



## Lack of transient receptor potential ankyrin 1 (TRPA1) retards cutaneous wound healing in mice: A preliminary study

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### ABSTRACT

Wound healing is an important process in various diseases, and elucidating the underlying mechanism is essential for developing therapeutic strategies. We investigated whether the loss of transient receptor potential ankyrin 1 (TRPA1) affects the cutaneous wound healing process in mice. We assessed the formation of granulation tissue by myofibroblasts and macrophages, re-epithelialization, and related gene expression. TRPA1-null (KO) and wild-type (WT) C57BL/6 mice were used for establishing the wound model. Two round full-thickness excision wounds (diameter, 5.0 mm) were produced in the dorsal skin of mice under general anesthesia. After specific intervals, healing was evaluated using macroscopic observation, histology, immunohistochemistry, and real-time reverse transcription-polymerase chain reaction (RT-PCR). TRPA1 KO retarded the formation of granulation tissue and re-epithelialization in the healing of cutaneous wound. Furthermore, TRPA1 KO suppressed the appearance of myofibroblasts, macrophage infiltration, and mRNA expression of  $\alpha$ SMA, F4/80, and Col-1 $\alpha$ 2. These findings indicate that TRPA1 is required for cutaneous wound healing in mice. The lack of TRPA1 retards macrophage infiltration and the subsequent fibrotic tissue formation, which might further impair the fibrogenic behavior of fibroblasts.

### 1. Introduction

Rapid healing of skin wounds is essential for maintaining good health as it prevents bacterial contamination, water loss, and scar formation. The healing of soft tissues, including skin, is an important factor influencing the success rate of treatment in patients with pressure ulcers, diabetic wounds, open fractures, extensive soft tissue damage, and infection. A complete understanding of cutaneous wound healing is necessary to establish strategies for overcoming wound healing disorders.

The cutaneous wound healing involves three phases—the inflammatory, proliferative, and reconstruction phases [1–3]. When deep skin damage occurs, platelets aggregate to close the wound. Inflammatory cytokines and growth factors are then released from the damaged tissue and broken platelets, increasing vascular permeability and inducing the entry of neutrophils and macrophages to the wound. These neutrophils and macrophages remove bacteria and minute foreign substances from the damaged tissues, and the wound is cleaned. The series of steps from

hemostasis to wound cleansing is collectively defined as the inflammatory reaction, constituting the inflammatory phase. As the internal cleansing of the wound progresses, the cells centered on the macrophages secrete growth factors that differ from those secreted during the inflammatory phase. Thereafter, fibroblasts and vascular endothelial cells migrate to the wound and start dividing. Fibroblasts produce collagen, and the period during which collagen forms a network structure, with capillaries extending into it, is defined as the proliferative phase. Subsequently, a relatively long-lasting reconstructive phase ensues in which the scar tissue is replaced with normal tissue. Keratinocytes and fibroblasts play important roles in the proliferative phase. Keratinocytes are the first to move to the damaged area, forming a primary coat that begins to proliferate and restore stratification. Fibroblasts transform into myofibroblasts that generate the extracellular matrix and contractile tissue. The potent transforming growth factor  $\beta$  (TGF- $\beta$ ) regulates the migration of keratinocytes and the conversion of fibroblasts to myofibroblasts [4].

Transient receptor potential (TRP) channels are polymodal receptors

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that mediate sensory transmission and are activated by various stimuli [5]. The TRP superfamily contains 28 genes that are subdivided into seven subfamilies [5]. Among these, transient receptor potential ankyrin 1 (TRPA1) is highly expressed in some nervous tissues and was initially considered a sensor that responds to temperatures below 17 °C; however, this contention is now disputed in mammals. In recent years, TRPA1 has been shown to respond to *in vivo* alkalis, endogenous prostaglandins, nitric oxide (NO), free radicals, and reactive oxygen species (ROS), such as H<sub>2</sub>O<sub>2</sub>, as well as to extrinsic stimulants, such as mustard oil, cinnamon, wasabi, and garlic [6–12]. Animal studies have shown that TRPA1 is also involved in the control of local tissue inflammation in inflammatory diseases and chemical reactions to drugs [6,12]. We previously reported that by regulating the inflammatory response, TRPA1 is closely involved in wound healing in the corneal epithelium [25]. Based on these findings, we hypothesized that TRPA1 may also play a major role in cutaneous wound healing because, similar to the cornea, the skin is also derived from the ectoderm.

