


# Persistent proliferation of keratinocytes and prolonged expression of pronociceptive inflammatory mediators might be associated with the postoperative pain in KK mice

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## Abstract

Epidermal keratinocytes play a vital role in restoration of the intact skin barrier during wound healing. The negative effect of hyperglycemia may prolong the wound healing process. Epidermal keratinocytes have been demonstrated to modulate and directly initiate nociceptive responses in rat models of fractures and chemotherapy-induced neuropathic pain. However, it is unclear whether epidermal keratinocytes are involved in the development and maintenance of incisional pain in nondiabetic or diabetic animals. In the current study, using behavioral tests and immunohistochemistry, we investigated the differential keratinocytes proliferation and expression of pronociceptive inflammatory mediators in keratinocytes in C57BL/6J mice and diabetic KK mice. Our data showed that plantar incision induced postoperative pain hypersensitivity in both C57BL/6J mice and KK mice, while the duration of postoperative pain hypersensitivity in KK mice was longer than that in C57BL/6J mice. Moreover, plantar incision induced the keratinocytes proliferation and expression of IL-1 $\beta$  and TNF- $\alpha$  in keratinocytes in both C57BL/6J mice and KK mice. Interestingly, compared to C57BL/6J mice, the slower and more persistent proliferation of keratinocytes and expression of IL-1 $\beta$  and TNF- $\alpha$  in keratinocytes were observed in KK mice. Together, our study suggested that plantar incision may induce the differential keratinocytes proliferation and expression of IL-1 $\beta$  and TNF- $\alpha$  in keratinocytes in diabetic and nondiabetic animals, which might be associated with the development and maintenance differences in diabetic and nondiabetic postoperative pain.

## Keywords

Keratinocytes, postoperative pain, diabetes, inflammation

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## Introduction

Clinical pain management after surgery is far from being successful despite dramatically increased attentions. Many patients develop chronic pain after surgery which might be, at least in part, a result of undertreated acute postoperative pain. The pathophysiology of postoperative pain is very different from the inflammatory or neuropathic pain<sup>1</sup> and thus it is necessary to gain new insights into the mechanisms of postoperative pain in experimental settings to develop therapeutic options with greater efficacy and less risk of adverse effects. Peripheral sensitization is a contributing factor for

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central sensitization. Continuous pathogenetic impulses from the periphery might further consolidate and aggravate central sensitization. In recent years, peripheral mechanisms in postoperative pain have been sharing the same degree of attention as central mechanisms. Accumulated evidences suggested that the inflammatory and ischemic-like conditions including increased lactate, NGF, IL-1 $\beta$ , and C5a in the incisional wound contribute to peripheral sensitization and pain behavior after incision.<sup>2,3</sup> However, the detailed cellular and molecular mechanisms underlying peripheral sensitization of postoperative pain are not fully understood.

Wound healing occurs as a cellular response to injury and involves activation of keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets.<sup>4</sup> Many growth factors and cytokines released by these cell types are needed to coordinate and maintain healing.<sup>5–8</sup> Keratinocytes, which comprise most of the epidermis, through terminal differentiation, develop a mechanical barrier against chemical stimulus and microorganism. During wound healing, due to the skin environmental changes, the function of keratinocyte also changes.<sup>9</sup> Previous studies showed that keratinocyte, through activation, proliferation, and release of proinflammatory mediators,<sup>10–13</sup> plays a critical role in the peripheral sensitization of pain in rat models of fracture and chemotherapy-induced neuropathic pain. Therefore, the keratinocyte is possibly involved in the development and maintenance of incisional pain, as a cellular response during wound healing.

Interestingly, during diabetic wound healing, the keratinocyte shows an absence of migration, hyperproliferation, and incomplete differentiation.<sup>4,14,15</sup> The evidence from clinical study indicates that diabetic patients have higher pain scores and need larger doses of morphine for effective postoperative pain treatment compared with nondiabetic patients.<sup>16</sup> However, it is unclear whether epidermal keratinocytes are involved in the differential development and maintenance of incisional pain in nondiabetic or diabetic animals. Therefore, in the current study, we aimed to determine the differential keratinocytes activation and proliferation as well as expression of pronociceptive inflammatory mediators in keratinocytes between C57BL/6J mice and KK mice.

## Methods

### Animals

Adult male C57BL/6J (9–11 weeks, 25–28 g) and KK mice (blood sugar >11.1 mmol/L, 9–11 weeks, 25–28 g) were purchased from Huafukang Company. All the mice were housed on a 12-h light/12-h dark cycle and maintained at 21°C  $\pm$  2°C with free access to food and water. High-fat diet is provided to KK mice and regular diet is provided to C57BL/6J mice. All experiments were

approved by the Ethical Committee of Beijing Friendship Hospital, Capital Medical University, China and were performed in compliance with the guidelines for animal experimentation of the international association for the study of pain.

