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ERK2 dependent signaling contributes to wound healing after a partial-thickness burn

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ABSTRACT

Burn healing is a complex physiological process involving multiple cell activities, such as cell proliferation, migration and differentiation. Although extracellular signal-regulated kinases (ERK) have a pivotal role in regulating a variety of cellular responses, little is known about the individual functions of ERK isoform for healing *in vivo*. This study investigated the role of ERK2 in burn healing. To assess this, $Erk2^{+/-}$ mice generated by gene targeting were used. The resultant mice exhibited significant delay in re-epithelization of partial-thickness burns in the skin in comparison to wild-type. An *in vitro* proliferation assay revealed that keratinocytes from $Erk2^{+/-}$ mice grew significantly slower than those prepared from wild-type. These results highlight the importance of ERK2 in the process of burn healing.

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Introduction

Healing of the dermal surface after burn requires migration, proliferation, and differentiation of skin cells. All of these steps involve the orchestrated regulation of cell signaling pathway that control the expression of many genes associated with diverse crucial functions. A disruption of these signaling pathways can lead to delayed healing, thus resulting in a patient's morbidity and mortality. Disrupted signaling can also accelerate the healing processes, leading to hypertrophic scars or keloids. Therefore, the optimal healing of burns remains a therapeutic challenge in spite of a variety of available treatment modalities.

Although the induction of signals that allow the cells to respond to these events is not well understood, ERK pathway may be implicated in such events [1,2]. Some reports suggested that ERK pathway play an important role in healing process [3–5]. In *Drosophila*, ERK activities are induced in epidermal cells near the wounds, and activated ERK is required for a robust wound response [3]. Mechanical injury of confluent cultures activated ERK in normal human keratinocytes [4]. Inhibition of ERK pathway causes a delay in wound healing in cultured rabbit corneal epithelial cells [5]. However, at present little is known about the specific functions of the individual ERK isoforms for burn healing *in vivo*. Although ERK1 and ERK2 have roughly 90% sequence identity, some studies suggested distinct physiological functions of each isoforms [6–8]. Most of studies investigating the ERK pathway have used inhibitors of upstream kinases. However, these drugs inhibited the activation of ERK1, 2 and 5 [9]. Therefore, to address the specific function of ERK2 *in vivo*, *Erk2* mutant mice were generated. While the lack of *Erk2* ($Erk2^{-/-}$) leads to early embryonic lethality (before E13.5) [7], $Erk2^{+/-}$ mice were viable, fertile and appeared relatively normal in adult. Thus $Erk2^{+/-}$ mice were used in this study.

We investigated the role of ERK2 activity in burn wound healing in the skin, a tissue where the ERKs seem to be particularly fundamental [10–12]. As a burn model in this study, only the epidermis and superficial part of skin appendages were injured, which was classified as partial-thickness burn. This burn wound was spontaneously epithelialized in contrast to full-thickness burn, and thus the role of ERK2 during the spontaneous healing process could be investigated.

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Materials and methods

Targeted disruption of Erk2. To delete ERK2, mice carrying the *Erk2*(floxN) allele were mated with EIIA-Cre mice [13]. Construction of the *Erk2*(floxN) allele (Fig. 1A) was described previously [7]. Mice with *Erk2*(⁻) were identified by a Southern blot analysis (Fig. 1B and C) and routinely genotyped by PCR. The primers used were mE2-F3 (5′-GATCTGATGCTTGCCAAAGCC-3′) and mE2-R4 (5′-TGTAAAGTAGCAGCAGATGC-3′) to detect wild-type allele and mE2-F3 and mE2-R1 (5′-CAGAGTTTCATTATGGAGTCCTCGC-3′) to detect *Erk2*(−) allele.

All experiments were conducted according to the institutional ethical guidelines for animal experiments and safety guidelines for gene manipulation experiments of the National Defense Medical College, RIKEN and OIST.

The mice used for the test were C57BL/6 and BalbC background and between 9 and 12 weeks of age. The mice were housed under standard laboratory conditions with a 12 h light/dark cycle; room temperature was maintained at 23 ± 1 °C. The mice had ad libitum access to water and food.