To test this hypothesis, we used C57BL/6 mice lacking *TRPA1* and assessed the impact of this deficiency on cutaneous wound healing. For quantitative assessment of the process of skin wound healing, we analyzed the closure rate of circular excision wounds at regular healing

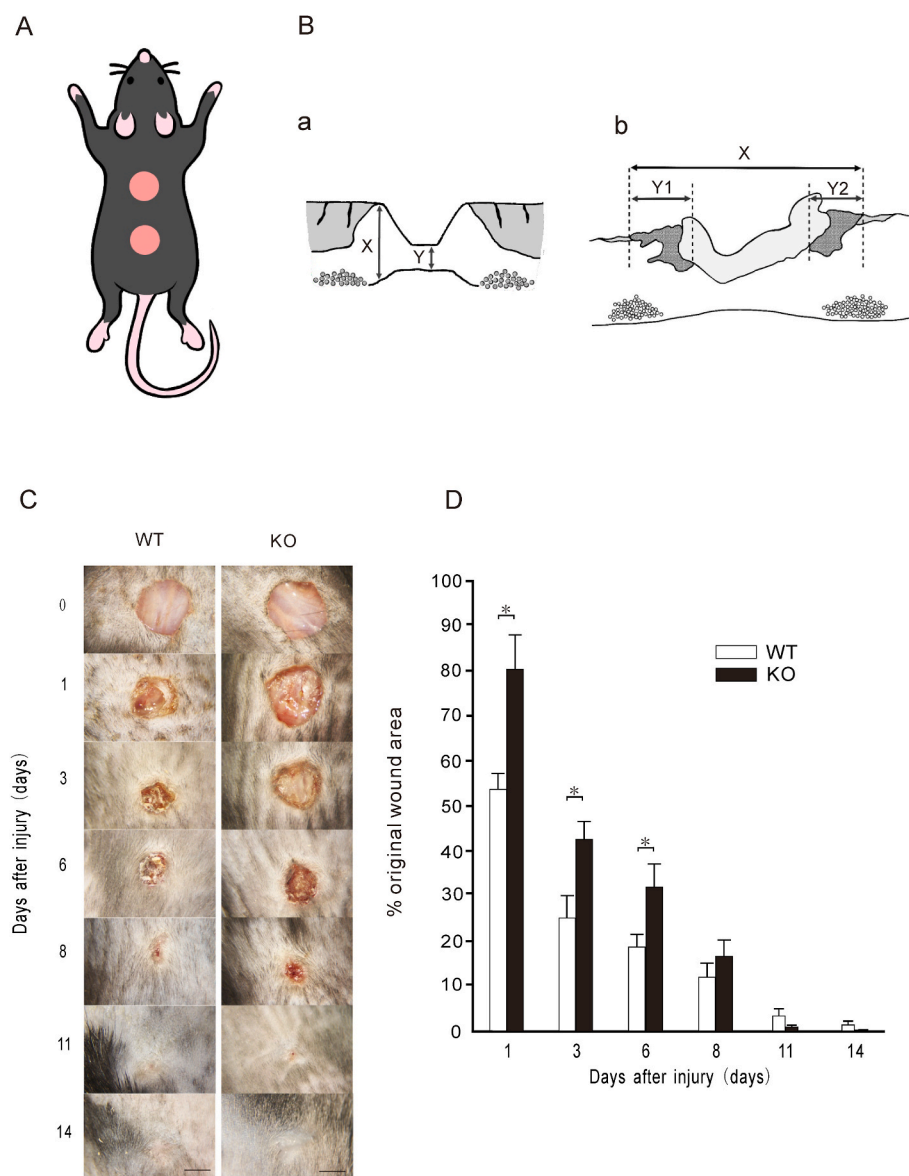
intervals employing macroscopic observations, histology, immunohistochemistry, and detection of mRNAs.

## 2. Materials and methods

This study design was approved by the Animal Care and Use Committee and DNA Recombination Experiment Committee of Wakayama Medical University, Japan.

### 2.1. Generation of full-thickness cutaneous wound in mice

TRPA1-knockout (KO) and wild-type (WT) mice in C57BL/6 background, aged 8–10 weeks, were used. We determined the minimum sample size requirement from an animal welfare perspective based on our previous studies [13,14]. Mice were anesthetized using an intraperitoneal injection of a mixture of three anesthetic agents (0.5 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol) [15]. We created two round (diameter, 5.0 mm), full-thickness excision wounds on the back skin of mice using sterile disposable biopsy trephines (Kai Industries, Gifu, Japan) and a surgical blade after shaving the back hair (Fig. 1A) [13,14]. To keep the wounds clean, an aseptic