### Plantar incision

The plantar incision in mice was performed as described previously.<sup>17</sup> We use 1.5% to 2% isoflurane to anesthetize the mice. A 5-mm longitudinal incision was made in right heel. The skin and muscle were incised by a No. 11 blade. The muscle origin and insertion were kept intact. In addition, 8–0 nylon was used to suture the skin. The wound was closed and covered with antibiotic ointment to be protected from infection.

### Behavior tests

The mice were put on an elevated iron mesh floor to acclimate for 20 to 30 min. Then the paw withdrawal threshold (PWT) and cumulative pain score (CPS) were assessed. PWT was assessed with the up-down method using von Frey filaments (North Coast Medical, USA) from 0.04 to 2.0 g (0.04, 0.07, 0.16, 0.4, 0.60, 1.0, 1.4, 2.0 g).<sup>18</sup> We first applied 0.04 g stimulus to an area near the wound for 5 s or stop when the animal withdrew the paw. We decreased the stimulus if the mice had a positive response, such as rapid lifting, shaking, or licking of the incised paw, or we increased the stimulus if the mice have a negative response. CPS was used to assess nonevoked pain behaviors. The mice were observed closely for a 1-min period every 5 min for 1 h. Based on the way that the right hind paw was positioned, a score of 0, 1, or 2 was given. Zero point was recorded when the right hind paw touched the mesh and made the mesh blanch or distort. One point was recorded when the right hind paw touched the mesh and did not make the mesh blanch or distort. Two point was recorded when the right hind paw did not touch the mesh. The sum of the 12 scores (0–24) was recorded as CPS.

### Antibodies

The sources of primary antibodies in this experiment were as follows: Cytokeratin (clone AE1/AE3) (ma-1316, 1:100, Thermo Fisher Scientific, Waltham, MA); IL-1 $\beta$  (ab2105, 1:100, Abcam Systems, Cambridge, UK), TNF- $\alpha$  (ab6671, 1:100, Abcam Systems, Cambridge, UK), Proliferating cell nuclear antigen (PCNA) (GB11010, servicebio, 1:200, Wuhan, China). The sources of secondary antibodies were as follows: Cy3 conjugated Goat Anti-rabbit IgG (GB21303, servicebio, 1:300, Wuhan, China), Alexa Fluor<sup>®</sup> 488-conjugated

AffiniPure Goat Anti-mouse IgG (GB25301, servicebio, 1:400, Wuhan, China).

### Immunofluorescence

The mice were euthanized by overdose of chloral hydrate.<sup>10</sup> Moreover, 4% paraformaldehyde and the saline were used for perfusion. The hindpaw skin of the incision margin was collected in 4% tissue fix solution (Soarbio, P1110) for 24 h. The tissue was removed from the tissue fix solution and was dehydrated in a stepwise gradient of alcohol and anhydrous ethanol. Melted paraffin was used to infiltrate tissue. The skin was embedded in melted paraffin and then kept at  $-20^{\circ}\text{C}$  to solidify the paraffin. Following embedding, 4- $\mu\text{m}$ -thick slices were made and baked in an oven at  $60^{\circ}\text{C}$ , and were then stored at room temperature. Paraffin section was blocked with BSA for 30 min after dehydration and antigen repair. The primary antibodies were applied, respectively, overnight at  $4^{\circ}\text{C}$  in phosphate-buffered saline (PBS). The glass slides were then washed in PBS buffer (PH 7.4) three times, 5 min each. The secondary antibodies were incubated for 50 min at room temperature, followed by washing in PBS buffer (PH 7.4) three times, 5 min each. The 4',6-diamidino-2-phenylindole was used to stain nuclear for 10 min at room temperature. The glass slides were covered with anti-fluorescence quenching tablets after washing in PBS buffer (PH 7.4) three times. Images were obtained using confocal microscopy (Nikon Eclipse C1).

### Statistical analysis

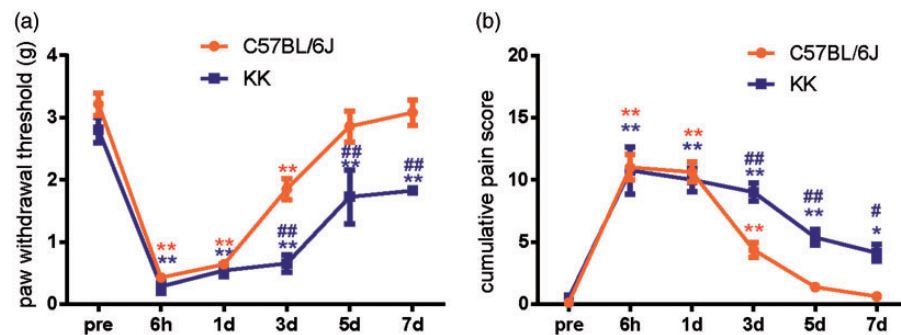
All data analysis was performed by GraphPad Prism 6. Quantitative analysis for Behavioral data was analyzed by one-way analysis of variance (ANOVA).