Partial-thickness burn models. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and their dorsal hair was clipped and depilated with a hair removal cream (Shiseido Co., Tokyo, Japan). The dorsal skin was exposed for 4 s to water heated to 70 °C through a Walker–Mason template [14] to induce a partial-thickness burn. Fig. 2D and E shows the histology of the skin. Only the epidermis and a superficial portion of the skin appendages were injured and the interwoven pattern of collagen fibers in most of the dermis was retained (Fig. 2E). Based on these findings, the wounds were classified as a partial-thickness burn.

Preparation of protein extracts. The mouse skin was quickly removed after euthanasia. The specimens were cut and homogenized in four volumes of 20 mM Tris–HCl (pH 7.4), 2 mM EDTA, protease inhibitor cocktail (Complete, Roche Diagnostics, Penzberg, Germany) and phosphatase inhibitors (20 mM glycerophosphate, 1 mM Na_3VO_4, 2 mM NaF). After homogenization, a portion of each sample was immediately frozen at −80 °C. The rest of the homogenate was centrifuged at 15,000*g* for 30 min at 4 °C. The supernatants were collected and stored at −80 °C until use. The amount of protein in each sample was measured using a BCA assay (Pierce, Rockford, IL).

Western blot analysis. The supernatants of the homogenates were separated by SDS–PAGE. The proteins in the SDS–PAGE were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA). The blots were immunoreacted with anti-ERK1/2 (1:1000; rabbit polyclonal #9102, Cell Signaling, Beverly, MA), anti-phospho-ERK1/2 (1:1000; rabbit polyclonal #9101, Cell Signaling), or anti-β-actin (1:5000, mouse monoclonal AC-15, Sigma, St. Louis, MO) antibodies and the protein bands were visualized by a chemiluminescence detection system (Super Signal West Pico; Pierce or ECL plus; Amersham, Arlington Heights, IL). The signals in the immunoblots were analyzed by an LAS3000 digital imaging system (Fujifilm, Tokyo, Japan). The total ERK activity was evaluated by adding the signals for phosphp-ERK1 and phospho-ERK2 as performed previously [12]. These values were divided by the amount

