tissue. After healing for 10 d (5 days after transplantation), the area of the wound was much smaller in the injected group. B, The comparison of the average area of wounds between injected and non-injected at different time points (*, $P<0.05$ as significance by Student's *t*-test; **, $P<0.01$ as significance by Student's *t*-test). C, Gene expressions, related to fibrosis (type-I collagen, α-SMA, MMP-9, and MMP13) in the scars and normal skin are measured by real-time qRT-PCR analysis. The levels of expression are normalized by mRNA of β-actin (*, $P<0.05$ as significance by ANOVA; **, $P<0.01$ as significance by ANOVA). D, The alternating variation of epithelial markers and mesenchymal markers of grafted cells *in vivo*. Grafted labeled cells were detected immediately after injection. These cells were E-cadherin and CSFE co-positive, but no stain with vimentin or SMA. However, grafted cells were negative for E-cadherin, and positive for vimentin (IF staining, red) and SMA (IF staining, red) at 3 days post-grafting. Scale bar, 30 μm.

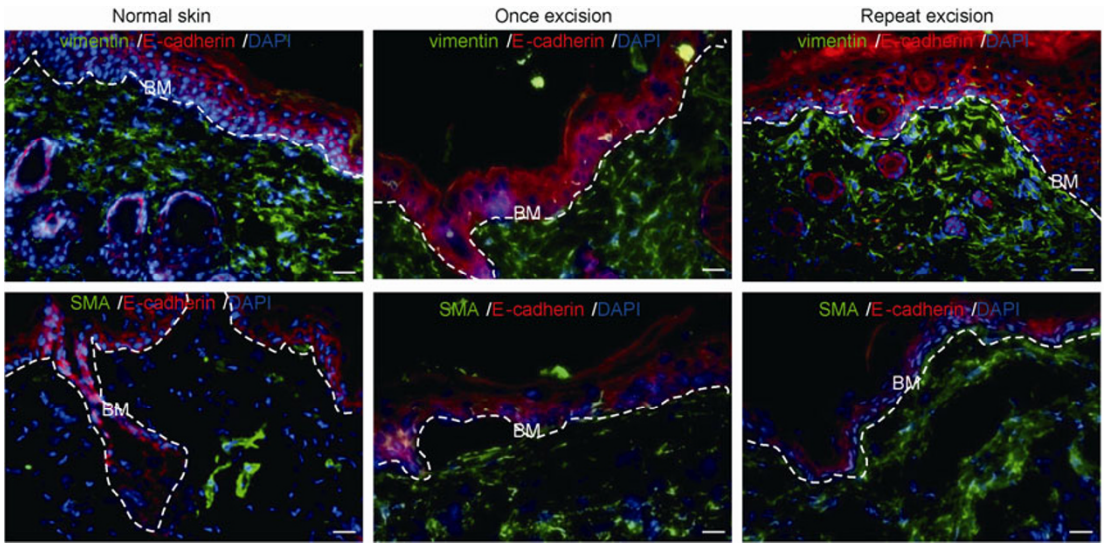

Figure 6 Mesenchymal features of keratinocytes at the tongue of the wound margin in a chronic injury model. A, The samples were stained by E-cadherin (IF staining, red) and vimentin (IF staining, green) or E-cadherin and SMA (IF staining, green). Mesenchymal features gain by the epithelial cells in the migration tongues because of vimentin expression. Nuclei DNA are stained by DAPI in blue. High magnitude images show the apparent SMA-positive cells only located in the dermis (green). BMZ was designated by dotted white lines. Scale bar, 30 μm.

for E-cadherin. After grafting, the vimentin- or α-SMA-positive cells increased (Figure 4B). We further examined the epithelial, fibrotic, and EMT-related genes to determine whether or not the SMA-positive cells were myofibrotic cells. Among the epithelial markers, E-cadherin and keratin 14 were significantly decreased, but keratin 10 was significantly increased over time. The classic fibrotic markers, type-I collagen and α-SMA, showed an opposite tendency. Finally, the EMT-related transcription factors of human, slug and twist-1, were significantly expressed in transplanted epidermis (Figure 4C). Taken together, these data suggested that keratinocytes in the grafted epidermal sheets transdifferentiated into myofibroblast-like cells *in vivo*. Moreover, we noticed that the α-SMA-positive cells were not limited to the basal layer keratinocytes. α-SMA-positive cells occupied the suprabasal and spinous/granular layers of the epidermis after transplantation. These results suggested that keratinocytes could transdifferentiate into myofibroblast-like cells in the proper microenvironment, and the transdifferentiated keratinocytes play a positive role for wound healing.

