Erythroid differentiation regulator 1 promotes wound healing by inducing the production of C-C motif chemokine ligand 2 via the activation of MAP kinases *in vitro* and *in vivo*

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Abstract. The erythroid differentiation regulator 1 (Erdr1) protein has been studied for its role in various inflammatory skin diseases, including skin cancer, actinic keratosis and psoriasis. However, the therapeutic effects of Erdr1 on wound repair and its underlying mechanisms remain unknown. The present study aimed to investigate the effects of Erdr1 on wound healing *in vitro* and *in vivo*. The results demonstrated that treatment with recombinant Erdr1 enhanced wound healing *in vivo* and *in vitro*. In addition, Erdr1 increased the proliferation and migration of human dermal fibroblasts (HDFs). Notably, Erdr1 significantly induced the production of the chemoattractant C-C motif chemokine ligand 2 (CCL2) and recruited immune cells involved in wound healing. Treatment with recombinant Erdr1 induced the activation of the ERK1/1, p38 and JNK1/2 mitogen-activated protein (MAP) kinases. Treatment with specific inhibitors for MAP kinase inhibitors markedly suppressed cell proliferation and migration, and inhibited the production of CCL2 in HDFs. Furthermore, the inhibition of CCL2 with a neutralizing antibody significantly suppressed the recombinant Erdr1-induced proliferation and migration of HDFs. The wound healing activity of Erdr1 was comparable to that of epidermal growth factor. Taken together, these results demonstrated that Erdr1 promoted the proliferation and migration of HDFs and exhibited potent wound healing properties mediated by CCL2. Therefore, the results of the present study suggested that Erdr1 may be a potential therapeutic target for promoting wound healing.

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Introduction

The processes that induce the restoration of injured tissues immediately after wound healing comprise three interrelated dynamic phases, namely, the inflammatory, proliferative and regeneration phases (1,2). A number of studies have demonstrated that the primary resident fibroblasts in the dermis are the key players that maintain skin homeostasis during wound healing (3-5). Dermal fibroblasts migrate to the wound site and are induced to proliferate by a variety of growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor β (3,6,7). Therefore, the migration and proliferation of fibroblasts need to be precisely regulated during wound healing.

Previous studies have reported that a variety of chemokines are expressed at the wound site, indicating the important role of chemokines in recruiting inflammatory cells to the wound site (6,8-10). Notably, the monocyte chemoattractant protein-1, also referred to as C-C motif chemokine ligand 2 (CCL2), is one of the chemokines that mediate the infiltration of monocytes and macrophages during wound healing (11-15). Additionally, treatment with CCL2 improves wound healing by enhancing the recruitment of myeloid cells in *db/db* mice, a Toll-like receptor 3-deficient mouse model in which the secretion of CCL2 is impaired (16,17). Therefore, understanding the role of CCL2 is important for aiding wound healing and developing novel therapeutic agents.

The erythroid differentiation regulator 1 (Erdr1) is a highly conserved autocrine factor that can induce the synthesis of hemoglobin in both human and murine erythroleukemia (18). Erdr1 is expressed in various tissues, including the placenta, liver, brain, lung, intestine, bone marrow, thymus, sebaceous glands, vessels, nerves, normal human epidermis and human keratinocytes (18-20). A number of studies have reported that Erdr1 exhibits anticancer effects in various types of cancer, including gastric cancer and melanoma. For example, Erdr1 is an antimetastatic factor that is negatively regulated by IL-18 via downregulation of the expression of heat shock protein 90 in melanoma (21). Additionally, recombinant Erdr1 has been

reported to suppress the invasiveness and motility of gastric cancer cells via the JNK pathway (19). Erdr1 exhibits a therapeutic potential for various inflammatory diseases, including psoriasis, rosacea, hair loss disorders and rheumatoid arthritis (20,22-26). We therefore hypothesized that Erdr1 may promote the migration and proliferation of fibroblasts involved in wound healing. The mechanisms underlying the therapeutic effects of Erdr1 in wound healing are yet to be elucidated. The present study aimed to investigate the effects of Erdr1 on wound healing.