For comparisons of the expression of cytokeratin at different time points, we used ANOVA followed by Bonferroni multiple comparison tests. The data of PCNA numbers at different time points were also analyzed with ANOVA followed by Bonferroni multiple comparison tests. All data are expressed as mean  $\pm$  standard error of the mean.  $P < 0.05$  was regarded as significant.

## Results

### Postoperative pain hypersensitivity was significantly prolonged in KK mice after plantar incision

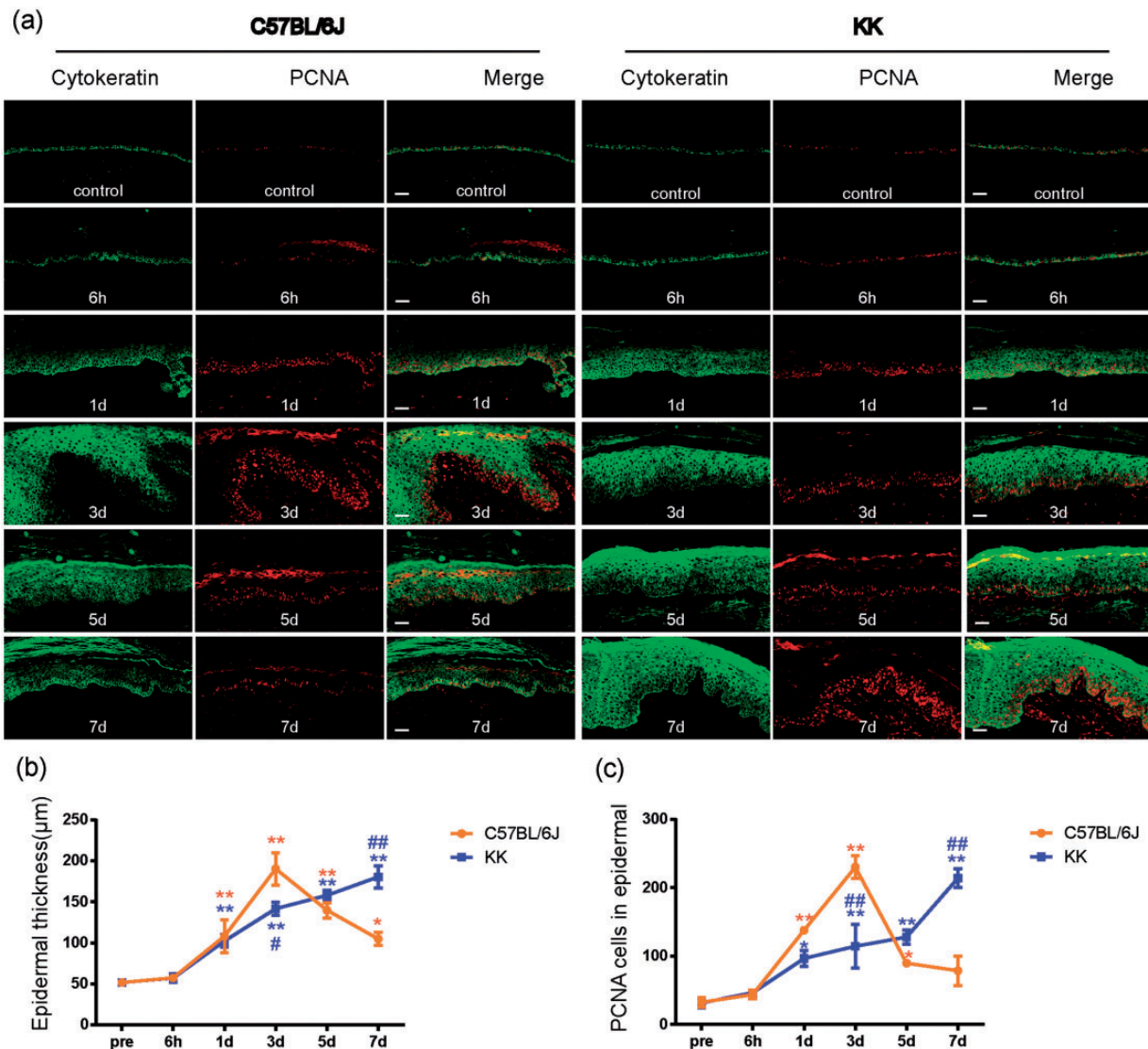
In C57BL/6J mice, the lowest PWT and highest CPS of the ipsilateral paw to the incision were observed at 6 h after incision and remained for one day ( $P < 0.01$ , Figure 1(a) and (b)). Then, the PWT and CPS gradually recovered, but remained different from the preoperative level at three days after incision ( $P < 0.01$ , Figure 1(a) and (b)). The PWT and CPS completely recovered to the preoperative level at five and seven days after incision ( $P > 0.05$ , Figure 1(a) and (b)). In KK mice, the lowest PWT and highest CPS of the ipsilateral paw to the incision were also observed at 6 h after incision and remained for three days ( $P < 0.01$ , Figure 1(a) and (b)). Then, the PWT and CPS gradually recovered but still remained different from the preoperative level at five and seven days after incision ( $P < 0.05$ , Figure 1(a) and (b)). Compared to the C57BL/6J mice, the PWT still remained significantly lower and CPS remained higher at three, five, and seven days after incision in KK mice ( $P < 0.01$ , Figure 1(a) and (b)).



