



Original Article

Involvement of RNA helicase p68 in skin wound healing process in rats

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ABSTRACT

Purpose: RNA helicase p68 plays an important role in organ development and maturation through tuning cell proliferation. However, the character and role of p68 in the whole wound healing process need more study. **Methods:** First, we characterize expression of p68 in normal rat skin development postnatal. Then, we assayed dynamic change of p68 in rat skin from different stage after injury, and explored the role of p68 in proliferation and migration of three types of wound healing related cells.

Results: p68 was down-regulated during skin developmental and maturation process, up-regulated after wound, peaked on day 14 and then significantly decreased. Wound fluid enhanced wound healing related cell proliferation and up-regulated expression of p68. Conversely, reducing p68 expression by RNA interference resulted in significantly slower proliferation and migration.

Conclusion: Our results define an important role of RNA helicase p68 in skin wound healing process.

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Introduction

As the largest organ of human body, skin serves as a protective barrier, any damage of it should be rapidly and efficiently repaired.¹ A major goal of wound healing biology is to figure out how skin can be induced to reconstruct the damaged parts more perfectly.² Wound healing is a complex and well-regulated at multiple levels process, which involves in proliferating, migrating and differentiating of cells around the wound margin.^{2–4} In histological level, the process of wound healing has been well documented, while the mechanisms involved in regulating the behavior of each cell types during the phases of proliferation and migration at wound site, needs further study.

RNA helicase p68, which is one of the prototypical members of amino acid sequence, Asp-Glu-Ala-Asp (DEAD) box, is important for a wide range of cellular processes, including pre-mRNA, rRNA, miRNA processing^{5–8} and transcription regulation.^{9,10} RNA helicase p68 is essential for organ development and maturation, and deletion of p68 could lead to embryo lethality.^{11,12} RNA helicase p68 regulates cell proliferation in both normal and pathological

conditions.^{13–16} In an early study, RNA helicase p68 was shown to be involved in the regulation of keratinocytes at the acute phase of skin wound healing.¹⁷ However, the characterization of p68 expression and role in the wound healing process has not been fully demonstrated. This prompted us to systematically study the expression pattern and role of RNA helicase p68 in wound healing process and uncover their important role in regulating cell proliferation and migration.

Material and methods

Animals

All experiments were approved by the Ethical Committee for Animal Research of the Third Military Medical University. Subjects were adult Sprague-Dawley (SD) rats (220–250 g) obtained from the Animal Facility of the Third Military Medical University (Chongqing, China). All animals were kept in a temperature-controlled environment (21 °C ± 1 °C) on a normal 12-h light/dark cycle (light 06:00–18:00) and had food and water available ad libitum.

Wound injury and sample collection

Firstly, normal rat skin on postnatal day 1(P1), P4, P8, and P30 (five animals each time point) were collected and fixed in 10%

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formalin. Twenty-five adult SD rats, were randomly divided into 5 groups. All rats were anesthetized with a single intra-peritoneal injection of ketamine (80 mg/kg body weight)/xylazine (10 mg/kg body weight). The hair on the back of each rat was shaved, and the back was subsequently wiped with 70% ethanol. Two full-thickness wounds (2 cm in diameter, 3–4 cm apart) were made on the back of twenty rats by excising the skin. Skin specimens were obtained from the animals 1, 7, 14 and 21 days after injury. At each time point, an area of the wound margin tissue was excised from each individual wound. Same amount of skin was taken from the back of non-wounded rats as control. All tissues were fixed in 10% formalin and embedded in paraffin for histological processing.