Materials and methods

Mice and cell culture. Female BALB/c mice (7-week-old; weight, 20-22 g) were purchased from Orient Bio, Inc. All experiments were performed following the ethical guidelines of the Korea University Institutional Animal Care and Use Committee (Seoul, Korea; approval no. KUIACUC-2018-0045). Human dermal fibroblasts (HDFs; Biosolution Co., Ltd.) were cultured in a mixture (3:1) of DMEM (Thermo Fisher Scientific, Inc) and F-12K with 10% fetal bovine serum (Capricorn Scientific GmbH), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific, Inc.) in an incubator at 37˚C with 5% CO_2. The passage number was <13 for all experiments.

Preparation of recombinant proteins. The recombinant mouse Erdr1 protein was prepared as previously reported (19,21). Briefly, the Erdr1-pCMV-SPORT6 plasmid was purchased from Open Biosystems, Inc. The region of the coding sequence was transferred into the bacterial expression plasmid pET22B (Merck KGaA). The 177 amino acid encoded Erdr1 protein was expressed in the *Escherichia coli* Top10 system (Invitrogen; Thermo Fisher Scientific, Inc.), purified using a Ni-NTA purification system according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.), quantified by Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Inc.), separated by 10% SDS-PAGE and visualized by Coomassie blue staining (Sigma-Aldrich; Merck KGaA) (purity >95%). The endotoxin level of the purified protein (<0.1 EU/ml) was measured using the LAL system (Associated of Cape Cod International, Inc.). The recombinant human EGF protein (purity >98%) was purchased from PeproTech, Inc.

Reagents and antibodies. Antibodies against tubulin (cat. no. sc-69969), JNK1/2 (cat. no. sc-571), phosphorylated (p-) JNK1/2 (cat. no. sc-6254), ERK1/2 (cat. no. sc-153), p-ERK1/2 (cat. no. sc-7383) and p38 (cat. no. sc-535) were purchased from Santa Cruz Biotechnology, Inc. Rabbit-HRP (cat. no. 7074), mouse-HRP (cat. no. 7076) and p-p38 (cat. no. 9216) antibodies were purchased from Cell Signaling Technology, Inc. Human CCL2 ELISA pair set (cat. no. SEK10134) was purchased from Sino Biological, Inc. Pluronic® F-127 (cat. no. P2443) for the preparation of 22% (w/v) hydrogel was purchased from Sigma Aldrich; Merck KGaA and dissolved in saline overnight on the rotator at 4˚C. Inhibitors for ERK (U0126; cat. no. 662005), p38 (SB203580; cat. no. S8307) and JNK (SP600125; cat. no. S5567) were purchased from Merck KGaA.

Proliferation assay. HDFs were seeded and pre-cultured into 96-well plates at a density of $5x10^3$ cells/well for 24 h at 37˚C in a 5% CO_2 incubator. Subsequently, the cells were cultured without serum for 16 h at 37˚C and pre-treated with 1, 10 or 25 μg/ml mitomycin-C (MMC) or PBS for 1 h at 37˚C. HDFs were washed and treated with 1, 10 or 50 ng/ml Erdr1 or EGF as a positive control for 24 h at 37˚C in serum-free culture medium. To evaluate the effects of the CCL2-neutralizing antibody (Sino Biological, Inc.) on cell proliferation, HDFs were treated with 50 ng/ml Erdr1 or PBS for 24 h following pretreatment with 1, 10 or 30 μg/ml CCL2-neutralizing antibody. After 24 h, the cells were treated with 20 μl of 100 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 2 h, and the medium was removed. The precipitated formazan was dissolved in 200 μl DMSO, and the absorbance at 560 nm was determined by a microplate reader.

In vitro migration assay. HDFs were seeded into 12-well plates at a density of $8x10^4$ cells/well and pre-cultured for 24 h, followed by culture with serum-free medium for 16 h at 37˚C in 5% CO_2 incubator. The monolayers of HDFs at 80-90% confluency were scratched with a 200-μl pipette tip, and the plates were washed with PBS to remove the cell debris. HDFs were pre-treated with 1, 10 or 25 ng/ml MMC for 1 h at 37˚C, washed and stimulated with 50 ng/ml Erdr1 or 50 ng/ml EGF. To evaluate the effects of the CCL2-neutralizing antibody on cell migration, HDFs were treated with 50 ng/ml Erdr1 or PBS for 24 h following pretreatment with 1, 10 or 30 μg/ml CCL2-neutralizing antibody. After 24 h, the cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min and incubated with Rhodamine phalloidin for 1 h at room temperature. Fluorescence images (magnification, x100) were acquired using an Olympus IX 71 fluorescence microscope (Olympus Corporation).

