Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing

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Summary

Members of the epidermal growth factor (EGF) family are the most important growth factors involved in epithelialization during cutaneous wound healing. Heparin-binding EGF-like growth factor (HB-EGF), a member of the EGF family, is thought to play an important role in skin wound healing. To investigate the in vivo function of HB-EGF in skin wound healing, we generated keratinocyte-specific HB-EGF-deficient mice using Cre/loxP technology in combination with the keratin 5 promoter. Studies of wound healing revealed that wound closure was markedly impaired in keratinocyte-specific HB-EGF-deficient mice. HB-EGF mRNA was upregulated at the migrating epidermal edge, although cell growth was not altered. Of the members of the EGF family, HB-EGF mRNA expression was induced the most rapidly and dramatically as a result of scraping in vitro. Combined, these findings clearly demonstrate, for the first time, that HB-EGF is the predominant growth factor involved in epithelialization in skin wound healing in vivo and that it functions by accelerating keratinocyte migration, rather than proliferation.

Key words: Conditional knockout, HB-EGF, Keratinocytes, Migration, Wound healing

Introduction

Cutaneous wound healing requires precise coordination of epithelialization, dermal repair and angiogenesis (Singer and Clark, 1999). Epithelialization is ultimately dependent on the migratory, proliferative and differentiation abilities of keratinocytes. The growth and differentiation of keratinocytes are regulated mainly by a variety of growth factors (Hashimoto, 2000), of which the members of the epidermal growth factor (EGF) family are the most important for skin wound healing.

The EGF family consists of EGF, transforming growth factor (TGF)-α, heparin binding EGF-like growth factor (HB-EGF), amphiregulin (AR), epiregulin (EPR), betacellulin (BTC), epigen and neuregulin (NRG)-1, NRG-2, NRG-3 and NRG-4 (Falls, 2003; Harari et al., 1999). The EGF receptor (EGFR) family consists of EGFR (also called ErbB1), ErbB2, ErbB3 and ErbB4 (Jorissen et al., 2003). The mammalian ligands that bind EGFR include EGF, HB-EGF, TGF-α, AR, BTC, EPR and epigen. Recent studies using gene targeting or transgenic models have revealed that EGFR is essential for epithelial development in the skin, lung and gastrointestinal tract, whereas ErbB2, ErbB3, ErbB4 and neuregulins are essential for the development of cardiac muscle and the central nervous system (Erickson et al., 1997; Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Miettinen et al., 1995; Murillas et al., 1995; Riethmacher et al., 1997; Sibilia and Wagner, 1995).

Previous reports have shown that TGF-α, AR, HB-EGF and EPR are autocrine growth factors in normal human epidermal keratinocytes (NHEK) (Coffey et al., 1987; Cook et al., 1991; Hashimoto et al., 1994; Shirakata et al., 2000). It has been reported that keratinocyte migration and proliferation are predominantly mediated by autocrine EGFR activation (Stoll et al., 1997). However, the importance of the role that the EGF family plays in skin wound healing has not been confirmed in vivo using knockout mice. Previously, Marikovsky et al. (Marikovsky et al., 1993) reported that HB-EGF is a major component of the mix of growth factors found in wound fluid. Therefore, we speculated that HB-EGF was an important member of the EGF family in cutaneous wound healing. To test this hypothesis, we generated keratinocyte-specific HB-EGF knockout mice, and clearly demonstrated that HB-EGF is an important growth factor for epithelialization in skin wound healing in vivo.

Materials and Methods

Cell culture

Normal human epidermal keratinocytes (NHEK) were prepared and cultured under serum-free conditions, as previously described (Shirakata et al., 2000; Shirakata et al., 2003). Third- or fourth-passage cells were used in this study.

Table 1. Primer sequences for PCR

Wild-type HB-EGF – upper	5′-CATGATGCTCCAGTGAGTAGGCTCTGATTAC
Wild-type HB-EGF – lower	5′-AGGGCAAGATCATGTGTCCTGCCTCAAGCC
lox HB-EGF – upper	5′-ATGGGATCGGCCATTGAACA
lox HB-EGF – lower	5′-GAAGAACTCGTCAAGAAGGC
cre-recombinase – upper	5′-TTACCGGTCGATGCAACGAGTGATG
cre-recombinase – lower	5′-TTCCATGAGTGAACGAACCTGGTCG
lox-out HB-EGF – upper	5′-CGGACAGTGCCTTAGTGGAACCTC
lox-out HB-EGF – lower	5′-GCTTCTTCTTAGGAGGGATCTTGGC

Table 2. Primer sequences for RT-PCR

hHB-EGF – upper	5′-CCACACCAAACAAGGAGGAG
hHB-EGF – lower	5′-ATGAGAAGCCCCACGATGAC
hEPR – upper	5′-TCGCCCGCTCCCATCGCCG
hEPR – lower	5′-GGTTCCACATATTATTTCTG
hTGF-α – upper	5′-GAGTGCAGACCCGCCCGTGGC
hTGF-α – lower	5′-CCAGGAGGTCCGCATGCTCAC
hAR – upper	5′-CCAAAACAAGACGGAAAGTGA
hAR – lower	5′-AGGATCACAGCAGACATAAAG
hGAPDH – upper	5′-ACCACAGTCCATGCCATCAC
hGAPDH – lower	5′-TCCACCACCCTGTTGCTGTA
mHB-EGF – upper	5′-GGAATTCTGGAGCGGCTTCGGAGAG
mHB-EGF – lower	5′-CAAGCTTTGCAAGAGGGAGTACGGAACT
mEPR – upper	5′-GGAATTCTGACGCTGCTTTGTCTAGGTT
mEPR – lower	5′-CAAGCTTTATGCATCCAGCGGTTATGAT
mTGF-α – upper	5′-GGAATTCCTAGCGCTGGGTATCCTGTTA
mTGF-α – lower	5′-CAAGCTTACCACCACCAGGGCAGTGATG
mAR – upper	5′-CAAGCTTACCACCACCAGGGCAGTGATG
mAR – lower	5′-CAAGCTTACCACCACCAGGGCAGTGATG

h, human; m, mouse; EPR, epiregulin; AR, amphiregulin.