Immunohistochemical staining

Sections were dewaxed, then, antigen retrieval was achieved by immersion in 10 mM citric acid buffer (pH 6.0), followed by microwaving for 15 min (at 1000 W) in a pressure cooker. Wash the sections for 3 times with PBS, 5 min each time. Incubate the sections with 3% of hydrogen peroxide, 10 min at room temperature (RT) and washed in PBS for 3 times. Block the sections with 10% normal goat serum for 30 min at RT and discard, not washed, then added the primary rabbit anti-p68 antibody (1:200 diluted) and incubated in 4 °C overnight. After washed with PBS for 3 times, slides were incubated with the secondary antibody (HRP-conjugated goat anti-rabbit antibody, Zhongshan, Beijing) for 30 min at 37 °C, washed, then were dyed with 0.1% DAB (Zhongshan, Beijing), after washing with distilled water, slides were counterstained with hematoxylin and then dehydrated and mounted. Negative control using secondary antibodies only was run concurrently. The pictures were taken with digital camera (Olympus, Tokyo, Japan).

Scoring of immunohistochemical staining

All of the immunostained slides were analyzed by two histopathologists, and eight fields in each slide were randomly selected and examined under a 40× objective lens. All fields were assessed using a semi-quantitative histological scoring system (H-score) described previously.^{18–21} Briefly, in each field, the staining intensity (I) was graded as 0–nostaining, 1–weak, 2–moderate, and 3–strong and the proportion (P) of cells with the observed intensity was recorded (from 0–none to 1.0–the entire population of examined cells). H-score for each field was determined by the sum of all $I \times P$ products. Therefore, an H-score of 0 was obtained if no staining ($I = 0$) was observed in the entire population of examined cells ($P = 1.0$). The maximum H score of 3.0 would be obtained if all of the examined cells ($P = 1.0$) in the field stained with maximal intensity ($I = 3$). According to this, H-scores of p68 expression were calculated.

Preparation of wound fluid

Wound fluid was obtained from 10 adult SD rats, which underwent a 10-cm midline dorsal skin incision and sponge implantation. Two strips of sterile, saline-moistened polyvinyl alcohol (PVA) sponges (Unipoint Industries, High Point, NC) were inserted into subcutaneous pockets, created on either side of the incision. The wound was then closed with surgical clips. Sponges were removed aseptically 1 day post-wounding and squeezed. The wound fluid was pooled to provide a constant and uniform supply for the assays. This pooled wound fluid was centrifuged at 400 g for 10 min. The harvested supernatant was then centrifuged at 1600 g for another 20 min and filtered through 0.2- μ m micro filters (Milipore, Bedford, MA) to remove any remaining cellular debris. Wound fluid was frozen at -70 °C immediately waiting for test.²²

Cell culture

Human epidermal keratinocyte cell line HaCaT and human umbilical vein endothelial cell line HUVEC were purchased from American Type Culture Collections (Rockville, MD) and were cultured in RPMI-1640 with 10% fetal bovine serum (FBS). Fibroblast cell was raised from rat skin by our laboratory, it was grown in IMDM with 10% FBS.

Wound fluid induction of p68

HaCaT, HuVEC and fibroblast were seeded at 1×10^4 /ml in culture flask or on the glass cover slips. After overnight incubation, the medium was changed to culture medium containing 0.1% serum for starvation. After 48 h, the cells will be quiescent, then the medium was changed to 10% wound fluid for stimulation. Control cells were left in 0.1% serum throughout this period. After 0 h, 6 h, 16 h and 24 h from the wound fluid stimulation, sample were taken for immunocytochemical staining and Western-blot analysis. In all cases, duplicate samples were taken and the experiment was repeated at least twice.

RNA interference for p68 knockdown

The siRNA oligonucleotides against p68 RNA helicase are designed as followed: Sense sequence, GCAAGUAGCUGCUGAAUUAUUU; anti-sense sequence, 5'-pAUUAUCAGCAGCUACUUGCUU.²³ For RNAi experiments, cells were grown to 50% confluence and transfected with small interfering RNA (100 nmol of RNA duplex, Invitrogen, USA).