Wound healing assay in vivo. Mouse dorsal hair was shaved and wiped with 70% ethanol before wounding. The mice were anesthetized with 65% N_2, 30% O_2 and 5% isoflurane and maintained with 2% isoflurane while a 5-mm wound puncture was performed. HG was not used for delivering the compounds to the wound sites after 4 days. The wounds were directly treated with saline, 1 μg EGF or 1 μg Erdr1 in 22% hydrogel (HG) once a day for 5 days. The wound closure was monitored by capturing images at the same position with a fixed height stand to ensure consistent image size. Measurement of wound area in the images was performed using ImageJ software (version 1.45; National Institutes of Health). The analysis of area measurement was performed by one person who was blinded to the grouping of the mice and the sampling day for each wound being measured to avoid measurement bias, and the percentage of wound size was calculated as follows (4,27): Wound closure (%)=(wound area on day 0-wound area on day of post-wounding)/wound area on day 0x100%.

Reverse transcription (RT)-PCR. HDFs were stimulated with 1, 10, 25, 50 or 100 ng/ml Erdr1 or EGF for 1, 3, 6, 9 or 12 h, and total RNA was extracted using a RiboEX total RNA kit (GeneAll Biotechnology Co., Ltd.). Total RNA (1 μg) was reverse-transcribed into cDNA by the AccuPower® RT Premix kit (BioNeer Corporation) according to the

manufacturer's instructions. The cDNAs were amplified with specific primers by PCR using Genie™ 32 Thermal Block (BioNeer Corporation). The specific primer sequences were as follows: CCL2, sense, 5'-CCTTGCCTTGCTGCTCTACC-3' and antisense, 5'-CCTATGTGCTGGCCTTGGTG-3'; and GAPDH sense, 5'-ACATCAAGAAGGTGGTGAAG-3' and antisense, 5'-ATTCAAGAGAGTAGGGAGGG-3'. The PCR products were separated on 1.5% agarose gels and stained using the StainingSTAR solution (Dynebio, Inc.).

ELISA. Human dermal fibroblasts were seeded at $2x10^5$ cells/well into 6-well plates and cultured overnight in serum-free medium for 24 h at 37˚C in a 5% CO_2 incubator. The cells were subsequently treated with 1, 10 or 50 ng/ml EGF or Erdr1 for 24 h at 37˚C. CCL2 protein levels were determined using a CCL2 matched ELISA kit (cat. no. SEK10134; Sino Biological, Inc.) according to the manufacturer's protocol. The absorbance at 450 nm was measured by a microplate reader.

Western blot analysis. Whole cell lysates were prepared using RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc.), and the protein concentration was quantified by BCA assay kit (Thermo Fisher Scientific, Inc.). A total of 30 μg of samples per lane were separated on 10% SDS-PAGE gels and transferred to a PVDF membrane. The membrane was blocked with 5% skim milk (Neogen Corporation) in 1X TBS-T (0.1% Tween-20) buffer. The membrane was washed twice with 1X TBS and incubated with the primary antibodies (1:1,000) in 5% BSA for 2 h at room temperature (Sigma-Aldrich; Merck KGaA)-TBS buffer. The membrane was washed six times with TBS-T buffer and incubated for 1 h at room temperature with the corresponding HRP-conjugated mouse or rabbit IgG secondary antibodies (1:5,000) in 5% skim milk-TBS. The target proteins were visualized using Amersham ECL Prime Western blotting Detection Regent (Cytiva).

Histologic analysis of wounds. Mice (n=5 mice/group) were sacrificed and skin tissues were harvest at day 6 post-wounding. Cross-sections (5 μm) of paraffin-embedded skin tissues were stained with hematoxylin for 5 min and eosin for 2 min (H&E). Stained tissues were mounted with mounting media (Thermo Fisher Scientific, Inc.) and observed under light microscopy (x100 magnification). The granulation tissue was identified by the presence of tissue matrix, immune cells, vascular tissue and fibroblasts, and the formation of granulation tissue area was defined as the area between underneath the neo-epithelium and above the subcutaneous fat tissue. The relative granulation tissue area ratios were calculated on the digital images using ImageJ based on the wound closure area, area of dermis and epidermis.