**Fig. 1.** Scheme of excision wound model and histological evaluation method. Macroscopic observation of the wounds. (A) Excision wound model in mice. (B) a; Histological scheme of skin wound. Thickness of newly generated granulation tissue was measured at the marginal area with the largest granulation tissue thickness (X) and in the central area with the smallest granulation tissue thickness (Y). b; Re-epithelialization in excised skin. The percentage of re-epithelialization in the original wound area was calculated as follows: Re-epithelialization (%) = [distance covered by epithelium (Y1+Y2)] × 100 / [distance between original wound edges (X)]. (C) Macroscopic observation of dorsal wound in knockout (KO) and wild-type (WT) mice. Bar, 1 mm. (D) Graph showing the ratios of unhealed wound area to the original area for each healing period. Significant differences were observed between the two groups at days 1, 3, and 6. The unhealed wound area in KO mice was significantly larger than that in WT mice. Open bars: WT, filled bars: KO (*n* = 7 animals in each group). Values represent the mean ± standard deviation. \**p* < 0.05.

procedure was performed for wound creation, and ofloxacin ointment (0.3%) was applied daily for 7 days after wounding. To avoid missing the signs of distress, such as weight loss, inappetence, vomiting, or diarrhea, we investigated the health of the mice before cleaning and feeding every morning. Signs of infection, such as abnormal bleeding and pus in the wound, were also carefully checked daily. We set humane endpoints with a particular emphasis on weight loss and signs of infection. Because of the meticulous care, none of the mice crossed the above endpoints during the study period.

## 2.2. Macroscopic observation

A total of 14 mice (7 KO and 7 WT) were macroscopically analyzed. We observed and pictured the wounds at various time points (0, 1, 3, 6, 8, 11, and 14 days after injury) using a stereomicroscope. The percentage of residual wound area to the original wound area was determined using the Photoshop software (version 19.1, Adobe Systems, Tokyo, Japan).

## 2.3. Histological evaluation

A total of 56 mice (28 KO and 28 WT) were used for histological evaluation. We euthanized mice by cervical dislocation at specific time points (3, 6, 8, and 11 days after injury) and then harvested wound tissue during the healing process using the procedure employed to create the wound. The harvested tissues were fixed in 4.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 48 h and embedded in paraffin. The thickness of the granulation tissue was measured after hematoxylin-eosin (HE) staining of paraffin sections (5  $\mu$ m thick) (Fig. 1B–a) [13,14].

Furthermore, the re-epithelialization of excisional skin wound healing was analyzed. Newly formed granulation tissue in the wounds is accompanied by re-epithelialization by keratinocytes. We evaluated the percentage of re-epithelialization relative to the original wound using Masson-Goldner trichrome-stained tissue (Fig. 1B–b) [16–19].

## 2.4. Immunohistochemical analysis

Immunohistochemical analysis was performed using a method to assess the cellular components of regenerating tissue at 6 and 8 days post-injury [20,21]. Rat monoclonal F4/80 anti-macrophage antigen antibody (Clone A3-1, 1:400; BMA Biomedicals, August, Switzerland) was used to evaluate the presence of monocytes and macrophages. The presence of myofibroblasts was confirmed using a mouse monoclonal anti- $\alpha$ -smooth muscle actin ( $\alpha$ SMA) antibody (Clone 1A4, 1:100; Neomarkers, Fremont, CA, USA). We selected two fields of view from the margins of the wound ( $\times$  400) and three fields of view from the center ( $\times$  400) and compared the average of the values for these five fields between KO and WT mice [22,23].

## 2.5. Evaluation of mRNA expression

With reference to macroscopic observations, mRNA expression was assessed on day 6 post-injury. Total RNA was extracted using the ISOGEN II reagent (Nippon Gene Co., LTD., Tokyo, Japan) and reverse-transcribed using PrimerScript RT Mix (Perfect Real time) (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. The mRNA expression of F4/80, collagen 1 $\alpha$ 2, TGF- $\beta$ 1, and  $\alpha$ SMA in regenerating tissues was evaluated using real-time reverse transcription-polymerase chain reaction (RT-PCR). Quantitative PCR was performed using the StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with SYBR Premix EX Taq II (Tli RNase H Plus) (Takara Bio) according to the manufacturer's instructions. The expression levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TGF- $\beta$ 1,  $\alpha$ SMA, F4/80, and Col-1 $\alpha$ 2 genes in newly-healed tissue on the dorsal side of the KO and WT mice were analyzed with standards using pre-designed primers (Supplement table 1).

## 2.6. Statistical analysis

For blinded evaluation, a person who was unaware about the labeling of the KO or WT mice was in charge of various measurements. Data obtained were statistically analyzed using JMP Pro version 16 (SAS Inc., Cary, NC, USA) and the statistical significance was set at  $p < 0.05$ .