Cell scratch assay

Cell scratch assay were used to test the effect of p68 knockdown by RNAi on cells migration. Seed cells at 50%–60% confluence before transfection. Transfect cells with the siRNA by Lipofectamine reagents (Invitrogen, USA), and the control cells transfect with negative RNA duplex. Incubate the dishes at 37 °C until cells reach 100% confluence to form a monolayer. Use a p200 pipet tip to create a scratch of the cell monolayer, and wash away cell debris. After 12 h of scratch, measure the scratch width, to calculate scratch closure rate between transfected siRNA and control cells.²⁴

Cell proliferation assay

HaCaT, HuVEC and fibroblast were distributed into 96-well plates (10^4 cells per well). As for assay for the effect of knock-down of p68 on cell proliferation, after the cells were transfected with siRNA for 48 h, compare the number of transfected cells with normal cells by CCK-8 kit (Kumamoto, Japan). As for the effect of wound fluid on cell proliferation, firstly, cells were starved for 48 h with medium containing 0.1% FBS, then changed to medium contained 1%, 5% and 10% wound fluid respectively, while the control was always cultured with 0.1% FBS. Compare the number of wound stimulated cells with control by CCK-8 kit.

Western-blot

Cells were harvested and protein was extracted. Total 40 μ g of protein from each sample was size fractionated on 10% SDS-PAGE gels for 100 min at 110 V. The proteins were transferred to polyvinylidene fluoride (PVDF) membrane and the membrane was blocked with 10% BSA (Roche, USA). The PVDF membrane was probed with an antibody directed against p68 (1:3000) and β -actin (1:1000). The membrane was washed and incubated with secondary antibody, goat anti-rabbit HRP (1:1000) or goat anti-mouse

HRP (1: 1000). The signal was developed with ECL Super Signal Chemiluminescent Substrate (Pierce, USA).

Statistical analysis

All quantitative values are presented as the means \pm SEM. Statistical comparisons were made by using the unpaired Student's *t* test with SPSS (version 15) software, as appropriate. $P < 0.05$ was considered to be statistical significant.

Results

Characterize p68 in rat skin during postnatal development

First, we characterize the expression and localization of p68 protein in skin of rat postnatal. There is highest expression of p68 in the skin of P1 rat, p68 mainly located in epidermis, dermis and hair follicle cells (Fig. 1 A). The epidermis of neonatal rat constituted of 3–4 layers of keratinocyte, as the skin maturing, the epidermis become thinner and the cells declined to only one layer. The expression of p68 down-regulated as the maturation of skin, the p68 is very weak in epidermis at P8 (Fig. 1B and C), and reached the lowest level in epidermis or hair follicle (Fig. 1 D) at P30. These results indicated that p68 protein expression might correlate with the proliferation and maturation of skin cells.

Expression of p68 in rat skin during wound healing process

Next, we assayed p68 expression pattern in skin of adult rat after excision wounding. To determine a potential role of RNA helicase p68 in the wound healing process, we generated full-thickness excision wound on the back of rat and analyzed the expression pattern of p68 by immunohistochemistry (IHC). We found that there was rarely p68 positive cells in the normal adult rat skin by IHC (Fig. 2A, G). On 1 day post-wound, p68 expression began to

emerge in epidermis and dermis near the wound margin (Fig. 2B, H). During wound healing process, the expression of p68 increased, reached its peak in new regenerated epidermis and granulation tissue on 14 days post-wound (Fig. 2C, D, I, J) and then markedly declined, but it was still present at 21 days post-wound (Fig. 2E, K). The expression of p68 was summarized (Fig. 2F, L) ($p < 0.01$). As the wound healing process involves proliferation and migration of cells around wound margin, and the expression pattern of p68 conformed to this process. Next, we investigated the role of p68 in cell proliferation and migration.