Statistical analysis. The data are presented as the mean ± standard deviation. All experiments were repeated at least three times independently. Statistical differences between the control and the treated groups were assessed by one-way analysis of variance followed by Tukey's post hoc test using SPSS 22 software (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Erdr1 promotes wound repair in vivo. To examine the effects of Erdr1 on wound closure, the wounds were treated with Erdr1-, EGF-, or saline-HG and analyzed. As demonstrated in Fig. 1A, the sizes of wounds in the Erdr1-HG or EGF-HG-treated mice were decreased between days 4 and 10 compared with those in the mice that were treated with saline-HG. The wound closure of the mice treated with Erdr1-HG or saline-HG was ~100% on day 10 (Fig. 1B). As previous studies have reported that the formation of granulation tissue in the early stage of a wound is an important step for supporting re-epithelialization and dermal reconstitution (28,29), the tissues from the wound site were collected on day 6 and stained with H&E for histological analysis; the results demonstrated that the formation of granulation tissues in the group that was treated with Erdr1-HG was similar to that of the group treated with EGF-HG and higher compared with that in the group treated with saline-HG (Fig. 1C). These results demonstrated that the promotion of wound healing of the Erdr1-HG-treated group was similar to that of EGF-HG treated group.

Erdr1 induces HDF proliferation and migration. To investigate the effects of Erdr1 on the proliferation and migration of HDFs, MTT and wound healing assays were performed. As demonstrated in Fig. 2A, 25-100 ng/ml Erdr1 significantly increased the proliferation of HDFs by ~25% compared with that of the PBS-treated control group. The proliferation of the HDFs that were treated with Erdr1 was comparable to that of the HDFs that were treated with 50 ng/ml EGF in the positive control group. To exclude the effects of Erdr1 on the proliferation of HDFs while evaluating the Erdr1-induced migration of HDF wound healing, subsequent assays were performed in the presence of MMC to block cell proliferation. As demonstrated in Fig. 2B, 25 μg/ml MMC completely inhibited cell proliferation, which was not observed at concentrations of 1 and 10 μg/ml. In addition, the proliferation of HDFs induced by 50 ng/ml EFG or 50 ng/ml Erdr1 was decreased by MMC (Fig. 2B).

The results of the wound healing assay demonstrated that Erdr1 increased the migration of HDFs (Fig. 2C). To further examine the effects of Erdr1 on the migration of HDFs, the wound healing assay was performed in the presence of MMC; as presented in Fig. 2D, 1 and 10 μg/ml MMC did not inhibit the migration of Erdr1-treated HDFs. However, 25 μg/ml MMC slightly inhibited the Erdr1-induced migration of HDFs (Fig. 2D). These results suggested that Erdr1 may promote wound healing by inducing the proliferation and migration of HDFs.

Erdr1 increases the production of CCL2 in HDFs. To examine the effects of Erdr1 on the expression of CCL2, HDFs were cultured with various concentrations of Erdr1 or EGF (positive control) for different durations. The mRNA and protein levels of CCL2 were determined using RT-PCR and ELISA, respectively. As presented in Fig. 3A, treatment with 50 and 100 ng/ml Erdr1 increased the mRNA expression levels of CCL2. In addition, the mRNA expression of CCL2 induced by 50 ng/ml Erdr1 appeared to increase over time (Fig. 3B). In order to confirm the effects of Erdr1 on the production