Generation of HB-EGF knockout mice using a gene targeting Cre-loxP strategy and PCR

The targeting construct has been described previously (Iwamoto et al., 2003). Homozygous $HB^{lox/lox}$ mice were bred with K5 promoter-driven Cre-recombinase transgenic mice to generate K5-Cre-$HB^{lox/+}$ mice (Takeda et al., 2000). Subsequently, K5-Cre-$HB^{lox/+}$ mice were bred with $HB^{lox/lox}$ mice to generate $HB^{lox/lox}$: K5-Cre ($HB^{-/-}$) mice. The genotype of each mouse was confirmed by PCR. Primers are shown in Table 1.

RT-PCR analysis

Keratinocytes were cultured in MCDB153 complete medium on type I collagen-coated dishes until they reached confluency. Keratinocytes were treated by tip scraping and total RNA was harvested at several time points. mRNA expression of HB-EGF, TGF-α, AR, EPR and GAPDH was analyzed by RT-PCR. The absence of HB-EGF mRNA in keratinocytes from $HB^{-/-}$ mice was confirmed by RT-PCR. Primers are shown in Table 2. The RT-PCR was performed using RT-PCR High Plus (Toyobo Co. Ltd, Osaka, Japan) according to the manufacturer's instructions. cDNA was reverse-transcribed from total RNA for 30 minutes at 60°C and heated to 94°C for 2 minutes. Amplification was performed using a DNA thermal cycler (Astec, Fukuoka, Japan) for 25 cycles. A cycle profile consisted of 1 minute at 94°C for denaturation, 1.5 minutes at 60°C for annealing and primer extension.

Wound healing studies

Wound healing experiments were performed in $HB^{-/-}$ and $HB^{lox/lox}$ mice. Under sodium pentobarbital anesthesia, two full-thickness wounds were created on the skin of the backs of each of nine 9- to 10-week-old female mice using 6-mm skin biopsy punches. Each wound diameter was determined as the average of longitudinal and lateral diameter. Wound closure was monitored, and skin sections were harvested at 3, 5, 7, 9 and 11 days after wounding. For BrdU labeling, mice received intraperitoneal injections of BrdU (250 μg/g; Sigma, Tokyo, Japan) 2 hours prior to sacrifice.

Histological analysis

Mouse tissues were fixed in 4% paraformaldehyde or formaldehyde, dehydrated and embedded in paraffin. Four-μm sections were stained with Hematoxylin and Eosin. For β-gal staining, after fixation with 0.2% glutaraldehyde and 1% formalin, the tissues were stained with 5-bromo-4-chloro-3-indol β-D-galactoside (X-gal). Skin sections were stained with rabbit anti-keratin IgG or anti-BrdU IgG, and immunopositive reactions were visualized using a streptavidin-biotin-peroxidase staining kit (Nichirei Co. Inc., Tokyo, Japan) according to the manufacturer's instructions. Morphometric analysis was performed using MacSCOPE Ver2.61 software. Statistical analysis was performed using Student's *t*-test.

Results

HB-EGF mRNA induction after in vitro scrape wound

To investigate the distinct role of HB-EGF in skin wound healing of the growth factors produced by NHEK, we first examined the induction of EGFR-ligand mRNA in NHEK in an in vitro wound-healing model. Confluent cultures of NHEK were scraped with a yellow pipette tip; total RNA was harvested at several time points, and the expression of growth-factor mRNAs was analyzed by RT-PCR. HB-EGF mRNA was rapidly induced after scraping, reaching a peak of 2.6-fold induction at 1 hour, whereas AR, TGF-α and EPR mRNAs were only slightly induced, with a maximum 1.5-fold increase (Fig. 1A). This indicates that HB-EGF is the most inducible gene of the EGFR ligands in NHEK. In normal mouse keratinocytes, HB-EGF was again the EGF family member that was induced predominantly after scraping, with a maximum 4.0-fold increase at 2 hours (Fig. 1B). EPR was also induced to a lesser degree, with a maximum 2.5-fold induction. TGF-α and AR were not induced after scraping. These results indicated that HB-EGF may play an important role in skin wound healing, and led us to investigate the in vivo function of HB-EGF.

Generation of keratinocyte-specific HB-EGF-deficient mice

Since germline targeting of the HB-EGF gene resulted in severe lethality (Iwamoto et al., 2003), we generated keratinocyte-specific HB-EGF-deficient mice ($HB^{lox/lox}$: K5-Cre, which we refer to as $HB^{-/-}$) using Cre/loxP technology in combination with the keratin 5 promoter (Takeda et al., 2000). $HB^{-/-}$ mice were identified by PCR analysis (Fig. 2A-C). The keratinocyte-specific absence of HB-EGF mRNA in $HB^{-/-}$