**Figure 1.** The duration of pain hypersensitivity after plantar incision was prolonged in KK mice as compared to C57BL/6J mice. In C57BL/6J mice, the lowest PWT and highest CPS of the ipsilateral paw to the incision were observed at 6 h after incision and remained for one day ( $P < 0.01$ ). Then, the PWT and CPS gradually recovered to the preoperative level on five and seven days after incision ( $P > 0.05$ ). In KK mice, the lowest PWT and highest CPS of the ipsilateral paw to the incision were also observed at 6 h after incision and remained for three days ( $P < 0.01$ ). Then, the PWT and CPS gradually recovered but still remained different from the preoperative level on day 5 and day 7 after incision ( $P < 0.05$ ). Compared to the C57BL/6J mice, the PWT was significantly lower and the CPS was significantly higher on days 3, 5, and 7 after incision in KK mice ( $P < 0.01$ ). PWT, paw withdrawal threshold; CPS, cumulative pain score; \*\* $P < 0.01$ , compared with preoperative values; ### $P < 0.01$ , # $P < 0.05$  compared with the C57BL/6J mice;  $n = 8$ .

### Plantar incision induced the differential proliferation of keratinocytes in the margin skin of the incision between C57BL/6J and KK mice

The keratinocyte proliferation in the margin skin of the incision was characterized by the epidermal thickness and the numbers of PCNA cells with positive cytokeratin labeling. In C57BL/6J mice, the epidermal thickness increased at one day after incision ( $P < 0.05$ , Figure 2(a) and (b)), peaked at three days after incision ( $P < 0.01$ , Figure 2(a) and (b)), and then gradually recovered. But even at seven days after incision, the epidermal thickness is still higher than that in normal control group ( $P < 0.05$ , Figure 2(a) and (b)). In KK mice, the epidermal thickness began to increase at one day after incision ( $P < 0.05$ , Figure 2(a) and (c)), and reached the peak at seven days after incision ( $P < 0.01$ , Figure 2(a) and (c)).



**Figure 2.** The differential proliferation of keratinocytes in the margin skin of the incision in C57BL/6J and KK mice. Representative confocal images of cytokeratin (a keratinocyte marker, green) and PCNA (red) immunoreactivity in the margin incision skin. Colocalization (merge) is shown in yellow/orange. Scale bars: 50 μm. In C57BL/6J mice, the epidermal thickness increased at one day after incision ( $P < 0.05$ ), peaked at three days after incision ( $P < 0.01$ ), and then gradually recovered. In KK mice, the epidermal thickness began to increase at one day after incision ( $P < 0.05$ ), and reached the peak at seven days after incision ( $P < 0.01$ ). In C57BL/6J mice, the number of PCNA cells with positive cytokeratin labeling increased at one day after incision ( $P < 0.05$ ), peaked at three days after incision ( $P < 0.01$ ), and then gradually returned to the level in control group at seven days after incision ( $P > 0.05$ ). In KK mice, the number of PCNA cells with positive cytokeratin labeling began to increase at one day after incision ( $P < 0.05$ ), and reached the highest level at seven days after incision ( $P < 0.01$ ). PCNA, proliferating cell nuclear antigen. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with preoperative values; ### $P < 0.01$ , # $P < 0.05$  compared with the C57BL/6J mice;  $n = 4-6$ .

In C57BL/6J mice, the number of PCNA cells with positive cytokeratin labeling increased at one day after incision ( $P < 0.05$ , Figure 2(a) and (c)), peaked at three days after incision ( $P < 0.01$ , Figure 2(a) and (c)), and then gradually returned to the level of normal control group at seven days after incision ( $P > 0.05$ , Figure 2(a) and (c)). In KK mice, the number of PCNA cells with positive cytokeratin labeling began to increase at one day after incision ( $P < 0.05$ , Figure 2(a) and (c)), and reached the highest level at seven days after incision ( $P < 0.01$ , Figure 2(a) and (c)). Both the epidermal thickness and the number of PCNA cells in contralateral hindpaw did not change at those time points (data not shown).