## 3. Results

### 3.1. Macroscopic observations suggest delayed healing of cutaneous wounds in TRPA1 KO mice

The wound area remaining at the time of evaluation on days 1, 3, and 6 was significantly different between the two groups ( $p < 0.0001$ ,  $p < 0.0001$ , and  $p = 0.048$ , respectively). The wound area was significantly larger in the KO mice than in the WT mice (Fig. 1C and D). The initial wound healing was delayed in KO mice compared with that in WT mice. On day 6, the remaining wound area in WT mice decreased to 19.0% of the original, whereas in KO mice, it was approximately 32.1% of the original. However, after day 8 of injury, no significant difference was observed between the two groups, and skin defects appeared to be almost healed in both the groups on day 11 after injury. These observations indicate that the lack of TRPA1 delayed the early processes in the healing of full-thickness cutaneous wounds.

### 3.2. TRPA1 KO affects the formation of new granulation tissue in skin wounds

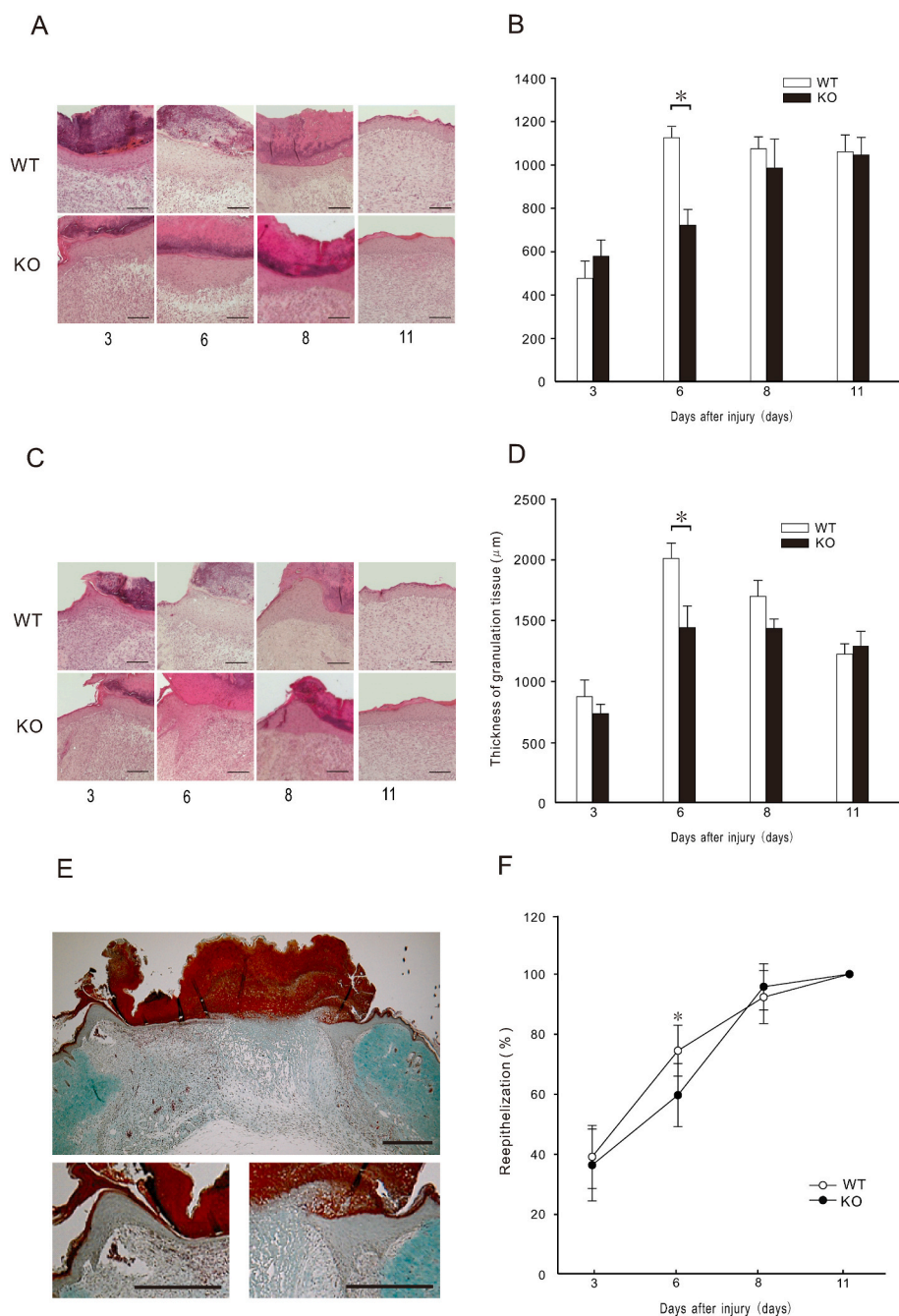
The process of wound closure through the formation of new granulation tissue was histologically observed using HE staining (Fig. 2A and C). The thickness of the granulation tissue formed in the center and at the margins of the wound was significantly different between the KO and WT mice on day 6. On day 6, the thickness of the granulation tissue in the center (KO, 720  $\mu$ m; WT, 1123  $\mu$ m;  $p = 0.001$ ) and at the margins (KO, 1437  $\mu$ m; WT, 2007  $\mu$ m;  $p = 0.023$ ) of the wound was significantly smaller in the KO mice than that in the WT mice (Fig. 2B and D). These results indicate that TRPA1 is critical in the early phase of healing of skin wounds.