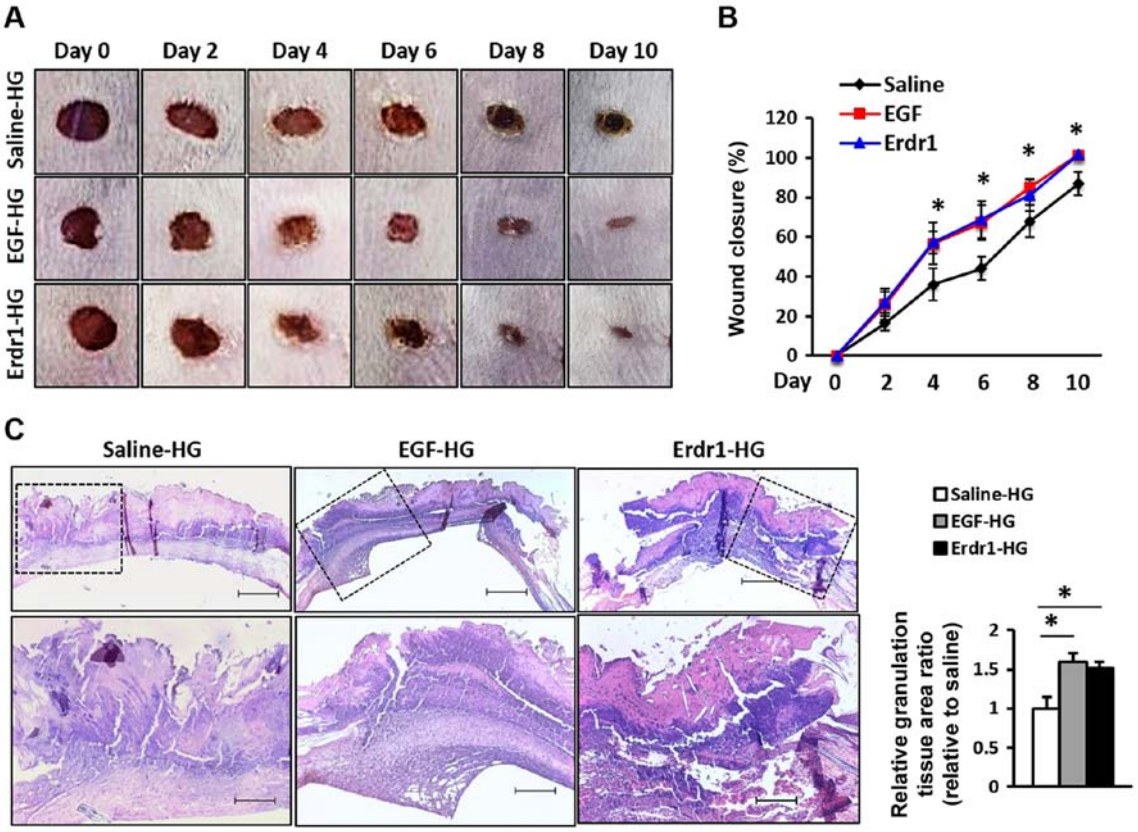

Figure 1. Treatment with Erdr1 accelerates wound healing *in vivo*. (A) Representative images of the wounds in mice treated with Erdr1-HG, EGF-HG or control saline-HG every 24 h for 5 days. The images represent the status of wound repair during 10 days of wound healing. (B) The wound closure in mice treated with saline-HG, Erdr1-HG, or EGF-HG was measured digitally on the indicated days after the infliction of injury, and the wound closure was calculated as a percentage. (C) Tissues obtained from the wound sites on day 6 after inflicting the wounds were stained with H&E, and the formation of granulation tissues was analyzed. The relative granulation in the tissues was calculated and compared with that of the group treated with saline-HG. Magnification, x40 (upper panel) and x100X (lower panel); scale bar, 200 μm. n=6. *P<0.01 vs. saline-HG. Erdr1, erythroid differentiation regulator 1; EGF, epidermal growth factor; HG, hydrogel.

of CCL2, ELISA was performed to detect the production of CCL2 in HDFs following stimulation with Erdr1 or EGF. As demonstrated in Fig. 3C, following HDF stimulation with Erdr1, the secretion of CCL2 significantly increased compared with that in the control cells. These results suggested that the Erdr1-induced production of CCL2 may serve a crucial role in wound healing in HDFs.

Erdr1 induces CCL2 production via the MAP kinase pathway in HDFs. Previous studies have demonstrated that the MAP kinase signaling pathway serves an important role in the migration and proliferation of skin fibroblasts and keratinocytes (5,30-33). Therefore, the effects of Erdr1 on the activation of the ERK1/2, p38 and JNK1/2 MAP kinases in HDFs were assessed in the present study. As demonstrated in Fig. 4A, treatment with Erdr1 induced the phosphorylation of ERK1/2, p38 and JNK1/2, and the phosphorylation of MAP kinases induced by Erdr1 at 5 min was comparable to that in the EGF-treated HDFs (Fig. 4A).