### *The differential expression of IL1 $\beta$ in keratinocytes between C57BL/6J and KK mice*

The plantar incision induced the co-expression of cytokeratin and IL1 $\beta$  in margin incision skin in both C57BL/6J mice and KK mice. In C57BL/6J mice, the co-expression of cytokeratin and IL1 $\beta$  gradually increased and reached the peak at three days after incision ( $P < 0.01$ , Figure 3(a) and (b)). And then the co-expression of cytokeratin and IL1 $\beta$  gradually decreased but still kept at higher level as compared to the control group at seven days after incision ( $P < 0.05$ , Figure 3(a) and (b)).

In KK mice, the level of co-expression of cytokeratin and IL1 $\beta$  was increased from ( $63.67 \pm 14.09$  cells/HPF,  $n = 6$ ) at one day after incision to ( $156.5 \pm 32.27$  cells/HPF,  $n = 4-6$ ) at seven days after incision. There was no difference in the level of co-expression of cytokeratin and IL1 $\beta$  at 6 h, one day, and three days after incision between C57BL/6J mice and KK mice ( $P > 0.05$ , Figure 3(a) and (b)). However, at five and seven days after incision, the co-expression of keratinocyte and IL1 $\beta$  in KK mice was significantly higher than that in C57BL/6J mice ( $P < 0.01$ , Figure 3(a) and (b)).

### *The differential expression of TNF $\alpha$ in keratinocytes between C57BL/6J mice and KK mice*

Plantar incision induced the co-expression of cytokeratin and TNF $\alpha$  in margin incision skin in both C57BL/6J and KK mice. In C57BL/6J mice, the co-expression of cytokeratin and TNF $\alpha$  began to increase at one day after incision ( $P < 0.05$ , Figure 4(a) and (b)) and reached the peak at three days after incision ( $P < 0.01$ , Figure 4(a) and (b)). Then, the level of co-expression of cytokeratin and TNF $\alpha$  returned to the baseline value at five and seven days after incision ( $P > 0.05$ , Figure 4(a) and (b)).

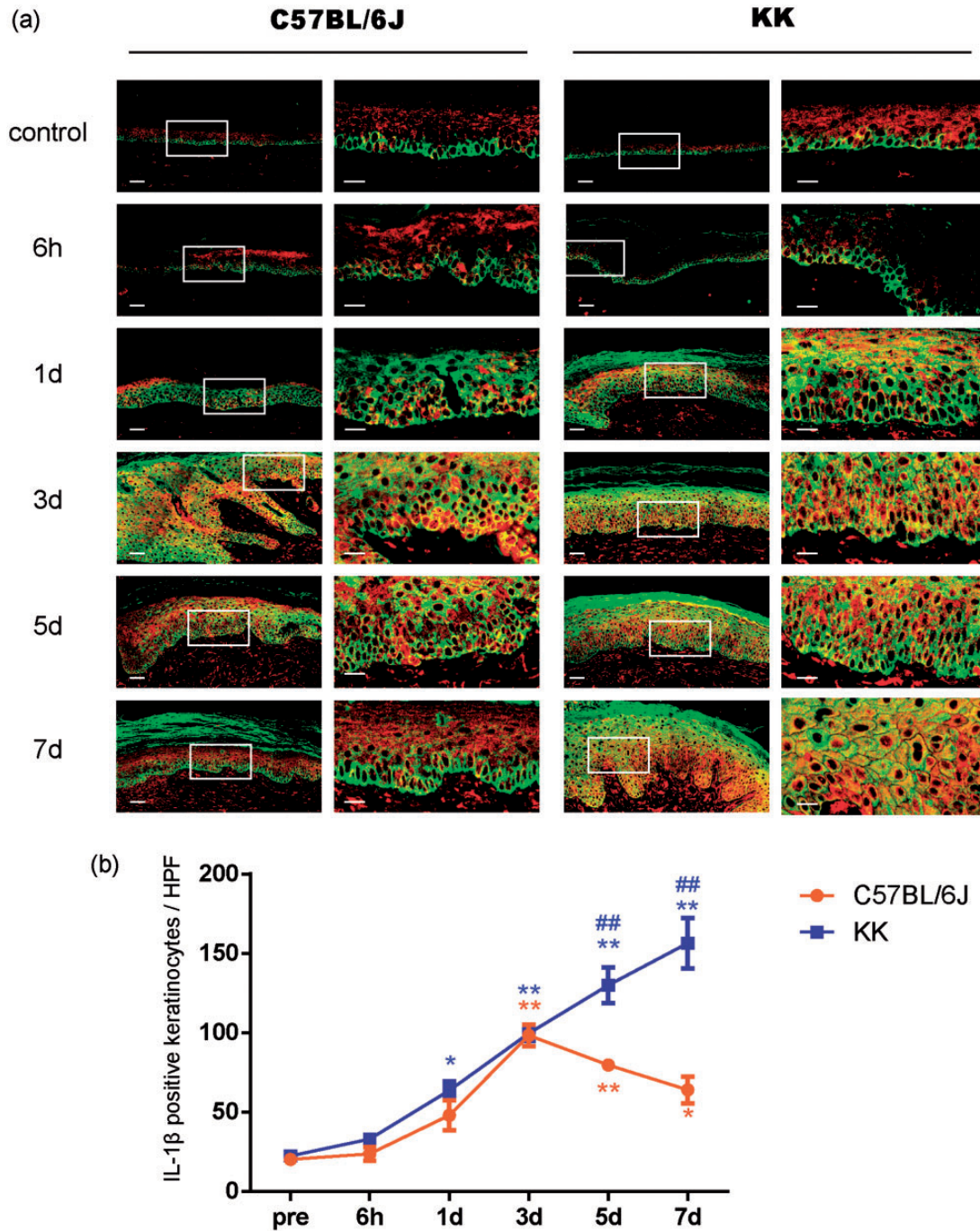
In KK mice, the level of co-expression of cytokeratin and TNF $\alpha$  was gradually increased from ( $73.33 \pm 8.48$  cells/HPF,  $n = 4-6$ ) at one day after incision to