### 3.3. Lack of TRPA1 retards re-epithelialization of cutaneous wounds by keratinocytes

No epithelial regeneration was observed in either the WT or KO mice on day 1 after injury (data not shown). Although re-epithelialization could be observed on day 3, there was no significant difference between the KO and WT mice. On day 6, re-epithelialization was significantly delayed in KO mice compared with that in WT mice (59.7% vs. 74.5%;  $p = 0.013$ ) (Fig. 2E and F). However, after day 8, the significant difference in re-epithelialization between the two groups disappeared, and on day 11, the wounds were completely re-epithelialized in both the WT and KO mice. These results suggest that the lack of TRPA1 retarded re-epithelialization by keratinocytes during cutaneous wound healing.

### 3.4. Lack of TRPA1 inhibits macrophage infiltration and myofibroblast formation

Macrophages play an important role in granulation tissue formation and angiogenesis via the expression of growth factors in the early stages of wound healing. Therefore, the properties of cellular components in the newly formed granulation tissue were evaluated immunohistochemically, focusing on macrophages and myofibroblasts. Immunohistochemical evaluation of F4/80-labeled macrophages showed a statistically significant difference between the two groups on day 8 ( $p = 0.017$ ) (Fig. 3A and B). Macrophage infiltration in the healing tissues was substantially reduced in KO mice compared with that in WT mice. Immunohistochemical evaluation of myofibroblasts revealed a



**Fig. 2.** Histological observation of the wounds. (A–D) Histological observation of the formation of new granulation tissue using hematoxylin and eosin (HE) staining at each healing interval in dorsal wound in knockout (KO) and wild-type (WT) mice. (A) HE staining of the granulation tissue at the center of the wound (Figure 1Ba–B). Bar, 1 mm. (B) Summary of Fig. 2A. On day 6, formation of new granulation tissue was significantly lower in the KO mice compared with that in the WT mice. Open bars: WT, filled bars: KO. Values represent the mean  $\pm$  standard deviation.  $*p < 0.05$ . (C) HE staining of the granulation tissue at the margin of the wound (Figure 1Ba–A). Bar, 1 mm. (D) Summary of Fig. 2C. On day 6, formation of new granulation tissue was significantly lower in the KO mice compared with that in the WT mice. Open bars: WT, filled bars: KO ( $n = 7$  animals in each group). Values represent the mean  $\pm$  standard deviation.  $*p < 0.05$ . (E–F) Histological observation re-epithelialization via Masson–Goldner trichrome staining at each healing interval in dorsal wound in KO and WT mice. (E) Masson–Goldner trichrome staining was observed at a low (upper) and high (lower) magnification to measure the original wound distance and re-epithelialization area, respectively. Bar, 1 mm. (F) Summary of the re-epithelialization ratio. Observation of re-epithelialization on day 6 revealed a significant delay in KO mice compared with that in WT mice. However, on day 11, the wound was completely covered with epithelium in both WT and KO mice.  $\circ$ : WT,  $\bullet$ : KO. Values represent the mean  $\pm$  standard deviation.  $*p < 0.05$ .

statistically significant difference in the expression of  $\alpha$ SMA between the two groups on days 6 and 8 ( $p < 0.0001$  for both) (Fig. 3C and D). The formation of myofibroblast populations in the healing tissue was substantially reduced in the KO mice compared with that in WT mice. These results suggest that the lack of TRPA1 inhibits macrophage infiltration and myofibroblast formation during the wound healing process, leading to diminished granulation tissue formation and epithelial regeneration.