In order to further investigate the role of the Erdr1-induced phosphorylation of ERK1/2, p38, and JNK1/2 in the proliferation and migration of HDFs, specific inhibitors of ERK1/2 (U0126), p38 (SB203580) and JNK1/2 (SP600125) were used. As presented in Fig. 4B and C, the proliferation and migration of HDFs induced by Erdr1 were significantly inhibited by the specific inhibitors of ERK1/2, p38 and JNK1/2 MAP kinases. To determine whether these MAP kinases were involved in the Erdr1-induced production of CCL2, HDFs were pretreated with specific MAP kinase inhibitors, and the production of CCL2 protein was measured by ELISA. The results demonstrated that the MAP kinase inhibitors significantly inhibited the production of CCL2 in Erdr1-treated HDFs compared with that in the Erdr1-HG and DMSO-treated group (Fig. 4D), consistent with the results of the proliferation and migration assays. Taken together, these results suggested that the Erdr1-induced production of CCL2 enhanced the proliferation and migration of HDFs by phosphorylating ERK1/2, p38 and JNK1/2.

Involvement of CCL2 in Erdr1-mediated wound healing. To determine the role of the Erdr1-induced production of CCL2 in wound healing, HDFs were treated with a CCL2-neutralizing antibody. As presented in Fig. 5A, the CCL2-neutralizing antibody inhibited the proliferation of HDFs in the Erdr1-treated group. Furthermore, the migration of the cells treated with only CCL2-neutralizing antibody did not increase compared with that in the negative control (PBS-treated) group (Fig. 5B). Taken together, these results suggested that Erdr1 promoted wound healing by inducing the production of CCL2 via the activation of the ERK1/2, p38 and JNK1/2 MAP kinase signaling pathways (Fig. 5C).