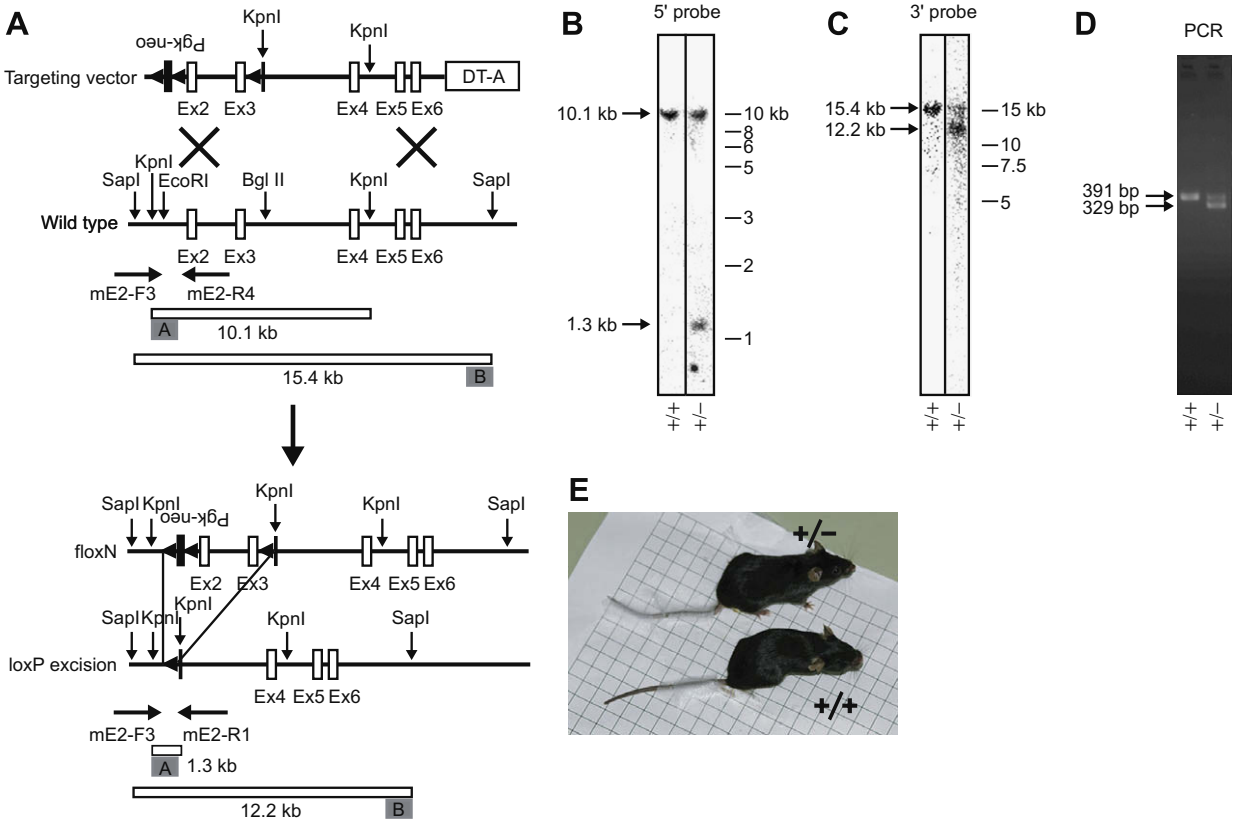

Fig. 1. Targeted disruption of *Erk2* in mice. (A) Schematic diagrams of the targeting vector, the wild-type allele, the targeted allele (*floxN*) and loxP excised (*Erk2*(−)) allele of the *Erk2* gene. White boxes represent *Erk2* exons and black boxes represent the *Pgk-neo* cassette. A *Kpn*I restriction site was generated in intron 3 (black bar). The 5′-(A) and 3′-(B) probes used for Southern blot analysis are shown as gray boxes. The primers used for PCR are shown as arrows. (B,C) A Southern blot analysis of wild-type and heterozygous adult mice genomic DNA. DNA samples were digested with *Kpn*I or *Sap*I, and then hybridized with the 5′- or 3′-probe, respectively. The positions and sizes of the wild-type and mutant mice. (D) PCR genotyping of wild-type and mutant mice. The positions and sizes of PCR fragments for wild-type and mutant mice are indicated. (E) General appearance of $Erk2^{+/+}$ and $Erk2^{+/-}$ mice at 10-week-old of age.