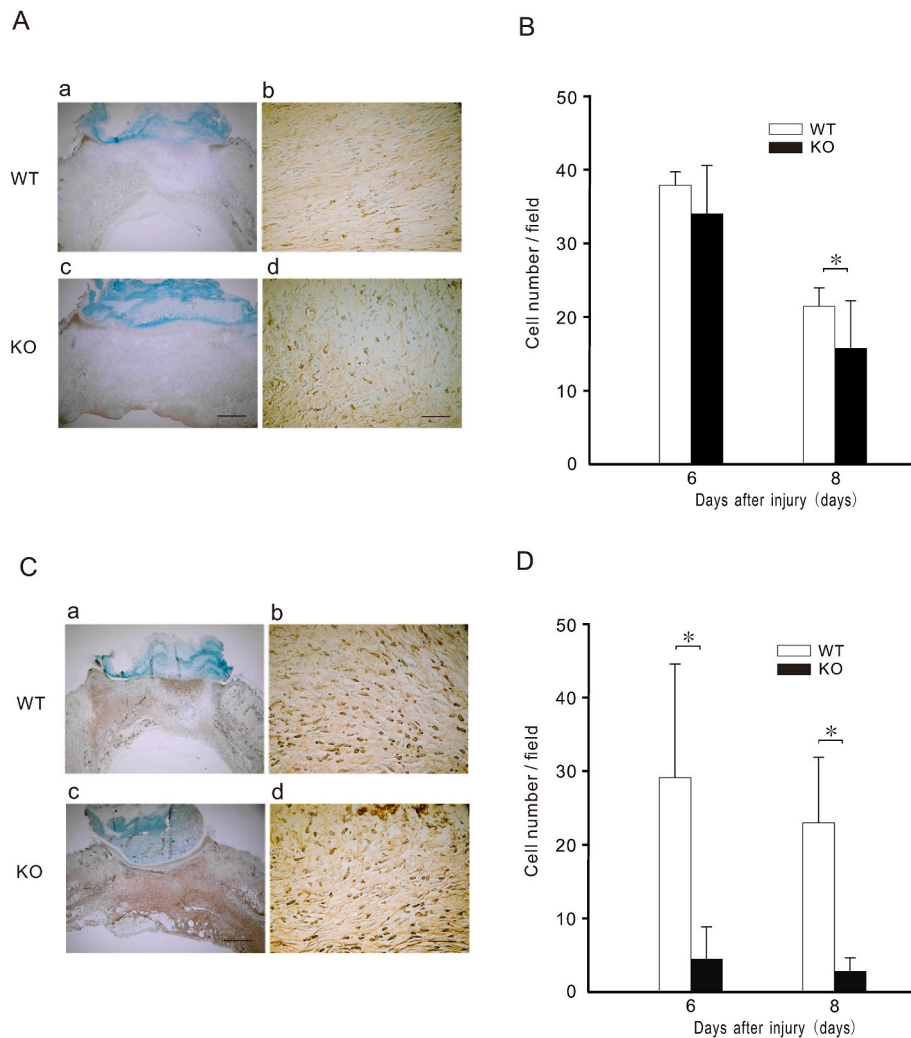
### 3.5. Lack of TRPA1 alters the expression of wound healing-related genes in regenerated tissues

We performed real-time RT-PCR to evaluate the expression of TGF- $\beta$ 1,  $\alpha$ SMA, F4/80, and collagen 1 $\alpha$ 2 mRNAs in the regenerated tissues of the KO and WT mice on day 6, when significant differences were observed between the two groups. Although the expression of TGF- $\beta$ 1 mRNA was not statistically different, it was more diminished in the

tissues of KO mice than in those of WT mice ( $p = 0.060$ ) (Fig. 4A). However, the expression of  $\alpha$ SMA mRNA was significantly lower in the tissues of KO mice than in those of WT mice ( $p = 0.049$ ) (Fig. 4B). Similarly, the expression of F4/80 and collagen 1 $\alpha$ 2 mRNAs was significantly suppressed in the tissues of KO mice compared with that in the tissues of WT mice ( $p = 0.003$  and  $p = 0.043$ , respectively) (Fig. 4C and D).