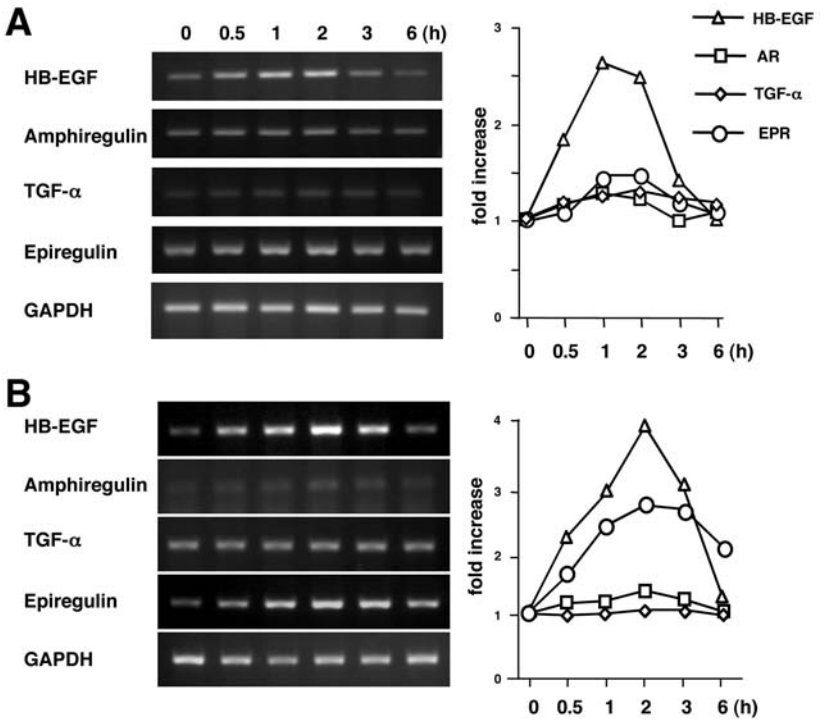

Fig. 1. Induction of expression of EGFR ligand mRNA, by scraping, in human and mouse keratinocytes. Confluent NHEK (A) and normal mouse epidermal keratinocytes (B) were scraped with a pipette tip; total RNA was harvested at several time points and mRNA expression was analyzed by RT-PCR. Right panels show densitometric analysis. In both cell types HB-EGF mRNA was rapidly and dramatically induced after scraping, whereas TGF-α, AR, and EPR mRNA were slightly induced in NHEK and the normal keratinocytes, although in the latter cells EPR was also increased.

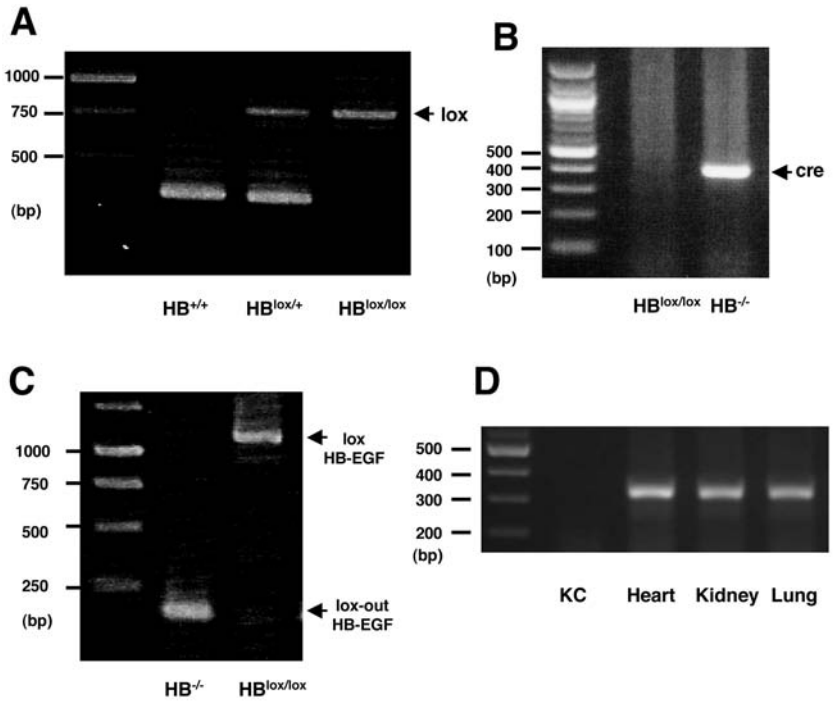

Fig. 2. Genotype of the keratinocyte-specific HB-EGF-deficient mice. (A-C) Keratinocyte-specific HB-EGF-deficient mice were confirmed by PCR as lox homozygous (A), Cre-recombinase positive (B) and lox-out (C). (D) Keratinocyte-specific disruption of HB-EGF mRNA in $HB^{-/-}$ mice was confirmed by RT-PCR. KC, keratinocytes.

mice was confirmed by RT-PCR (Fig. 2D). No apparent abnormalities were observed in the $HB^{-/-}$ mice.

Impaired wound healing in keratinocyte-specific HB-EGF-deficient mice

To examine the role of HB-EGF in skin wound healing in vivo, we performed a wound-healing assay using $HB^{lox/lox}$ and $HB^{-/-}$ mice. Two 6-mm punch skin biopsies were made in the back of each mouse and the wound diameter was measured at various times after wounding as a measure of healing. There was no difference in wound diameter up to day 3 post-wounding; however, wound healing was noticeably retarded from day 5 to 11 in the $HB^{-/-}$ mice. Wound closure was delayed significantly in $HB^{-/-}$ mice compared with $HB^{lox/lox}$ mice on day 8 (Fig. 3A). The wound diameter was reduced to 34% in $HB^{lox/lox}$ mice on day 8, whereas it was still 58% in the $HB^{-/-}$ mice (Fig. 3B). These results indicate that HB-EGF expression by keratinocytes is important for skin wound healing in vivo.

Cell proliferation was not impaired at the wound site in HB–/– mice

Since EGFR ligands promote NHEK proliferation and migration (Hashimoto, 2000), we investigated whether proliferation or migration was predominantly impaired in $HB^{-/-}$ mice. We measured the cell numbers in the leading edge of the biopsy wound and in the peripheral skin (1.2 mm from the wound margin) in $HB^{lox/lox}$ and $HB^{-/-}$ mice (Fig. 4A). After 48 hours, the total cells numbers were 90±13 and 65±19 in $HB^{lox/lox}$ and $HB^{-/-}$ mice, respectively, and after 72 hours 170±20 and 140±41 in $HB^{lox/lox}$ and $HB^{-/-}$, respectively (Fig. 4B). Since these small observed differences in cell numbers were not statistically significant, we investigated keratinocyte proliferation in $HB^{lox/lox}$ and $HB^{-/-}$ mice using a BrdU incorporation assay. Two hours before sacrifice, the mice received intraperitoneal injections of BrdU (250 μg/g). Skin samples were harvested, sectioned and stained with anti-BrdU antibody. Three days after wounding, there were no differences in the number or distribution of BrdU-positive cells between the $HB^{lox/lox}$ and $HB^{-/-}$ mice (Fig. 4C). These results suggest that delayed wound healing in $HB^{-/-}$ mice is not due to impaired cell proliferation in the epidermis.