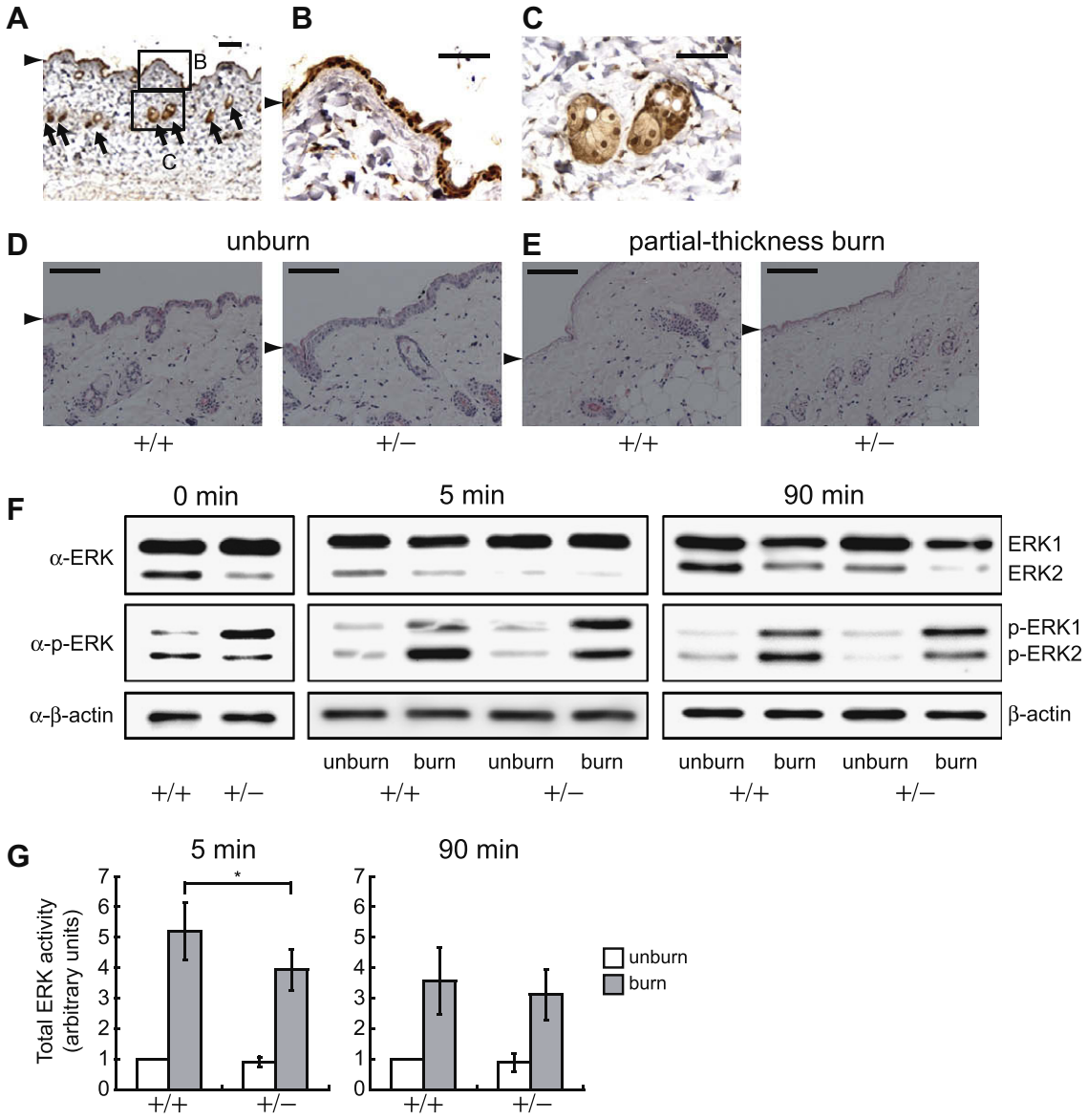

Fig. 2. (A–C) ERK2 localization in the skin. Immunostaining of ERK2 in normal skin from wild-type mice shows abundance of ERK2 in hair follicle formation (arrow) and epidermis (arrowhead). (B,C) High-power magnification of boxed segments indicated in (A). Scale bars; 100 μm in (A), 50 μm in (B) and (C). (D,E) Animal model of partial-thickness burn. Histopathological analysis (H&E-stained section) of skin 12 h after a burn from wild-type (++) or *Erk2*$^{+/-}$ (+/−) mice. Note that while epidermis (arrowhead) was observed in unburned area (D), those were disrupted in the partial-thickness burn (E). Scale bars; 100 μm. (F) The increased phosphorylation of ERK2 in mice skin after burning. The expression of ERK2, ERK1, phospho-ERK2 and β-actin in an extracts obtained from the adult skin are shown. The total protein lysates (3 μg) were analyzed by western blotting with the indicated antibodies. The blots shown are representative examples before burn (*Erk2*$^{+/+}$, $n = 5$; *Erk2*$^{+/-}$, $n = 5$), 5 min after burning (*Erk2*$^{+/+}$, $n = 5$; *Erk2*$^{+/-}$, $n = 5$) and 90 min after burn (*Erk2*$^{+/+}$, $n = 5$; *Erk2*$^{+/-}$, $n = 5$). β-Actin served as controls for protein loading. The unburned area in the skin of the same mouse was used as the control. (G) Quantification of the total ERK activity in burned or unburned area from wild-type and *Erk2*$^{+/-}$ mice in (F).

of β-actin and the total activity of ERKs in unburned area from wild-type mice was defined as 1.