## 4. Discussion

In the present study, we observed that the loss of TRPA1 in mice significantly impaired early healing of wound in the dorsal skin. Macroscopic and histological analyses showed that the loss of TRPA1 delayed the formation of new granulation tissue and re-epithelialization until day 6 of the wound healing process. Immunohistochemical analysis suggested that the inhibition of macrophage invasion and myofibroblast



**Fig. 3.** Immunohistochemical observation of the wounds. (A–B) Immunohistochemical observation of F4/80-labeled macrophages. (A) Immunostained specimens of tissue from wild-type (WT) and knockout (KO) mice are shown at low magnification (a, c; bar, 1 mm) and high magnification (b, d; bar, 10  $\mu$ m). The number of F4/80-positive macrophages within a wound bed was enumerated in five visual fields. (B) Summary of Fig. 3A. The infiltration of F4/80-labeled macrophages in the wound tissue in KO mice was significantly lower than that in the wound tissue in WT mice at day 8 after injury. Open bars: WT, filled bars: KO. Values represent the mean  $\pm$  standard deviation. \* $p < 0.05$ . (C–D) Immunohistochemical observation of myofibroblasts using detection of  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) expression. (C) Immunostained specimens of tissue from WT and KO mice are shown at low magnification (a, c; bar, 1 mm) and high magnification (b, d; bar, 10  $\mu$ m). The number of myofibroblasts within a wound bed was enumerated in five visual fields. (D) Summary of Fig. 3C. The appearance of myofibroblasts as detected by  $\alpha$ SMA expression was significantly reduced in KO mice than in WT mice at 6 and 8 days after injury. Open bars: WT, filled bars: KO. Values represent the mean  $\pm$  standard deviation. \* $p < 0.05$ .

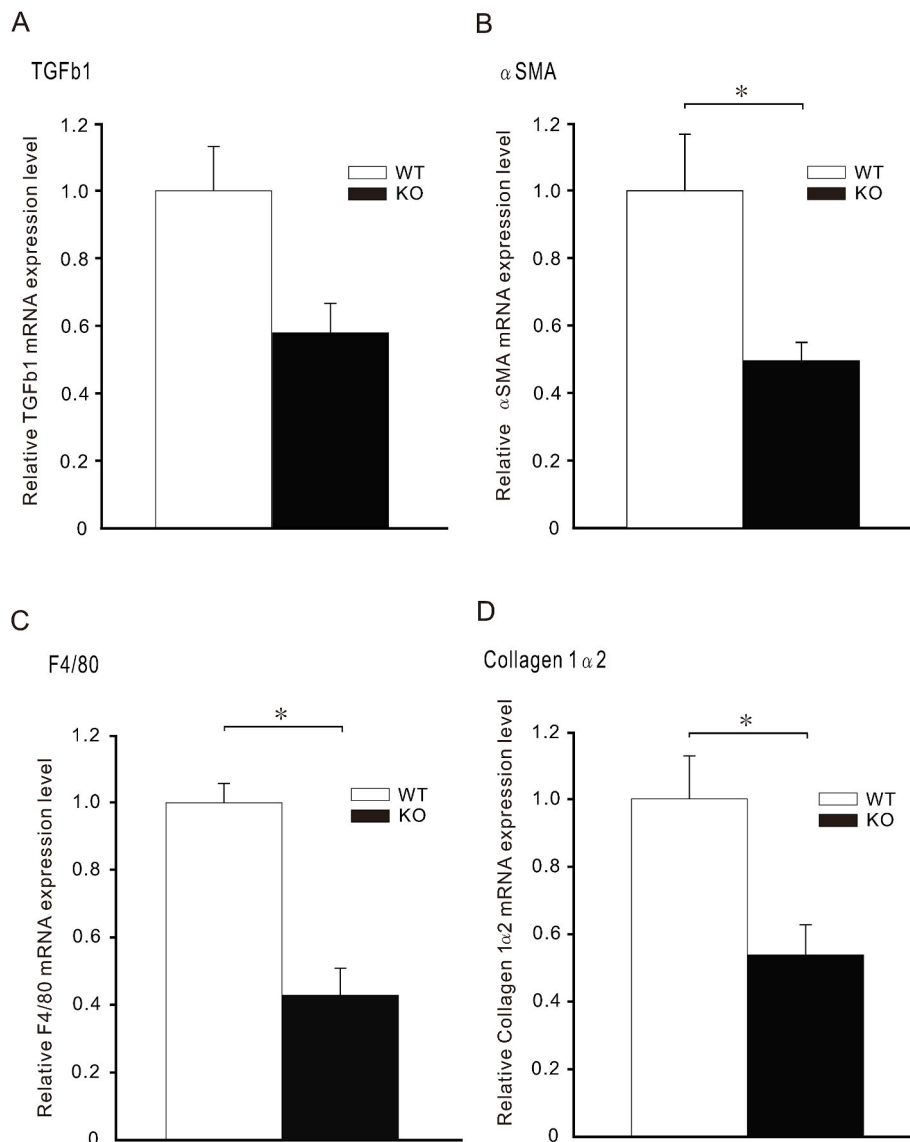
emergence may impair the formation of new granulation tissue and the epithelial resurfacing of wounds until day 6 in TRPA1-deficient mice. RT-PCR revealed that the mRNA expression levels of TGF- $\beta$ 1,  $\alpha$ SMA, F4/80, and collagen 1 $\alpha$ 2 involved in wound healing were significantly lower in KO mice than in WT mice. This result is consistent with the histological findings.