Cell migration was impaired at the wound site in $HB^{-/-}$ mice

Since no impairment of proliferation was found, we next investigated whether migration was impaired in $HB^{-/-}$ mice. To quantify the migration

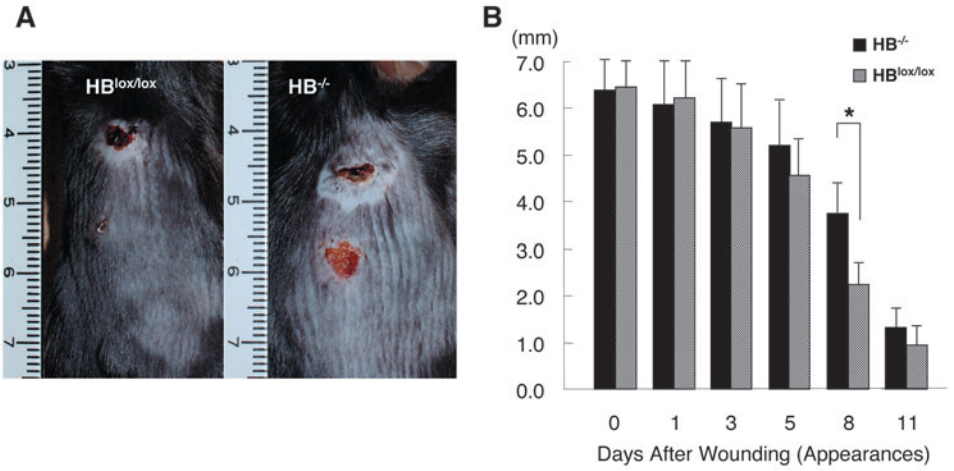

Fig. 3. Impaired wound healing in $HB^{-/-}$ mice. Two 6-mm punch biopsies were made in the skin of the backs of $HB^{lox/lox}$ and $HB^{-/-}$ mice, and wound diameter was monitored. (A) Macroscopic view of wound healing assay in $HB^{lox/lox}$ and $HB^{-/-}$ mice at day 8. (B) Measurements of wound diameter during healing. $^{*}P<0.05$.

of keratinocytes in wound healing, we measured the length of the leading edge in each wound of $HB^{lox/lox}$ and $HB^{-/-}$ mice in the wound-healing assay. Sections of skin from the wound area were stained with anti-keratin IgG (Fig. 5A). On day 7 post-wounding, the epidermis had migrated toward the center of the wound in $HB^{lox/lox}$ mice, whereas keratinocytes remained near the wound margin and the epidermis had not spread in $HB^{-/-}$ mice, suggesting that keratinocyte migration was impaired in $HB^{-/-}$ mice (Fig. 5B). We then prepared skin sections from all the samples from the wound-healing assay and calculated the ratio of leading edge to initial wound length, using computer-assisted morphometric analysis. On day 3 post-wounding, there was no difference in the leading edge ratio between $HB^{lox/lox}$ and $HB^{-/-}$ mice. However, the leading edge ratio was decreased markedly in $HB^{-/-}$ mice after day 3. The ratio was 30.7% in $HB^{-/-}$ and 44.5% in $HB^{lox/lox}$ on day 5, and 38% in $HB^{-/-}$ and 65% in $HB^{lox/lox}$ mice on day 7 (Fig. 5C). The difference on day 7 was statistically significant. These results suggest that endogenous HB-EGF is an important growth factor for the migration of epidermis in skin wound healing.

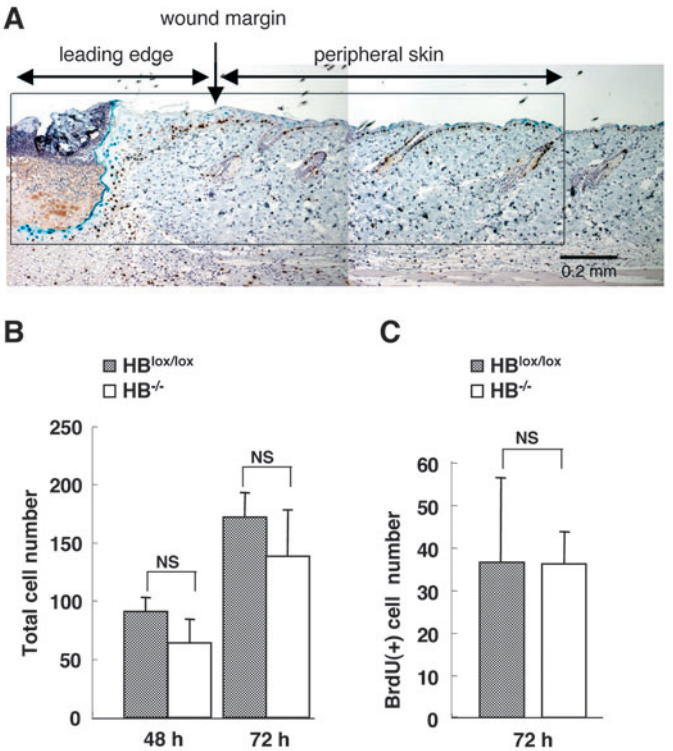

Fig. 4. BrdU-positive cell distribution at the leading edge and in the peripheral skin in $HB^{lox/lox}$ and $HB^{-/-}$ mice. (A) Skin sections were stained using anti-BrdU antibody, and cell numbers in the leading edge and in the peripheral skin were counted as indicated. (B) The total cell numbers in the peripheral skin (1.2 mm from the wound margin) and in the leading edge. (C) BrdU-positive cell number in the peripheral skin and in the leading edge. There were no differences in BrdU-positive cell numbers between $HB^{lox/lox}$ and $HB^{-/-}$ mice.