Histological analysis. The skin specimens were histologically analyzed using paraffin-embedded sections (4 μm thick). Prior to use, sections were dewaxed in xylene and hydrated using a graded series of ethanol. Antigenic retrieval was carried out by immersing mounted tissue sections in antigen unmasking solution (Vector Laboratories, Burlingame, CA) and heating in an autoclave (121 °C) for 5 min. Deparaffinized sections were blocked with a nonspecific staining blocking reagent (Dako, Glostrup, Denmark) for 1 h to reduce background staining. The sections were incubated overnight at 4 °C with anti-ERK2 (1:500, mouse monoclonal, Transduction laboratories, Lexington, KY) or anti-ERK1 (1:100, mouse monoclonal, Zymed laboratories, South San Francisco, CA) antibodies in antibody diluent (Dako). Subsequently, slides were incubated with peroxidase-conjugated secondary antibody (DAKO En Vision+ system, Dako). Staining was detected with 3,3-diaminobendine-tetrachloride (DAB, Vector Laboratories) followed by counterstain with hemotoxylin. Hematoxylin–eosin (H&E) stainings were performed according to standard procedures.

Keratinocyte cellcultures. Keratinocytes culture was carried out as described previously [15]. Briefly, they were obtained from newborn mutant and wild-type littermates by CELLnTEC Advanced Cell Systems (Bern, Switzerland). Full-thickness skin was treated with 500 U/mL of dispase (Godo Shusei, Tokyo, Japan) overnight at 4 °C, after which the epidermis was peeled off from the dermis. By treating with TripLE Select (Invitrogen, Carlsbad, CA), epidermis was dissociated to single cells. They were cultured in CnT-07 medium (CELLnTEC). Keratinocytes (8×10^4) were plated in each well

of a collagen IV-coated 6-well plate. Each day, the cells were trypsinized and counted.

Statistics. Statistical analysis was carried out using Statview software (SAS, Cary, NC). Data were analyzed by Student's *t*-test and two-way repeated ANOVA. The values are presented as means ± SD.

Results

Generation of Erk2$^{+/-}$ mice

To investigate the genetic and physiological functions of ERK2 in mice, the *Erk2* gene was disrupted in exons 2 and 3 using the Cre-loxP system (Fig. 1A) as described previously [7]. Genotypes were confirmed by Southern blotting and assessed by PCR (Fig. 1B–D). *Erk2*$^{+/-}$ mice were viable, fertile, and of normal size in adult (Fig. 1E). To focus on the implication of ERK2 in the burn repair, firstly we have examined the structure of *Erk2*$^{+/-}$ mice skin. No major change of skin structure was detected including the thickness of the epidermis and dermis in *Erk2*$^{+/-}$ mice in comparison to wild-type at least at the light-microscopic level (Fig. 2D).

Phosphorylation level of ERK2 increased upon burn wound

First, we investigated the localization of ERK2 in mouse skin. We observed a strong staining pattern of ERK2 in epidermis and hair follicle cells in the mouse skin (Fig. 2A–C). Stem cells are implicated in skin wound healing. Stem cells are known to exist in epidermis and hair follicle cells [16]. In this sense, it is noteworthy that ERK2 is abundant in epidermal and hair follicular cells.

To examine the involvement of the ERK pathway in burn healing *in vivo*, partial-thickness burns were created in the mid-dorsal region of the mice as an animal model. Both *in vivo* and *vitro*, an epithelial wound is known to induce activation of ERKs [5,17,18]. ERK activation is involved in epithelial injury response [17] and increased upon mechanical injury of keratinocytes [5]. We therefore examined the ERK activation following partial-thickness burn.