In a previous report, Wei et al. [24] evaluated the role of TRPA1 in the process of cutaneous wound healing in mouse ears and stated that the absence of TRPA1 was detrimental to wound healing. Additionally, they reported that TRPA1 deletion attenuated the inflammatory cytokine response. These results are similar to those of the present study. However, delayed wound healing and diminished inflammatory response associated with TRPA1 deletion were reported to occur at 4 weeks after the injury, which was partially different compared to our results. Okada et al. reported the role of TRPA1 in the wound healing process in the cornea, a tissue that is different from the epithelium, although it is also derived from the ectoderm [25]. They reported that wound healing following TRPA1 deletion was significantly delayed to 3 weeks after wound creation, and the expression of various the mRNAs was significantly different until day 10 after wound creation [25]. Although the delayed wound healing associated with TRPA1 deletion in our study was consistent with the findings in these previous studies, the time duration in days of delayed wound healing was different. We speculate that this variation in post-injury days to healing is related to the difference in the sizes of the wounds created; the body parts used for wound creation, such as the skin on the back, cornea, and ear; and the

physiological turnover period.

Furthermore, the wound healing process in the TRPA1 KO mice in our study was similar to that in the inducible nitric oxide synthase (iNOS) KO mice reported by Kitano et al. [14]. NO is associated with biological functions, such as vasodilation, angiogenesis, inflammation, and tissue fibrosis, and with the immune response required for wound healing [26,27]. The aforementioned studies, NO synthesis was concluded to be essential for uncomplicated skin wound healing. It is established that TRPA1 is activated by temperatures of 17  $^{\circ}$ C or less, allyl isothiocyanate, and ROS, such as NO, free radicals, and hydrogen peroxide [6–12]. Delayed macrophage infiltration and expression of fibrogenic components in TRPA1 KO mice were similar to those observed in iNOS KO mice. NO is one of the ligands for TRPA1, and the wound healing process in TRPA1 KO and iNOS KO mice is very similar. These facts suggest that TRPA1 and NO may form a series of signaling cascades in cutaneous wound healing. Therefore, we hypothesize that TRPA1 agonists may be therapeutic agents for intractable skin ulcers.

We focused on the phenomenon and found that the lack of TRPA1 retards macrophage infiltration and the subsequent fibrotic tissue formation. However, this study has several limitations that need to be overcome to further elucidate the underlying mechanism. First, in this study, we compared TRPA1 KO and WT mice. Future studies using agonists or antagonists are needed to demonstrate the comprehensive role of TRPA1. Second, the mRNA expression was evaluated only on day 6 because the outcomes of macroscopic observation, histology, and immunohistochemistry were all significantly different on this day. For



**Fig. 4.** Expression pattern of wound healing-related genes in the dorsal wound of knockout (KO) and wild-type (WT) mice. mRNA expression of TGF- $\beta$ 1 (A),  $\alpha$ SMA (B), F4/80 (C), and collagen 1 $\alpha$ 2 (D) in the healing tissues of WT and KO mice on day 6. Open bars: WT, filled bars: KO. Values represent the mean  $\pm$  standard deviation. \* $p < 0.05$ .

comprehensive elucidation of the relationship between TRPA1 and gene expression in the wound healing process, it is necessary to perform the analysis after day 1 itself. Although keratinocytes, macrophages, and fibroblasts, which express TRPA1 and play an important role in wound healing, were evaluated histologically, we believe that additional evaluations at more time points, are needed to clarify the details of these interactions and the magnitude of the effect of TRPA1 KO. Moreover, to elucidate the mechanism underlying the involvement of TRPA1 in the infiltration and activation of macrophages, future studies should include culturing cutaneous monocytes or macrophages from WT mice and evaluating changes in the expression of TRPA1 induced by agonists and antagonists. However, this is the first report to quantitatively prove that TRPA1 deletion impairs macrophage invasion and fibrogenic gene expression and delays cutaneous wound healing. Detailed investigations at genetic levels using agonists and antagonists are warranted for the development of therapeutic agents for intractable skin ulcers.

## 5. Conclusions

TRPA1 is required for cutaneous wound healing. A lack of TRPA1

retards macrophage infiltration and the subsequent formation of fibrotic tissue, which might further impair the fibrogenic behavior of fibroblasts.

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## Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2022.101322>.

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