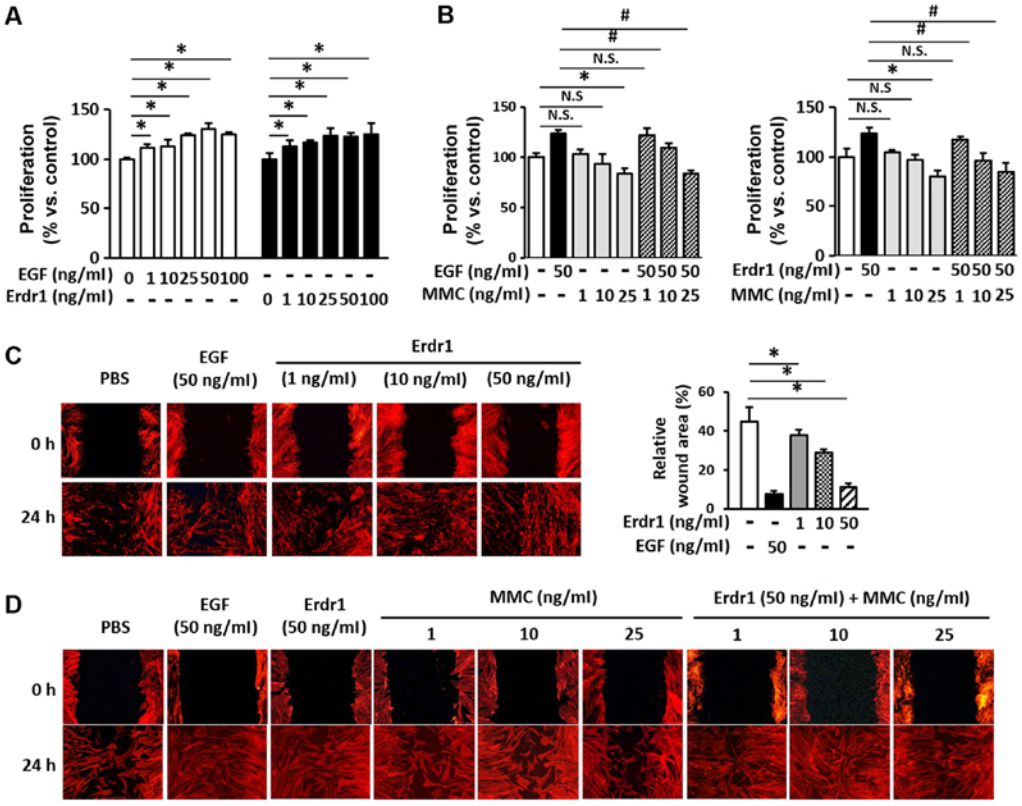

Figure 2. Erdr1 stimulates the proliferation and migration of HDFs. (A) The proliferation of the HDFs was determined using MTT assay. n=6. (B) The proliferation of the HDFs pre-treated with 1, 10 or 25 μg/ml MMC for 1 h and subsequently treated with 50 ng/ml EGF, Erdr1 or PBS for 24 h was determined by MTT assay. n=6. (C) Wound healing assay in HDFs treated with Erdr1, EGF or PBS at various concentrations for 24 h. n=3. (D) Wound healing assay in HDFs pre-treated with the indicated concentration of MMC for 1 h and subsequently treated with 50 ng/ml Erdr1, EGF or PBS for 24 h. *P<0.01 vs. PBS; #P<0.05 vs. EGF or Erdr1 as indicated; N.S., not significant; Erdr1, erythroid differentiation regulator 1; EGF, epidermal growth factor; HDFs, human dermal fibroblasts; MCC, mitomycin C.

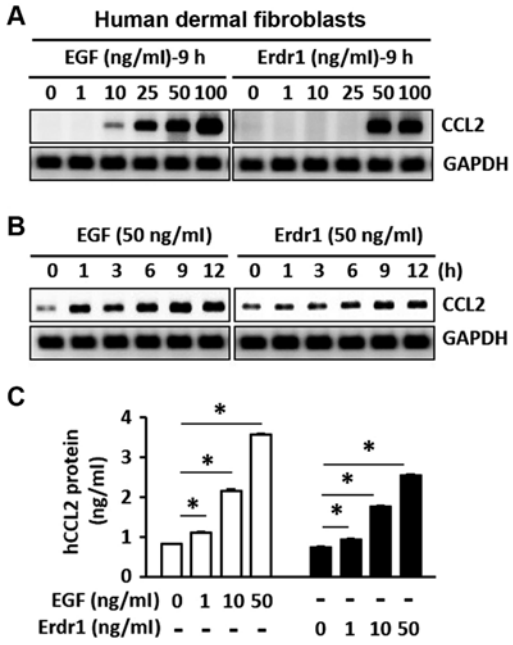

Figure 3. Erdr1 induces the production of CCL2 in HDFs. (A and B) The mRNA expression levels of CCL2 mRNA in HDFs treated with Erdr1, EGF or PBS (A) at various concentrations and (B) at different time points were determined using reverse transcription-PCR. (C) The protein levels of CCL2 in HDFs treated with Erdr1, EGF or PBS for 24 h were determined using ELISA. n=6. *P<0.01 vs. PBS. Erdr1, erythroid differentiation regulator 1; EGF, epidermal growth factor; HDFs, human dermal fibroblasts; CCL2, C-C motif chemokine ligand 2.

Discussion

Wound healing is an intricate, interactive biological process (34,35). Successful wound healing involves inflammation, tissue formation and remodeling (2,5,36). The various behaviors of skin cells, including the migration and proliferation of fibroblasts, are crucial events for wound contraction (2,5,36,37). Additionally, dermal fibroblasts serve an important role in process of wound healing via extracellular modulation (2,5,36,37). The migration and proliferation of dermal fibroblasts serve an essential role in tissue regeneration and repair; fibroblasts and keratinocytes are involved in the proliferation, migration and deposition of the extracellular matrix as well as the formation of granulation tissue (2,6,8). Erdr1 is expressed in various tissues and cells, including the normal human epidermis, $CD4^+$ T cells, skin tumors and human keratinocytes (18-20,38). Our previous study demonstrated that the sequence of human Erdr1 identified from the cDNA from human tissues exhibited 100% homology with the sequence of Erdr1 from murine tissues (21). However, the sequence of human Erdr1 was not identified in the present study; the sequence of human Erdr1 will be analyzed in future studies. Our recent study reported that Erdr1 is a potential therapeutic agent for inflammatory skin diseases including psoriasis (25), rosacea (26) and hair loss disorders (39); therefore, we hypothesized in the present study that Erdr1 may also promote wound healing.