Expression of HB-EGF at wound sites

It has been reported that HB-EGF was upregulated in burn wound healing and that topical application of HB-EGF accelerated re-epithelialization of partial-thickness burns (Cribbs et al., 2002; Cribbs et al., 1998; McCarthy et al., 1996). It has been also reported that addition of HB-EGF into c-jun null keratinocyte growth medium can rescue the migration defect and induce phosphorylation of EGF receptor (Li et al., 2003). Since HB-EGF may play an important role in skin wound healing, we investigated the HB-EGF expression and keratinocyte proliferation pattern in skin wound healing using $HB^{lox/+}$:K5-Cre ($HB^{+/-}$) mice. With the targeting vector containing the *lacZ* gene as a reporter for the expression of HB-EGF, it is possible to ascertain the expression of HB-EGF by staining for β-gal in $HB^{+/-}$ mice. HB-EGF was expressed at the leading edge of the epithelium at day 2 post-wounding, and was predominantly expressed at the tip of the leading edge until day 7 (Fig. 6A). Unlike the HB-EGF expression pattern, BrdU-positive (BrdU+) cells were detected mainly within the peripheral skin on days 2 and 3. On days 5 and 7, BrdU+ cells were found toward the leading edge, although they were preferentially located near the wound margin. To quantify the distribution of HB-EGF-expressing cells and proliferating cells, we counted the β-gal-positive (β-gal+) cells and BrdU+ cells in 0.2 mm ranges in the leading edge and in the peripheral skin in $HB^{+/-}$ mice. On day 2 the peak of the β-gal+ cells was between 0 +0.2 mm into the leading edge, whereas the peak of the BrdU+ cells was –0.2 to –0.4 mm into the peripheral skin. On day 3, the peak of the β-gal+ cells was between +0.4 and +0.6 mm, whereas the peak of the BrdU+ cells was between 0

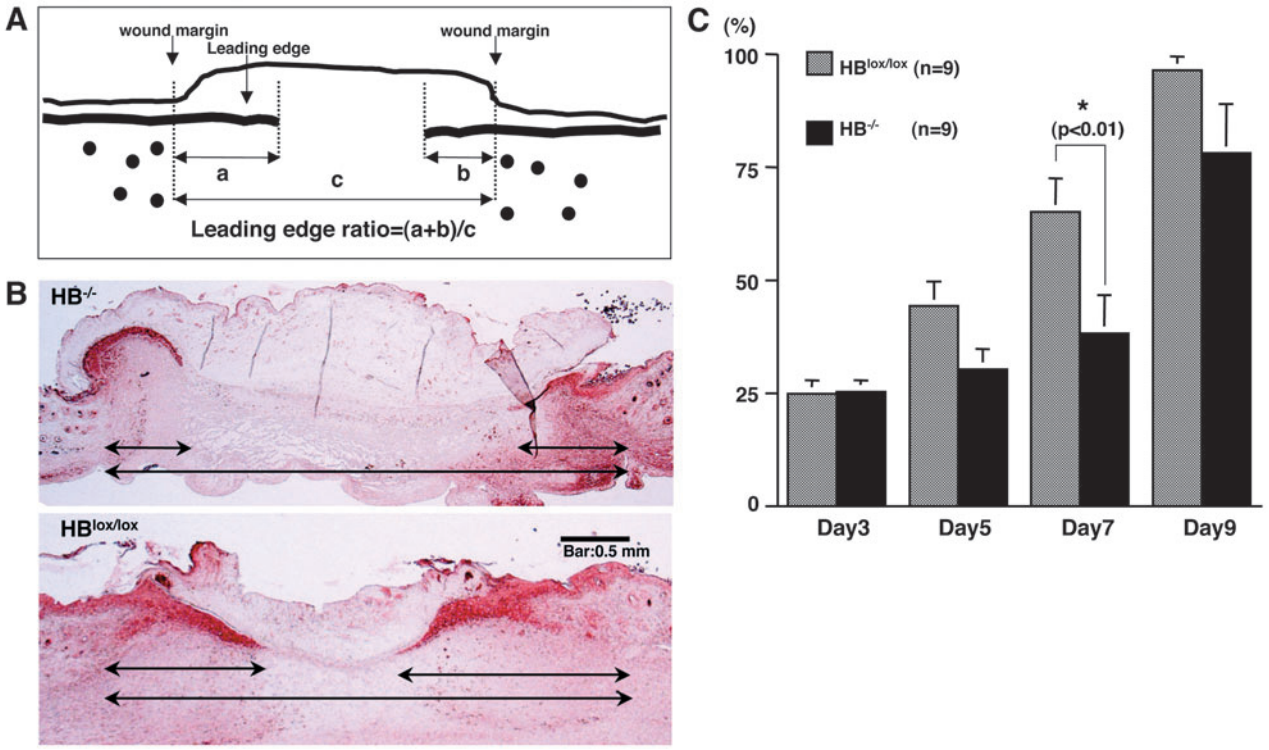

Fig. 5. Impaired keratinocyte migration in $HB^{-/-}$ mice. (A) Serial sections were prepared, and the epidermis was stained with anti-keratin antibody. Computer-assisted morphometric analysis was performed and the ratio of the leading edge to initial wound length was calculated. (B) Immunohistochemical staining of wound healing assay at day 7. Scale bar: 500 μm. (C) Measurements of leading edge ratio in $HB^{lox/lox}$ and $HB^{-/-}$ mice. The leading edge ratio was significantly decreased in $HB^{-/-}$ mice (n=9) at day 7. *P<0.01.

and +0.2 mm. From day 5 to day 7, BrdU+ cells were located at the leading edge, although they always appeared just behind the β-gal+ cells (Fig. 6B,C).

We also examined β-gal+ and BrdU+ cells in the wound-healing assay using $HB^{-/-}$ mice. As in $HB^{+/-}$ mice, in $HB^{-/-}$ mice, on day 2 post-wounding, β-gal+ cells were localized mostly 0 to +0.2 mm into the leading edge, whereas BrdU+ cells were detected mostly between –0.2 and –0.4 mm into the peripheral skin. On day 3, the peak of the β-gal+ cells were localized at 0 to +0.2 mm, whereas the peak of the BrdU+ cells were between 0 and –0.2 mm. On days 5 and 7, the peak aggregation of BrdU+ cells in $HB^{-/-}$ mice was at almost the same location as the β-gal+ cells, at the leading edge (Fig. 6D,E). The overlapping distribution patterns of BrdU+ and β-gal+ cells in these mice may be due to the impaired cell migration (Fig. 5B,C). However, the total counts of BrdU+ cells were similar in $HB^{lox/lox}$ and $HB^{-/-}$ mice (Fig. 4B,C).