( $114.00 \pm 28.95$  cells/HPF,  $n = 4-6$ ) at seven days after incision. There was no difference in the level of co-expression of cytokeratin and TNF $\alpha$  at 6 h, one day, and three days after incision between C57BL/6J mice and KK mice ( $P > 0.05$ , Figure 4(a) and (b)). However, at five and seven days after incision, the co-expression of cytokeratin and TNF $\alpha$  in KK mice was significantly higher than that in C57BL/6J mice ( $P < 0.01$ , Figure 4(a) and (b)).

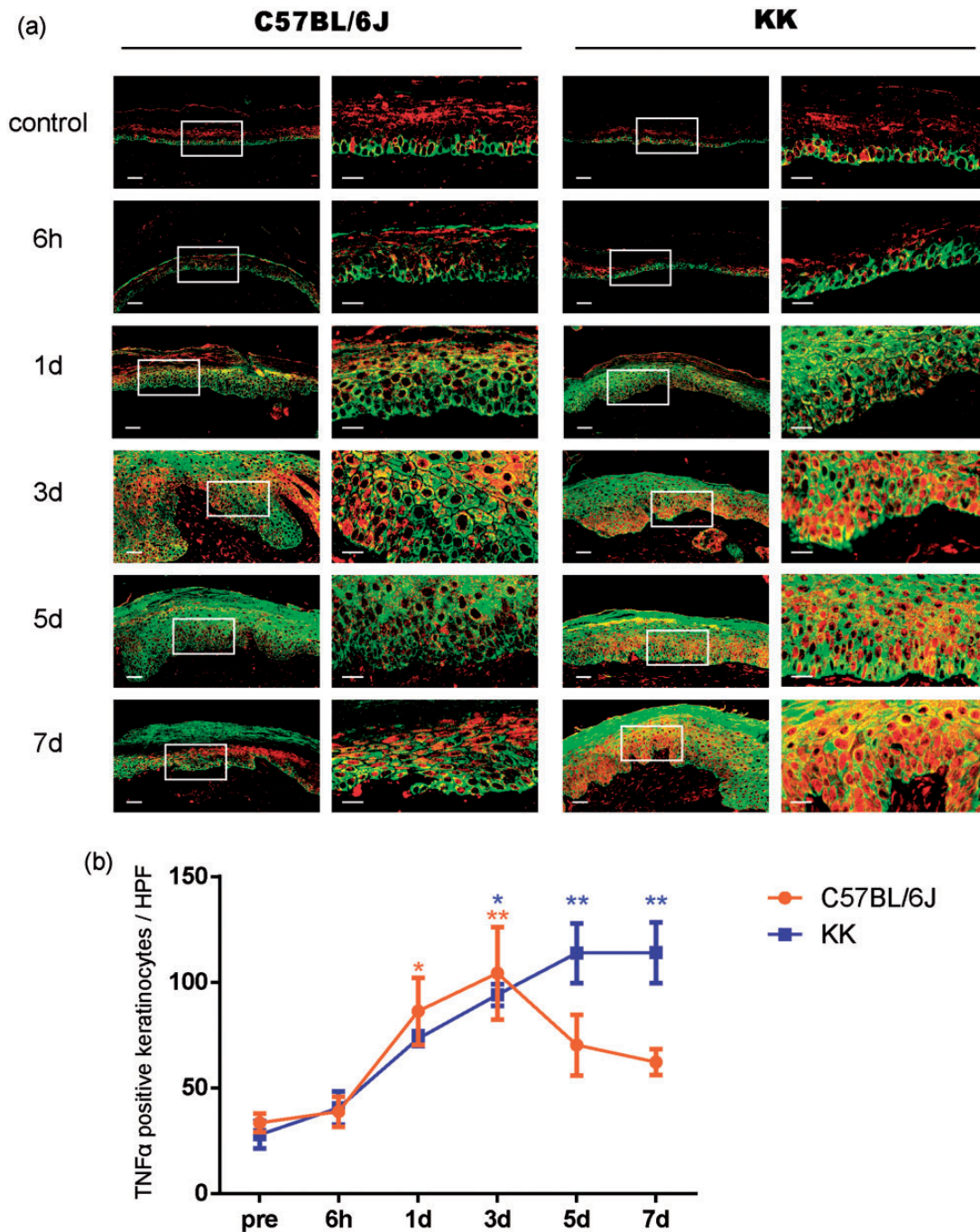
## **Discussion**

The current study has demonstrated that plantar incision induced the prolonged duration of pain hypersensitivity in diabetic animals. Additionally, the keratinocyte proliferation after plantar incision in diabetic mice is slower and more persistent as compared to that in nondiabetic mice. Furthermore, the expression of IL1 $\beta$  and TNF $\alpha$  in keratinocytes of nondiabetic mice reached the peak at three days after incision and gradually returned to the baseline. In contrast, in diabetic mice, the expression of IL1 $\beta$  and TNF $\alpha$  in keratinocytes persistently increased from one day after incision and reached the peak at seven days after incision.

Keratinocytes are the most common type of cells in the skin and play an important role in wound healing. Wound healing is a complicated process that requires several distinct components, including hemostasis, inflammation, proliferation, and remodeling. During proliferation process, epidermal re-epithelialization and dermal repair are coordinated.<sup>19</sup> The involvement of keratinocytes proliferation in nociception has been reported in a rat model of tibial fracture.<sup>10</sup> KK mouse we used in this paper is a good obesity-associated diabetes model. The hyperglycemia and glucose intolerance in KK mice are similar to that in type 2 diabetes patients.