2.5 Primary human UKs undergo KMfT by accumulated induction

The microenvironment is critical for cell reprogramming. The first phase in wound healing is the inflammatory phase, which is triggered by capillary damage and leads to the formation of a blood clot composed of fibrin and fibronectin and serum. The cells located in and around the wound are soaked in serum. Therefore, we speculated that serum may have the driving force to provoke an EMT in skin. EMT is an intricate process by which epithelial cells lose their epithelial characteristics and acquire a mesenchymal-like phenotype. In this part of the study, the dynamic change of epithelial and mesenchymal markers with time was detected.

Some keratinocytes began to lose their original morphology within one week, then typically appeared as myofibroblasts at 1–2 weeks (Figure 1). The original keratinocytes served as a negative control and were 100% confluent without morphological changes (Figure 1). Based on the change in morphology of the cell, we selected four different time points to observe related gene expression. Among the epithelial markers, E-cadherin and keratin 14 had a tendency to decline, but keratin 10 showed an increased tendency with time (Figure 1B). The classic fibrotic markers, type-I collagen and α-SMA, and EMT-related markers showed a gradual tendency to increase (Figure 1B). To determine whether keratinocytes expressed myofibroblast markers after induction, immunochemical analysis was performed to examine the phenotypes. As shown in Figure 1C, under stimulation most of the UKs expressed vimentin, an intermediate filamentous cytoskeleton involved in the migration of mesenchymal cells. Vimentin fibers were observed in the perinuclear regions; moreover, α-SMA is a hallmark for myofibroblasts and was found in the cytoplasm (Figure 1C). These features are prominent characteristics of mesenchymal cells, especially myofibroblasts, demonstrating plasticity of the keratinocytes under this condition. The original keratinocytes in the control group showed no staining for vimentin and α-SMA, but intense staining for E-cadherin. All these results suggested that UK-derived myofibroblastic cells were similar to myofibroblasts in morphology and function.

2.6 Gain of myofibroblastic characteristics by epidermis derived from foreskin under accumulated induction

Epidermis isolated from foreskin was incubated in accumulated induction medium at 37°C in CO_2. The samples were collected at 1, 2, 3, and 4 d, and assessed for KMfT. In the control skin, SMA- and vimentin-positive cells were absent from the epidermis (Figure 3B). Under stimulation for 2 d, a small amount of keratinocytes in the epidermis expressed vimentin, illustrating EMT induction (Figure 3B). In addition, we noted α-SMA-positive cells in the epidermis after induction for 2 d, which were located in nearly the entire basal layer of the epidermis (Figure 3B). We further examined the genes related to KMT and KMfT. The dynamic change in the expression of these genes was similar to the *in vivo* results (Figure 3A).

2.7 Gain of KMT features by normal full-thickness human foreskin under accumulated induction

Compared with the change in epidermis *in vitro*, normal full-thickness human skin slices were incubated in accumulated induction medium at 37°C in CO_2. The samples collected at 1, 2, 3, and 4 d were then assessed for EMT characteristics. In the control skin, vimentin-positive cells were largely absent from the epidermis, but found in the dermis as expected (Figure 2). Under the stimulation, several keratinocytes in the epidermis in proximity to the basement membrane zone (BMZ) express vimentin, illustrating EMT induction (Figure 2), but no α-SMA-positive cells were noted in the epidermis at any time. At higher magnification, we noticed that vimentin-positive cells occupied the suprabasal and spinous/granular layers of the epidermis under accumulated incubation (Figure 2); however, these findings were consistent with the *in vivo* findings.

3 Discussion

Epidermis and keratinocyte suspensions have been assessed as treatment methods for chronic non-healing wounds [22,23]. Keratinocytes grafted on full-thickness wounds have yielded satisfactory results owing to rapid healing [24]. However, the underlying mechanisms have not been fully described.

In our study, we established two animal models to mimic the transplanted strategies in the clinical setting, which showed that wounds transplanted with keratinocytes were healed more rapidly and formed a smaller scar compared with wounds without cell transplantation; however, the former is associated with wound shrinking. A number of studies have emphasized that myofibroblasts play a pivotal role in wound contraction [13]. Therefore, we speculated that transplanted keratinocytes may transdifferentiate into myofibroblasts or promote the formation of myofibroblasts in wounds to enhance wound repair. In the current study, we found that a significant fraction of the inoculated cells spread in the granulation tissues and transdifferentiated into myofibroblast-like cells expressing myofibroblast markers, and contained only one normal-appearing nucleus, indicating a genuine transdifferentiation process induced by the host wound environment. We further detected the expression level of healing-related genes in mice. The expression level of repair-related genes in murine granulation tissue between the wounds with or without cell transplantation had a clear difference, showing that the healing-related genes were more highly expressed in the transplanted group. However, these transplanted keratinocytes did not survive permanently on the wound bed, which cannot be found eight weeks after transplantation (Figure S3 in Supporting Information). It may be concluded that through switching into functional myofibroblastic cells or through a paracrine manner, transplanted keratinocytes play a role in enhancing the expression level of repair-related genes to accelerate wound healing. A previous study [25] using an animal model demonstrated that the re-epithelization capacity of non-cultured keratinocytes was four times greater in the treatment group than that in the control group, which was confirmed by our study.