The expression of p68 in cell proliferation and migration

We used three kinds of wound healing related cells to study the role of p68 in wound healing *in vitro*. The wound micro-environment, as reflected by wound fluid, has been shown to modulate repair cells function during wound repair.^{25–27} In our study, we used the acute wound fluid to stimulate quiescent cells, including HaCaT, HuVEC and fibroblast, to assay the changes of p68 level in cell proliferation and migration process. The cells were made quiescent by 48 h serum withdrawal and then stimulated by adding wound fluid. We found wound fluid dose-dependently increased all three cell lines proliferation (Fig. 3A) ($p < 0.01$). Western-blot bands showed that 10% of acute wound fluid could induce the expression of p68, which peaked at 16 h (Fig. 3B and C) ($p < 0.01$). Immunocytochemistry has confirmed weak expression of p68 in quiescent cell (Fig. 3 D, E, F), after 16 h stimulation with wound fluid, the induced p68 protein mainly located in nuclei of these three cells (Fig. 3 G, H, I). Serum could also induce cells growth and increase of p68 expression (data not shown). We also assayed p68 expression during cells migration in an *in vitro* wound model. After cell scratch, the cells around wounding margin became activated and migrated, and the expression level of p68 in these cells was higher than the quiescent cells (Fig. 3J, K, L). These results indicated p68 might play important role in wound healing process.

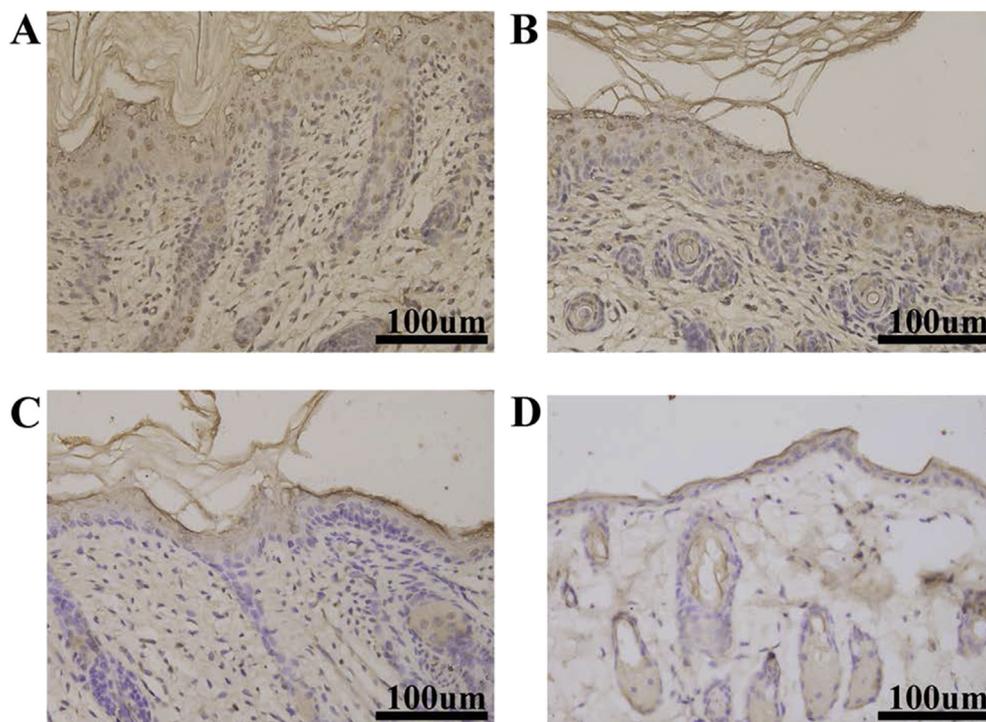


Fig. 1. Immunohistochemical staining of p68 in rat skin during postnatal development. A: 1 day postnatal; B: 4 days postnatal; C: 8 days postnatal; D: 30 days postnatal. Note decreases of p68 in the dermis as skin maturation.