In the basal state, the expression level of ERK2 in the skin from *Erk2*$^{+/-}$ mice was about half of those of wild-type (Fig. 2F). We found no differences in ERK1 expression levels between *Erk2*$^{+/-}$ and wild-type mice skins indicating that there were no compensatory changes in ERK1 expression. In addition, the *in vivo* basal phosphorylation state of ERK was evaluated in skin extracts with an antibody against phospho-ERK1/2 (Fig. 2F). In *Erk2*$^{+/-}$ mice, the density of the phospho-ERK2 band was significantly smaller than those of wild-type. On the other hand, the density of phospho-ERK1 band was higher than those of wild-type in *Erk2*$^{+/-}$ mice, thus suggesting the existence of partial compensatory changes in phosphorylation level.

The phosphorylation level of ERK1 and 2 in partial-thickness burn in the skin was significantly increased at 5 min after the burn wound both in the wild-type and *Erk2*$^{+/-}$ mice (Fig. 2F), thus indicating that the ERK pathway was quickly activated in the tissue that experienced a partial-thickness burn. However, the phosphorylation level of ERK2 from burned area of *Erk2*$^{+/-}$ mice was approximately half of that in the wild-type. ERK1 isoform from mutant samples were activated higher than that from wild-type, which is likely to compensate partially for the decrease of ERK2 phosphorylation level. Curiously, the expression levels of ERKs decreased in the burned area in comparison to the unburned area (for example, in the wild-type animals, lanes 3 and 4). It is likely that the epidermis in which ERK2 is abundant (as shown in Fig. 2A) was specifically distinguished after partial-thickness burn. At 90 min after the burns, the phosphorylation level of ERKs in tissue that experienced a partial-thickness burn was still enhanced in both mice in comparison to the unburned area (Fig. 2F). Since the total

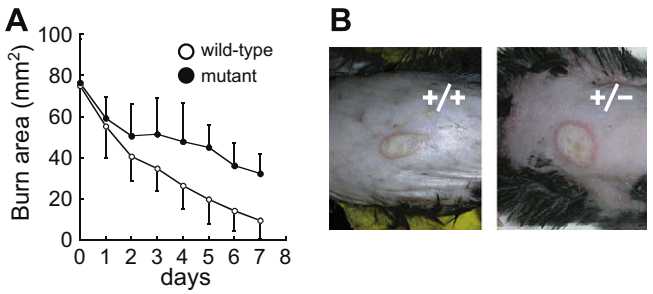

Fig. 3. Delayed burn healing in *Erk2*$^{+/-}$ mice (*Erk2*$^{+/+}$, $n = 12$; *Erk2*$^{+/-}$, $n = 8$). (A) The unhealed area is plotted versus the time after burn. (B) General appearance of partial-thickness burn in wild-type and *Erk2*$^{+/-}$ mice at day 5.

activity may be linked to mitogenic potential [12], activity of ERKs (ERK1 + ERK2) was measured by analyzing the levels of the phosphorylated forms of ERKs (Fig. 2G). A reduction of total ERK activity may be accompanied by a drastic reduction of many genes following stimulation [12]. In the tissue that experienced a partial-thickness burn, total ERK activity is higher in wild-type compared with those in *Erk2*$^{+/-}$ mice 5 min following partial-thickness burn (Fig. 2G). But it declined to reach almost equivalent levels in both genotypes 90 min following partial-thickness burn (Fig. 2G).

Delayed burn healing in the Erk2$^{+/-}$ mice

As described above, the inhibition of ERK was suggested to cause a delay in wound healing [5,19,20]. To study the specific role of ERK2 in skin burn healing, the effect of reduced ERK2 on the healing rate of partial-thickness was monitored using *Erk2*$^{+/-}$ mice. No significant difference between *Erk2*$^{+/-}$ and *Erk2*$^{+/+}$ mice was observed in the gross appearances of the wounds injured immediately after burns. A gradual reduction of burn area was observed during healing (Fig 3) and the burn area was reduced to 1/10 of the original area for the wild-type mice in 7 days. On the other hand, the *Erk2*$^{+/-}$ mice exhibited a significant delay in the healing process in comparison to the wild-type mice (Fig. 3; ANOVA, $F = 16.16$, $p < 0.001$ (genotype), $F = 50.11$, $p < 0.0001$ (periods), $F = 3.32$, $p < 0.005$ (interaction between genotype and periods)). This result further indicated that ERK2 signaling was involved in the healing process after burns and highlight a specific role of ERK2 in burn healing.