Discussion

Wound healing is a complex process involving a number of coordinated events including inflammation, cell migration, cell proliferation, matrix production and angiogenesis (Singer and Clark, 1999). A complex array of cells, growth factors, cytokines and matrix components are involved in wound healing, and a number of transgenic and knockout mouse models have revealed the contribution of several molecules to wound healing (Scheid et al., 2000). Impaired wound healing was observed in mice transgenic for several molecules, such as BMP-6, follistatin, truncated FGF receptor and thorombospondin-1, as well as in mice with activin, β1 integrin, and TGF-β1 knockouts, among others (Grose and Werner, 2003; Scheid et al., 2000; Werner and Grose, 2003). EGF family members such as EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, epiregulin and their receptor EGFR mainly regulate migration, proliferation and differentiation of many cell types involved in wound healing. EGFR knockout mice showed striking abnormalities, such as wavy hair and thin skin (Miettinen et al., 1995; Sibilia and Wagner, 1995). In contrast to this striking phenotype in EGFR knockout mice, EGF-disrupted mice showed no phenotypic abnormalities (Luetteke et al., 1999). No differences in wound healing were found in TGF-α knockout mice with either excisional dorsal wounding or ear-punch wounding (Luetteke et al., 1993; Mann et al., 1993). These unexpectedly minor differences in phenotypes in wound healing in EGFR-ligand knockout mice are probably due to the known functional redundancy among the EGF family members, including HB-EGF.

HB-EGF is produced and secreted by human keratinocytes and acts as an autocrine growth factor (Hashimoto et al., 1994). HB-EGF mRNA was rapidly and dramatically induced after scrape-wounding, although slight increases in TGF-α, amphiregulin and epiregulin mRNAs were observed. Furthermore, blocking HB-EGF by addition of neutralizing antibody to the medium inhibited keratinocyte migration (Tokumaru et al., 2000). In contrast, the addition of recombinant HB-EGF to the medium accelerates keratinocyte migration (Tokumaru et al., 2000). These results indicate that

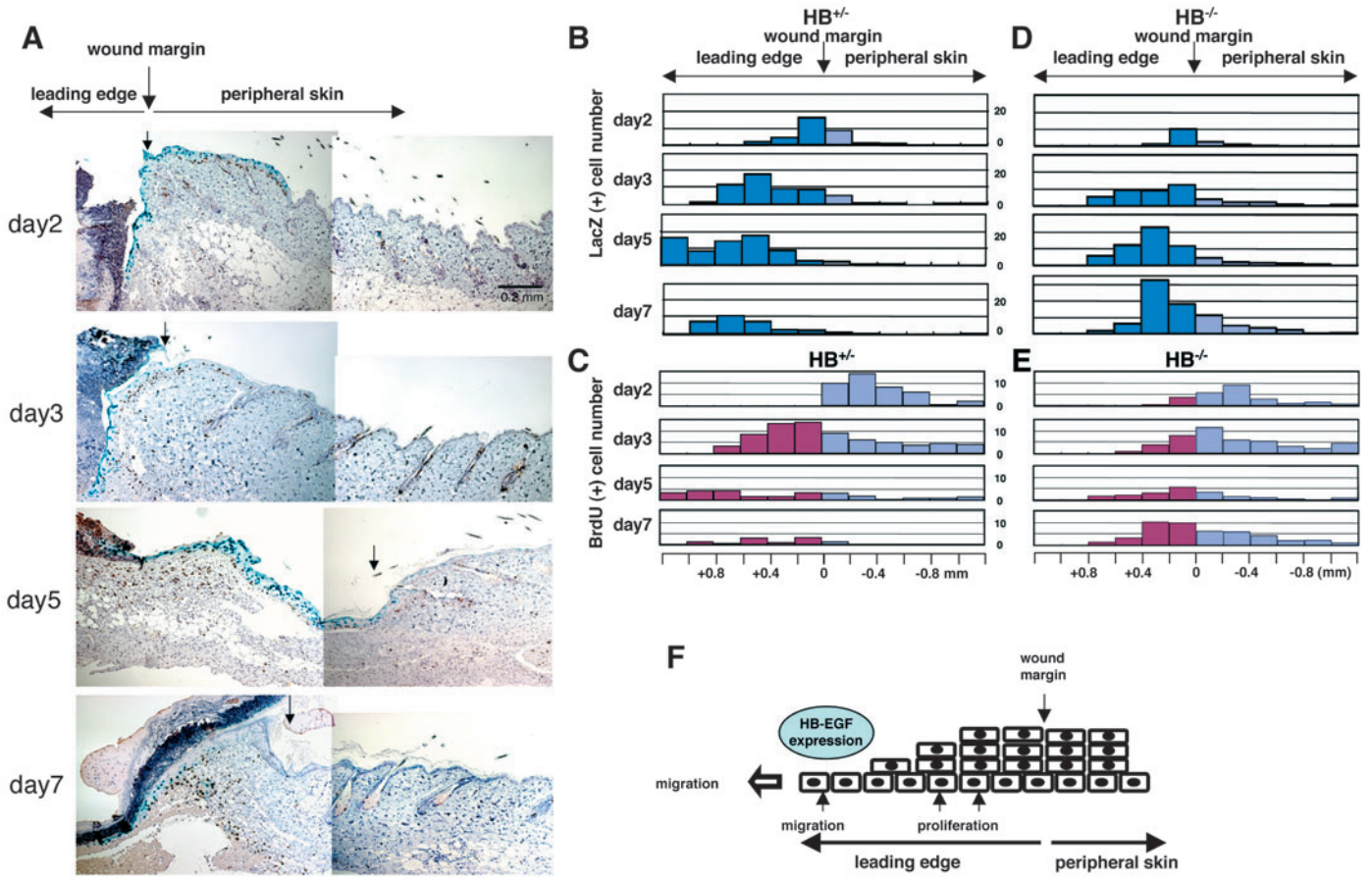

Fig. 6. HB-EGF expression in skin wound healing. The targeting vector contained the *lacZ* gene as a reporter for the expression of HB-EGF. When HB-EGF cDNA is deleted by Cre-recombinase, HB-EGF expression can be identified by X-gal staining in $HB^{+/-}$ mice. (A) Double staining for X-gal and BrdU at the wound healing stage. Scale bar: 0.2 mm. Arrow, wound margin. (B-E) Distribution of β-gal-positive [lacZ(+)] cells (B) and BrdU-positive cells (C) from day 2 to 7 in $HB^{+/-}$ mice. HB-EGF is expressed predominantly at the tip of the leading edge, whereas BrdU-positive cells were distributed mainly at wound margin. Distribution of β-gal-positive cells (D) and BrdU-positive cells (E) in $HB^{-/-}$ mice. There was no significant difference in the number of BrdU-positive cells between $HB^{+/-}$ and $HB^{-/-}$ mice at any stage of wound healing. (F) A proposed schematic illustration of skin wound healing. After injury, keratinocytes at the wound margin begin to migrate and express HB-EGF without proliferation. Next, focal release of HB-EGF may signal further migration and up-regulate HB-EGF expression in an autocrine manner. Values in B-E are number of cells per indicated area.