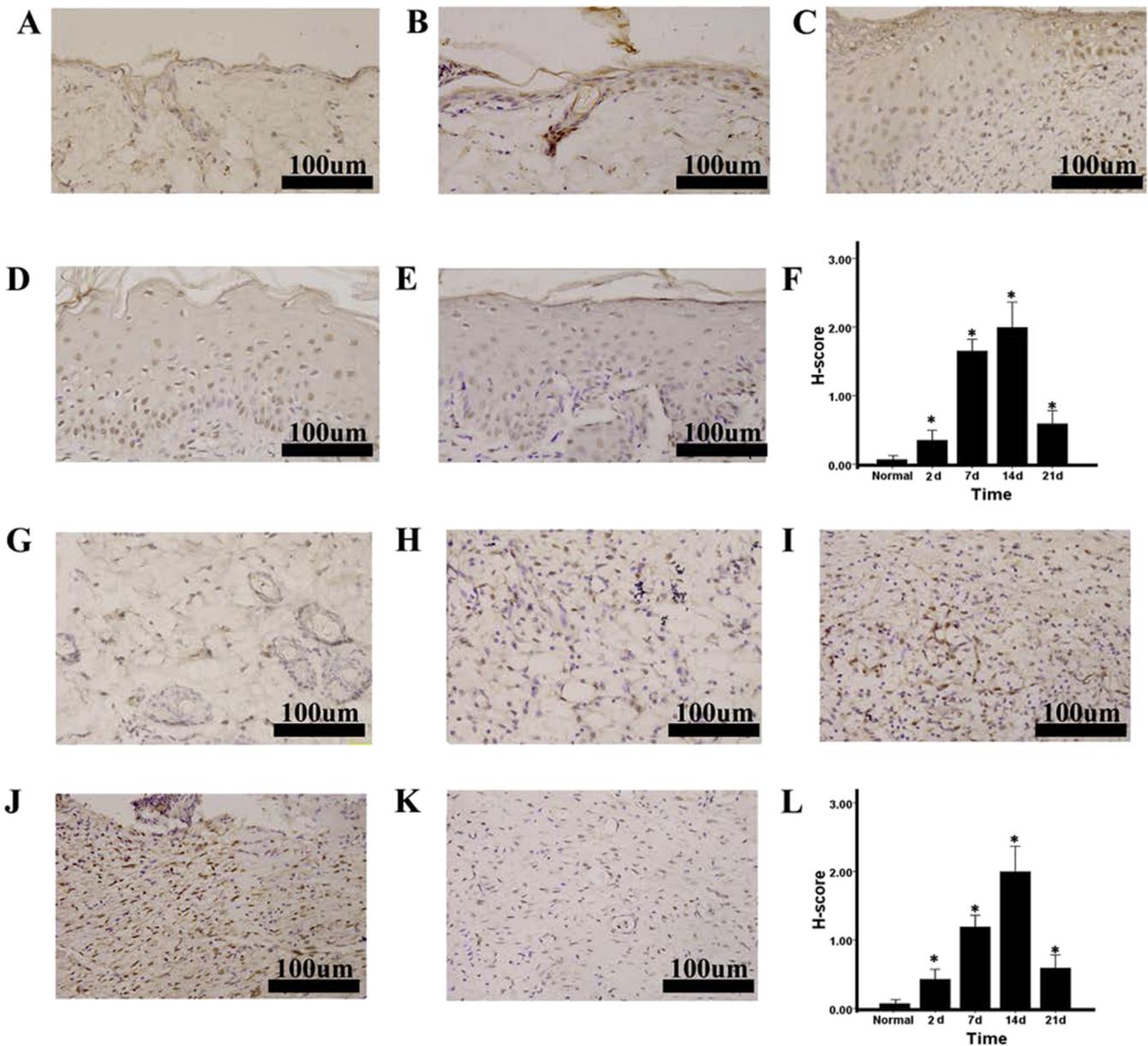


Fig. 2. Characterization of p68 in rat skin during wound healing. A: Normal epidermis; B: 1 day post wound epidermis; C: 7 days post wound epidermis; D: 14 days post wound epidermis; E: 21 days post wound epidermis; F: H-score of p68 in epidermis during wound healing process; G: normal dermis; H: 1 day post wound dermis; I: 7 days post wound dermis; J: 14 days post wound dermis; K: 21 days post wound dermis; L: H-score of p68 in dermis during wound healing process (original magnification 400 \times).