We achieved similar results in an epidermis transplanted model. Keratinocytes located in or near the basal layer were found to transdifferentiate into SMA-positive cells. It also provides evidence that epidermal grafting in clinical promotes wound repair. Further, keratinocytes not only terminally differentiate, but also transdifferentiate into other cell lineages *in vivo*, which is the evidence about supplementing the multipotency of keratinocytes discovered *in vitr*o [26].

In our study, we found that when keratinocyte suspension or epidermis was put into a wound microenvironment, keratinocytes underwent a myofibroblast-like transition. We further examined the keratinocytes at the tongue of the margin of the wound in samples of normal and abnormal wound healing. Keratinocytes at the margin of the wound underwent a mesenchymal-like transition rather than KMfT. These findings are inconsistent with the experimental results of keratinocyte transplantation or epidermal grafting. This may be caused by two main reasons. First, in our study, keratinocyte or epidermis derived from human is transplanted onto mouse wound bed. The central message is that heterologously transplanted keratinocytes may undergo KMfT while endogenous keratinocytes in the wound do not show such change. The wound environment of different species might be the prerequisite for KMfT. Therefore, in further research, we will emphasize that isolated keratinocytes or epidermis will cover up the wound bed of the same species. Second, the same cells from different species have different biological characteristics. That is to say, the capability of KMfT is owned by keratinocytes from human rather than mice. Therefore, difference in biological behaviors of the same cells derived from different species needs to be

further explored.

Moreover, *in vitro* induction study showed that the original keratinocytes and keratinocytes in epidermis developed KMfT, while keratinocytes in full-thick skin gained the characteristics of KMT. These results are consistent with the findings *in vivo*. These results suggested that the accumulated induction model might be a suitable experimental model for studying the mechanism of KMfT. In addition, we noticed that the keratinocytes were inclined to myofibroblastic transition when they detached from their original dermis. We speculated that the components of dermis, especially the BM, or the cells in dermis prevent the keratinocytes from KMfT. Many studies have proved that the keratinocyte and mesenchymal interaction occurs in wound healing. The epidermal-mesenchymal interaction, completely different from the concept of the EMT, refers to proximate paracrine cross-talk between tissue epithelia and stromal components [13]. The molecular mechanisms underlying epithelial-mesenchymal interactions for preventing KMfT are more complex and need further investigation. In the research, we revealed a possible mechanism of how transplanted keratinocytes and epidermal grafting promote wound healing. Nevertheless, the critical factors for promoting KMfT need further study.

It is noteworthy that human skin is composed of a variety of cells. We need to appreciate the diversity of cellular residents in the human skin, which limits our capability to make conclusive remarks about the lineage and/or origin of cells. Further, melanocytes and Langerhans cells have also been shown to express vimentin [27,28]. Moreover, the extent and spectrum of induction of mesenchymal markers in EMT is highly variable, mainly relying on the subtypes of epithelial cells. Therefore, we approached our interpretations and conclusions with cautions herein, particularly for the KMfT in wound healing.

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Supporting Information

Figure S1 KMT feature detection in re-epithelization of normal and abnormal wound healing. A, Gain of mesenchymal features by the epithelial cells in the migration tongues is demonstrated by vimentin and SMA expression (IF staining, green). Nuclei DNA are stained by DAPI in blue. B, Gain of mesenchymal features by the epithelial cells in keloid is demonstrated by vimentin and SMA expression. Vimentin-positive cells (IF staining, green) detected in the epidermis of keloid. The EMT features are absent in the epithelial cells of spinous and granular layers of keloid. It shows vimentin-positive cells adjacent to the BMZ. There are rare SMA-positive cells in the epidermis of foreskin or keloid. The BMZ is designated by dotted white lines. Scale bar, 30 μm.

Figure S2 CSFE labeling of the initial separation UKs. The CSFE mark rate was approximately 99% of the initial separation UKs (green).

Figure S3 Tracking of CSFE labeling UKs in healed skin of cell-injected groups at 8 weeks. The samples were stained by vimentin, or E-cadherin and SMA. There were no CSFE (green) and vimentin (IF staining, red), or CSFE (green) and SMA (IF staining, red) co-positive cells. Scale bar, 30 μm.

Table S1 Primer sequences used for real time RT-PCR

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