HB-EGF plays an important role in skin wound healing, and led us to investigate the in vivo function of HB-EGF. Since germline targeting of the HB-EGF gene resulted in embryonic lethality (Iwamoto et al., 2003), we generated keratinocyte-specific HB-EGF-deficient mice ($HB^{-/-}$) using Cre/loxP technology in combination with the keratin 5 promoter (Takeda et al., 2000). There was no difference in wound closure between $HB^{-/-}$ and $HB^{lox/lox}$ mice on day 3. However, wound closure was markedly retarded in $HB^{-/-}$ mice compared to $HB^{lox/lox}$ mice. We clearly demonstrated for the first time that endogenous HB-EGF is the most important growth factor in the epithelialization of skin wound healing in vivo, using keratinocyte-specific HB-EGF-deficient mice.

EGF family members are well known to promote keratinocyte growth in vitro (Hashimoto, 2000). It has been reported that TGF-α, amphiregulin, HB-EGF and epiregulin are autocrine growth factors in normal human keratinocytes (Coffey et al., 1987; Cook et al., 1991; Hashimoto et al., 1994; Shirakata et al., 2000). In vitro observation suggests that these EGF family members play important roles in development, epidermal morphogenesis, skin homeostasis and wound healing. In this study, we investigated HB-EGF function in cell migration and proliferation. HB-EGF stimulates keratinocyte migration in vitro and in vivo. However, there was little difference in proliferation between $HB^{lox/lox}$ and $HB^{-/-}$ mice. HB-EGF promoter activity was up-regulated at the migrating epidermal edge, whereas the distribution of proliferating cells (BrdU-positive) was not identical to that of HB-EGF mRNA-positive cells. Interestingly, the wound margin of normal epidermis expressed HB-EGF mRNA and was positive for BrdU, although HB-EGF promoter activity could not be detected in normal skin far from the wound margin. Therefore, normal skin does not require much HB-EGF, but after injury HB-EGF is induced and plays a crucial role in wound healing by up-regulating keratinocyte migration but not proliferation.

Combined, the evidence suggests that the synthesis of HB-EGF at the leading epithelial edge stimulates cells, via an autocrine loop, to migrate towards the center of the wound rather than to proliferate. Interestingly, there were few β-gal-positive cells and little HB-EGF expression in normal skin far

from the wound margin. Therefore, HB-EGF expression induced by wounding might itself stimulate further expression of HB-EGF at the leading edge via an autocrine loop. Fig. 6F shows a schematic illustration of our proposed skin wound healing mechanism. After injury, keratinocytes at the wound margin begin to express HB-EGF and migrate toward the wound site without proliferating. Next, the focal release of HB-EGF may trigger the migration of additional cells, rather than cell proliferation at the leading edge. Therefore, we conclude that HB-EGF is rapidly induced after injury and plays an important role in wound healing by up-regulating keratinocyte migration.

Nuclear transcription factors play important roles in almost all biological events resulting from growth factor signaling, and several nuclear transcription factors are thought to be involved in skin wound healing. Several mouse models with gene-targeted disruption of nuclear transcriptional factors have been analyzed for skin wound healing. Sano et al. (Sano et al., 1999) reported severe retardation of wound healing in keratinocyte-specific STAT3 knockout mice. D'Souza et al. (D'Souza et al., 2002) reported impaired skin wound healing in E2F-1 knockout mice. Recently, the development of keratinocyte-specific c-jun knockout mice was reported (Li et al., 2003; Zenz et al., 2003). These mice showed retarded wound healing, and the activation of EGFR was greatly decreased (Li et al., 2003). Since EGF itself is not produced by keratinocytes, the autocrine loop consisting of HB-EGF, EGFR and c-jun might be one of the major regulatory signal transduction mechanisms in skin wound healing.

In conclusion, HB-EGF is an important growth factor in epithelialization during skin wound healing in vivo, and acts mainly by stimulating migration, rather than proliferation, of keratinocytes.

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References

Coffey, R. J., Jr, Derynck, R., Wilcox, J. N., Bringman, T. S., Goustin, A. S., Moses, H. L. and Pittelkow, M. R. (1987). Production and auto-induction of transforming growth factor-alpha in human keratinocytes. *Nature* **328**, 817-820.

Cook, P. W., Mattox, P. A., Keeble, W. W., Pittelkow, M. R., Plowman, G. D., Shoyab, M., Adelman, J. P. and Shipley, G. D. (1991). A heparin sulfate-regulated human keratinocyte autocrine factor is similar or identical to amphiregulin. *Mol. Cell. Biol.* **11**, 2547-2557.

Cribbs, R. K., Luquette, M. H. and Besner, G. E. (1998). Acceleration of partial-thickness burn wound healing with topical application of heparin-binding EGF-like growth factor (HB-EGF). *J. Burn Care Rehabil.* **19**, 95-101.

Cribbs, R. K., Harding, P. A., Luquette, M. H. and Besner, G. E. (2002). Endogenous production of heparin-binding EGF-like growth factor during murine partial-thickness burn wound healing. *J. Burn Care Rehabil.* **23**, 116-125.

D'Souza, S. J., Vespa, A., Murkherjee, S., Maher, A., Pajak, A. and Dagnino, L. (2002). E2F-1 is essential for normal epidermal wound repair. *J. Biol. Chem.* **277**, 10626-10632.