The impact on cell proliferation and migration after p68 knock-down by RNAi

To further investigate the regulating role of p68 in cell proliferation and migration, we used p68 siRNA to knockdown p68. Compared with the control group, the Western-blot results showed that p68 was effectively knockdown in all three cells (Fig. 4A). The cell proliferation was significantly inhibited (Fig. 4B) ($p < 0.01$). And the cell scratch closure rate decreased obviously in the RNAi group (Fig. 4C) ($p < 0.01$). These results demonstrated p68 regulated cells proliferation and migration during wound healing.

Discussion

Wound healing is a complex process requiring the collaborative efforts of clusters of gene function turn on and off. Previous studies on skin tissue have demonstrated a role of RNA helicase p68 in the

regulation of keratinocyte at the early process of cutaneous wound healing.¹⁷ In this study, we further examined the of p68 expression in all phases of the repair process by using a rat skin wound model and demonstrated the regulation of p68 on cell proliferation and migration.

Wound healing is an essential biological process that comprises sequential steps aimed at restoring the architecture and function of damaged tissues.²⁸ Healing a wound is, no matter how simple the organization of a cellular system, always the result of sequential steps, each one taking the damaged area one step closer to restoring cellular architecture and function.²⁹ The perfect healing result needs precise regulation of the cells function. The fundamental nature of wound healing makes it a highly informative process to study. If the cell function were out of control, non-healing injury, scar or even skin cancer might occur. So, it is worth to study the mechanism of wound healing in molecular levels. Studies have highlighted the similarities between wound healing and developmental