<sup>20,21</sup> In current study, the keratinocyte proliferation peaked at three days after incision in C57BL/6J mice, while it peaked at seven days after incision in KK mice. Usui et al. reported that a proliferative burst of keratinocytes occurs between 24 and 72 h after injury in normal acute wounds,<sup>22</sup> which is consistent with our present study in C57BL/6J mice. Additionally, our study also indicated that the incision-induced keratinocytes proliferation develops slowly and lasts longer in diabetic animals. The maximal epidermal thickness and the highest amount of PCNA cells with positive cytokeratin labeling are almost the same in C57BL/6J mice and KK mice. However, it takes more days to reach the peak in KK mice, indicating that keratinocytes proliferation is inhibited in diabetic animals. Some studies demonstrated that keratinocytes contact with the sensory afferent nerves in function.<sup>23-25</sup> Keratinocytes contribute to the initial transduction process by regulating the release of many neuroactive substances after tissue injury.<sup>26,27</sup> In our



**Figure 3.** The plantar incision induced the IL1 $\beta$  expression in keratinocytes in margin incision skin in both C57BL/6J mice and KK mice. Representative confocal images of cytokeratin (green) and IL1 $\beta$ (red) immunoreactivity in the margin incision skin. Colocalization (merge) is shown in yellow/orange. The left columns of both groups are shown in lower magnification. The right columns of both groups are shown in higher magnification. Scale bars: 50  $\mu$ m (lower magnification) and 20  $\mu$ m (higher magnification). In C57BL/6J mice, the co-expression of cytokeratin and IL1 $\beta$  gradually increased and reached the peak at three days after incision ( $P < 0.01$ ). Then the co-expression of cytokeratin and IL1 $\beta$  gradually decreased but still kept at a higher level in contrast to the control group at seven days after incision ( $P < 0.05$ ). There was no difference in the co-expression of cytokeratin and IL1 $\beta$  at 6 h, one day, and three days after incision between C57BL/6J mice and KK mice ( $P > 0.05$ ). However, at five or seven days after incision, the co-expression of cytokeratin and IL1 $\beta$  in KK mice was significantly higher than that in C57BL/6J mice ( $P < 0.01$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , compared with preoperative value; ## $P < 0.01$ , compared with the C57BL/6J mice;  $n = 4-6$ .