Erickson, S. L., O'Shea, K. S., Ghaboosi, N., Loverro, L., Frantz, G., Bauer, M., Lu, L. H. and Moore, M. W. (1997). ErbB3 is required for normal cerebellar and cardiac development: a comparison with ErbB2-and heregulin-deficient mice. *Development* **124**, 4999-5011.

Falls, D. L. (2003). Neuregulins: functions, forms, and signaling strategies. *Exp. Cell Res.* **284**, 14-30.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.

Grose, R. and Werner, S. (2003). Wound healing studies in transgenic and knockout mice. A review. *Methods Mol. Med.* **78**, 191-216.

Harari, D., Tzahar, E., Romano, J., Shelly, M., Pierce, J. H., Andrews, G. C. and Yarden, Y. (1999). Neuregulin-4: a novel growth factor that acts through the ErbB-4 receptor tyrosine kinase. *Oncogene* **18**, 2681-2689.

Hashimoto, K. (2000). Regulation of keratinocyte function by growth factors. *J. Dermatol. Sci.* **24**, S46-S50.

Hashimoto, K., Higashiyama, S., Asada, H., Hashimura, E., Kobayashi, T., Sudo, K., Nakagawa, T., Damm, D., Yoshikawa, K. and Taniguchi, N. (1994). Heparin-binding epidermal growth factor-like growth factor is an autocrine growth factor for human keratinocytes. *J. Biol. Chem.* **269**, 20060-20066.

Iwamoto, R., Yamazaki, S., Asakura, M., Takashima, S., Hasuwa, H., Miyado, K., Adachi, S., Kitakaze, M., Hashimoto, K., Raab, G. et al. (2003). Heparin-binding EGF-like growth factor and ErbB signaling is essential for heart function. *Proc. Natl. Acad. Sci. USA* **100**, 3221-3226.

Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P., Ward, C. W. and Burgess, A. W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp. Cell Res.* **284**, 31-53.

Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C. and Hauser, C. (1995). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398.

Li, G., Gustafson-Brown, C., Hanks, S. K., Nason, K., Arbeit, J. M., Pogliano, K., Wisdom, R. M. and Johnson, R. S. (2003). c-Jun is essential for organization of the epidermal leading edge. *Dev. Cell* **4**, 865-877.

Luetteke, N. C., Qiu, T. H., Peiffer, R. L., Oliver, P., Smithies, O. and Lee, D. C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* **73**, 263-278.

Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A. and Lee, D. C. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* **126**, 2739-2750.

Mann, G. B., Fowler, K. J., Gabriel, A., Nice, E. C., Williams, R. L. and Dunn, A. R. (1993). Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* **73**, 249-261.

Marikovsky, M., Breuing, K., Liu, P. Y., Eriksson, E., Higashiyama, S., Farber, P., Abraham, J. and Klagsbrun, M. (1993). Appearance of heparin-binding EGF-like growth factor in wound fluid as a response to injury. *Proc. Natl. Acad. Sci. USA* **90**, 3889-3893.

McCarthy, D. W., Downing, M. T., Brigstock, D. R., Luquette, M. H., Brown, K. D., Abad, M. S. and Besner, G. E. (1996). Production of heparin-binding epidermal growth factor-like growth factor (HB-EGF) at sites of thermal injury in pediatric patients. *J. Invest. Dermatol.* **106**, 49-56.

Meyer, D. and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390.

Miettinen, P. J., Berger, J. E., Meneses, J., Phung, Y., Pedersen, R. A., Werb, Z. and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376**, 337-341.

Murillas, R., Larcher, F., Conti, C. J., Santos, M., Ullrich, A. and Jorcano, J. L. (1995). Expression of a dominant negative mutant of epidermal growth factor receptor in the epidermis of transgenic mice elicits striking alterations in hair follicle development and skin structure. *EMBO J.* **14**, 5216-5223.

Riethmacher, D., Sonnenberg-Riethmacher, E., Brinkmann, V., Yamaai, T., Lewin, G. R. and Birchmeier, C. (1997). Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nature* **389**, 725-730.

Sano, S., Itami, S., Takeda, K., Tarutani, M., Yamaguchi, Y., Miura, H., Yoshikawa, K., Akira, S. and Takeda, J. (1999). Keratinocyte-specific ablation of Stat3 exhibits impaired skin remodeling, but does not affect skin morphogenesis. *EMBO J.* **18**, 4657-4668.

Scheid, A., Meuli, M., Gassmann, M. and Wenger, R. H. (2000). Genetically modified mouse models in studies on cutaneous wound healing. *Exp. Physiol.* **85**, 687-704.

Shirakata, Y., Komurasaki, T., Toyoda, H., Hanakawa, Y., Yamasaki, K., Tokumaru, S., Sayama, K. and Hashimoto, K. (2000). Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes. *J. Biol. Chem.* **275**, 5748-5753.

Shirakata, Y., Tokumaru, S., Yamasaki, K., Sayama, K. and Hashimoto, K. (2003). So-called biological dressing effects of cultured epidermal sheets

are mediated by the production of EGF family, TGF-beta and VEGF. *J. Dermatol. Sci.* **32**, 209-215.

Sibilia, M. and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**, 234-238.

Singer, A. J. and Clark, R. A. (1999). Cutaneous wound healing. *New Engl. J. Med.* **341**, 738-746.

Stoll, S., Garner, W. and Elder, J. (1997). Heparin-binding ligands mediate autocrine epidermal growth factor receptor activation In skin organ culture. *J. Clin. Invest.* **100**, 1271-1281.

Takeda, J., Sano, S., Tarutani, M., Umeda, J. and Kondoh, G. (2000). Conditional gene targeting and its application in the skin. *J. Dermatol. Sci.* **23**, 147-154.

Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J. I., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y. et al. (2000). Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. *J. Cell Biol.* **151**, 209-220.

Werner, S. and Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiol. Rev.* **83**, 835-870.

Zenz, R., Scheuch, H., Martin, P., Frank, C., Eferl, R., Kenner, L., Sibilia, M. and Wagner, E. F. (2003). c-Jun regulates eyelid closure and skin tumor development through EGFR signaling. *Dev. Cell.* **4**, 879-889.