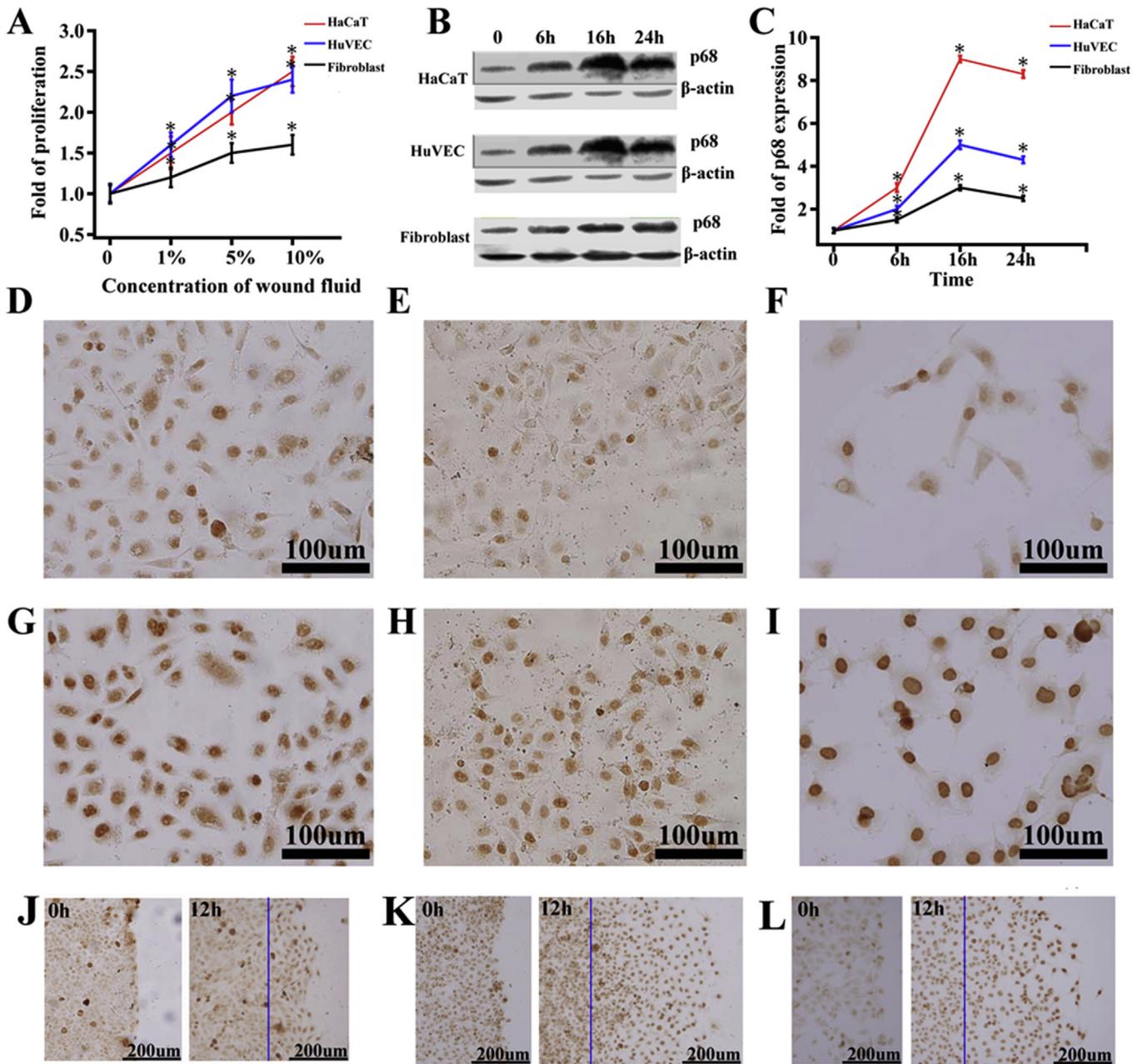


Fig. 3. Expression of p68 in wound fluid stimulation and cell scratch. **A:** The proliferation rate of three cells (HaCaT, HuVEC, Fibroblast) cultured with 1%, 5%, 10% wound fluid for 24 h. **B:** Western-blot of cells stimulated with 10% wound fluid for 0 h, 6 h, 16 h and 24 h. **C:** Linegraph showing the relative intensity in the levels of p68 expression in cells with wound fluid stimulation. **D, E, F:** Expression of p68 in HaCaT, HuVEC, Fibroblast cells starved 48 h with culture medium contained 0.1% FBS. **G, H, I:** Expression of p68 in the three starved cells stimulated with culture medium contained 10% wound fluid for 16 h (original magnification 400×). **J, K, L:** Expression of p68 in the three cells 12 h after scratch (original magnification 200×). After scratch, expression of p68 in the migrated cells is higher than the quiescent cells.

processes.^{30,31} Moreover, crucial developmental genes are essential for proper wound healing.³² Therefore, unravelling how cells and tissues react to injury may contribute insights into the basic molecular mechanisms of life and disease.^{33–35}

p68 has been highly conserved in evolution and appears to be essential for normal growth. p68 expression is developmentally regulated and appears to correlate with organ maturation.¹² RNA helicase p68 might be an important messenger molecule in normal skin biology and malfunction of p68 leads to diseased skin.^{17,36} We have found RNA helicase p68 is a novel tissue repair-related gene. There is very weak expression of p68 in normal adult rat skin. The level of p68 protein is up-regulated during the major stages of proliferation in wound healing, and then declined when

the healing process finished, but not totally disappeared. The change of p68 expression is consistent with the wound healing process. These results suggest that regulating of p68 activity might be a novel means for promotion and regulation of wound healing. And the expression change of p68 in wound repair process is very similar to that in the development of skin. But the expression of p68 should be precisely control, because over-expression p68 in normal fibroblasts causes their tumorigenic transformation.³⁷ Furthermore, p68 has been demonstrated to be abnormally over-expressed in colorectal adeno-carcinoma, colon cancer and prostate cancer.^{38–41} Our previous work has found over-expression of RNA helicase p68 protein in human cutaneous squamous cell carcinoma.³⁶