Impaired proliferation potential of keratinocytes from Erk2$^{+/-}$ mice

While the ERK pathway induces a variety of downstream responses, one of key genes regulated by this pathway is *cyclin D1*, which is responsible for G_1-S transition [21]. Thus to evaluate a

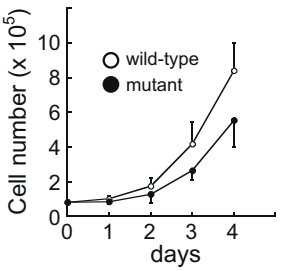

Fig. 4. Slow growth of mutant keratinocytes (*Erk2*$^{+/+}$, $n = 5$; *Erk2*$^{+/-}$, $n = 5$). Primary mouse keratinocytes from newborn *Erk2*$^{+/-}$ mice and wild-type littermates were tested for their ability to proliferate *in vitro*. Keratinocyte isolates were obtained independently from five mutant and five wild-type newborn mice.

proliferation capacity in *Erk2*$^{+/-}$ mice, we evaluated growth curve of keratinocytes obtained from the mutant mice. We observed a reduced proliferation capacity in kertinocytes from *Erk2*$^{+/-}$ mice (Fig. 4; two-way repeated ANOVA, $F = 8.92$, $p < 0.05$ (genotype), $F = 115.57$, $p < 0.0001$ (periods), $F = 5.63$, $p < 0.01$ (interaction between genotype and periods)) in consistence with the notion that ERK may be implicated in growth control. These results suggest that ERK2 haploinsufficiency was not compensated by ERK1 in growth regulation.

Discussion

The current results indicated that the ERK pathway was activated after partial-thickness burns and might be implicated in the healing process. Partial-thickness burn to the dorsal skin caused rapid activation of ERK (within 5 min, Fig 2F,G) and the healing was delayed in *Erk2*$^{+/-}$ mice (Fig 3). Both results indicate that ERK2 signaling was involved in this response although the mechanisms of burn healing have only been partially elucidated.

An *in vitro* proliferation assay in this study showed that disruption of ERK2 led to an abnormal proliferation in mouse skin keratinocytes (Fig 4). This result is consistent with a previous study that showed that ERK phosphorylation is required for proliferation in epithelial wound healing [5]. However, we could not rule out the possibility that other mechanisms were also impaired in the healing process in mutant mice. For instance, the mutation would cause abnormal cell migration since ERK activation also plays an important role in cell migration [22,23]. Although the exact function in cell migration remains to be elucidated, phospho-ERK is localized to the leading edge of the lamellipodia in migrating keratinocytes [21]. Furthermore, it was reported that ERK directly interact with β integrins [24], thus suggesting that ERKs may play an important role in integrating cell adhesion and receptor-mediated intracellular signaling system in the control of cell migration. Further investigation is necessary to determine the role of ERKs in proliferation and migration. Recently, Fitsialos et al. reported that inhibition of the ERK pathway completely blocks wound closure *in vitro* human keratinocyte and inactivates many early transcription factors and EGF-type growth factors [25]. They also reported that the ERK pathway controls a majority of early genes, suggesting that potential physiological role of ERKs in the early steps of the healing process [25].

Defining the mechanism that regulates the burn healing will provide the potential for developing new therapeutic approaches. The current findings strongly support the notion that the ERK pathway has an important regulatory role in burn healing. Further investigation of the ERK pathway in skin should improve the understanding of the burn healing process and might lead to the development of therapies to enhance burn wound healing. Conditional ERK2 knockout in the skin would clarify this question.

Acknowledgments

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