**Figure 4.** Plantar incision induced the TNF $\alpha$  expression in keratinocytes in margin incision skin in both C57BL/6J and KK mice. Representative confocal images of cytokeratin (green) and TNF $\alpha$  (red) immunoreactivity in the margin incision skin. Colocalization (merge) is shown in yellow/orange. The left columns of both groups are shown in lower magnification. The right columns of both groups are shown in higher magnification. Scale bars: 50  $\mu$ m (lower magnification) and 20  $\mu$ m (higher magnification). In C57BL/6J mice, the co-expression of cytokeratin and TNF $\alpha$  began to increase at one day after incision ( $P < 0.05$ ) and reached the peak at three days after incision ( $P < 0.01$ ). Then, the level of co-expression of cytokeratin and TNF $\alpha$  at five and seven days after incision returned to the baseline value ( $P > 0.05$ ). There was no difference in the co-expression of cytokeratin and TNF $\alpha$  at five and seven days after incision between C57BL/6J mice and KK mice ( $P > 0.05$ ). However, at five or seven days after incision, the co-expression of cytokeratin and TNF $\alpha$  in KK mice was significantly higher than that in C57BL/6J mice ( $P < 0.01$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , compared with preoperative value;  $n = 4-6$ .

current study, the number of keratinocytes and the expression of cytokines in keratinocytes at three days after incision did not match the changes of pain behaviors in C57BL/6J and KK mice. It indicates that the pain behaviors at three days after incision did not uniquely/exclusively result from the increased number of keratinocytes and the increased expression of cytokines in keratinocytes. There might be other molecules which are mainly responsible for the development of pain at three days after incision in nondiabetic mice. We will investigate this interesting phenomenon in the future.

Interleukin-1 $\beta$  (IL-1 $\beta$ ) is a cytokine with multifunctional biological activity in the interleukin family. TNF- $\alpha$  is one of the cytokines involved in systemic inflammation. IL-1 $\beta$  and TNF- $\alpha$  can be synthesized by a variety of cells, such as macrophages, monocytes, and keratinocytes.<sup>28</sup> Previous studies reported that there were two peaks of the expression IL-1 $\beta$  and TNF- $\alpha$  after incision. The first peak is related to the acute inflammation, which appeared within 3 h after incision.<sup>29</sup> The second peak is present around 72 h and is recognized as a response to wound remodeling.<sup>29,30</sup> In our research, we did not test the level of cytokine at 3 h after incision. So we did not capture the first peak of the increase of cytokine. Previous studies showed that activated keratinocytes proliferate and express IL-1 $\beta$  and TNF- $\alpha$  after fracture and that excess amounts of inflammatory mediators in the skin cause sustained nociceptive sensitization.<sup>10</sup> In the setting of skin incision, the neuropeptide substance P and CGRP released from peripheral nociceptive nerve ends can activate keratinocytes to release IL-1 $\beta$ , TNF $\alpha$  and other inflammatory cytokines and then contribute to the maintainance of peripheral sensitization.<sup>31</sup>

We found the peak of IL-1 $\beta$  (TNF- $\alpha$ ) expression in keratinocytes appeared at three days after incision in C57BL/6J mice, which is in agreement with previous studies.<sup>29,30</sup> But in diabetic mice, our data showed the peak time of IL-1 $\beta$  and TNF- $\alpha$  expression in keratinocytes was significantly delayed. Some studies reported that the healing impairment in the diabetic chronic wound is mainly related to a state of persistent hyperinflammation characterized by decreased macrophage function.<sup>32–35</sup> Hyperglycemia stimulate cytokine production and promote inflammation through activating MAP kinase or PKC pathways.<sup>36,37</sup> Advanced glycation end products that accumulate in chronic hyperglycemia also promote inflammation.<sup>38,39</sup> In our study, we also found that the expression of IL-1 $\beta$  (TNF- $\alpha$ ) in keratinocytes maintained in a high level from three to seven days after incision in KK mice. The prolonged duration of pain hypersensitivity in KK mice may be attributed to the high level of IL-1 $\beta$  and TNF- $\alpha$  in keratinocytes.

In summary, our findings demonstrated that plantar incision may induce the slow and persistent proliferation

of keratinocytes and expression of IL-1 $\beta$  and TNF- $\alpha$  in keratinocytes, which might be associated with the prolonged pain hypersensitivity in diabetic animals. Our study may provide a new insight into the mechanisms underlying diabetic or nondiabetic postoperative pain.

### Author Contributions

RG and YW conceived the idea and acquired funding. RG, JH, DM, HL, and KL acquired and analyzed the data. RG drafted the paper. YW revised the article critically for intellectual content. All authors discussed the results on the manuscript.


### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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