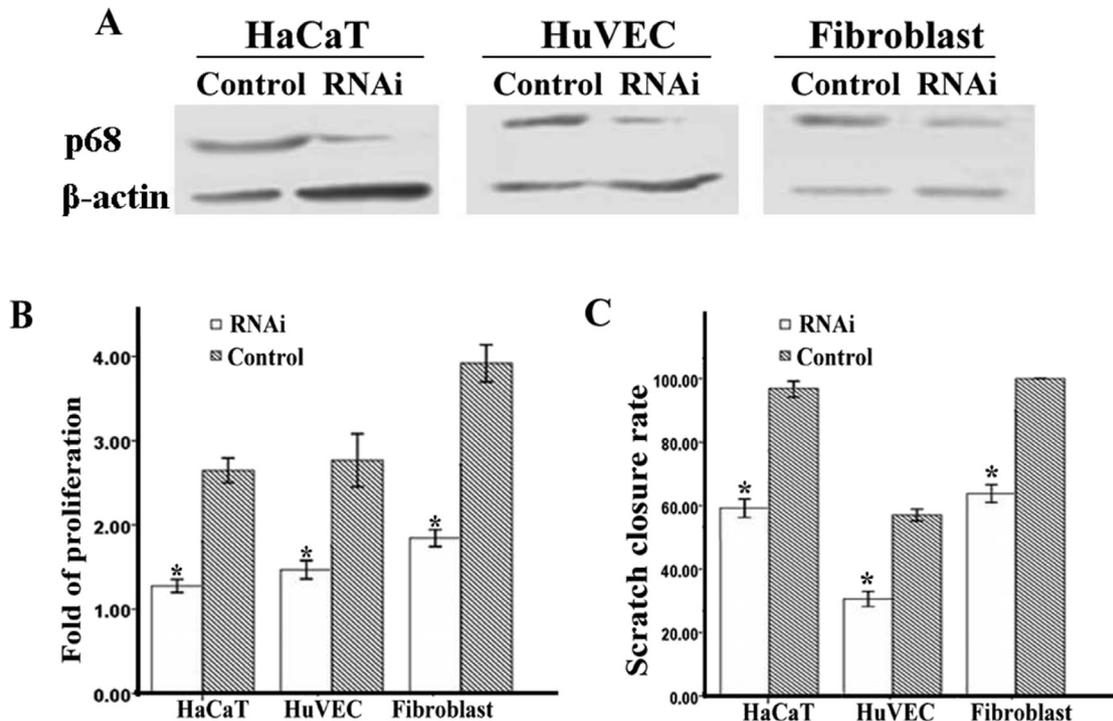


Fig. 4. Knock-down p8 inhibits cell proliferation and migration. A: Levels of p8 in HaCaT, HuVEC, Fibroblast were analyzed by western-blot. Immunoblots of β -actin are loading controls. B: Proliferation rate of cells was inhibited after knock-down of p8. C: Scratch-wound closure rate was compared between p8 RNAi group and control group, the migration of cell was inhibited after knock-down of p8.

How does p8 regulate repair cells and participate in wound healing needs further study. Lots of evidence demonstrate that p8 could control many genes in transcriptional level.⁴² p8 interacts with transcriptional co-activators, such as CBP/p300 and RNA Pol II.⁹ Moreover, p8 was shown to be recruited to the promoter of the ERa target gene, pS2, in the presence of estrogen, suggesting an involvement for p8 in transcriptional initiation.⁴³ Tyrosine phosphorylation of p8 could mediate the effect of platelet-derived growth factor and could also promote cell proliferation by activating the transcription of cyclin-D1 and c-Myc genes. In this study, we haven't found phosphorylation of p8 in the wound healing process (Data not shown). Recently, one group has found p8 could interact with Ca^{2+} -calmodulin in promoting cell migration.⁴⁴

In summary, RNA helicase p8 involved in skin tissue repair by regulating cell proliferation and migration. Therefore, p8 may be a target molecule to promote wound healing, but constantly high expression of it might lead to scar and tumor formation. We will verify this potential in the near future work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.cjtee.2017.10.001>.

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