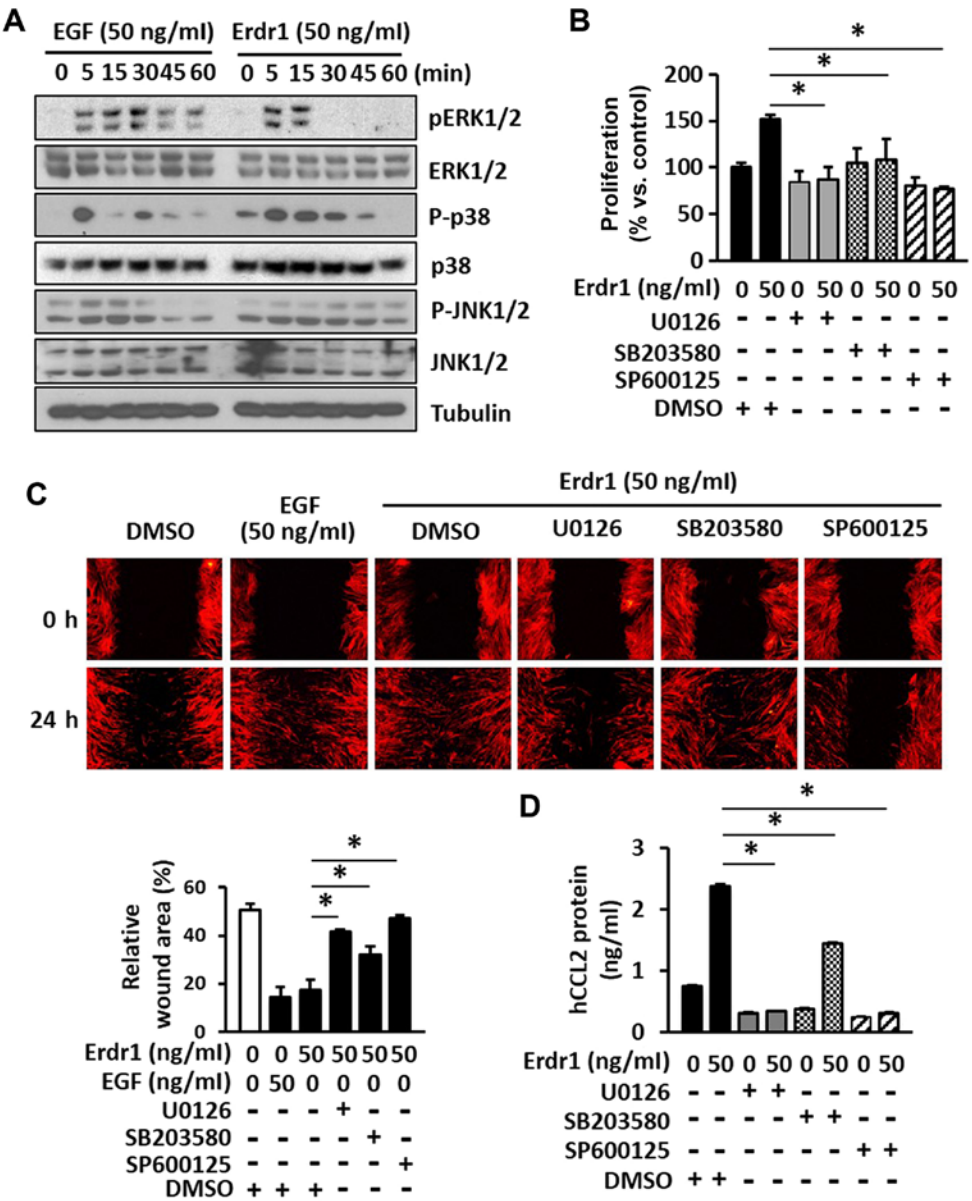

Figure 4. Erdr1 enhances the proliferation and migration of HDFs by inducing the production of CCL2 via the activation of MAPK kinases. (A) The levels of ERK1/2, p-ERK1/2, p38, p-p38, JNK1/2 and p-JNK1/2 in HDFs treated with 50 ng/ml Erdr1, EGF or PBS for 5, 15, 30, 45 and 60 min were analyzed by western blotting. Tubulin was used as the loading control. The (B) proliferation and (C) migration of HDFs treated with 50 ng/ml Erdr1 or DMSO (control) for 24 h after pretreatment with the ERK1/2 inhibitor U0126 (20 μM), the p38 inhibitor SB203580 (20 μM) or the JNK1/2 inhibitor SP600125 (20 μM) for 1 h was determined using the MTT and wound healing assays, respectively. n=6. (D) The levels of CCL2 protein were determined in HDFs pre-treated with 20 μM U0126, SB203580 or SP600125 for 1 h and subsequently treated with 50 ng/ml Erdr1 for 24 h using ELISA. n=6. *P<0.01 vs. Erdr1. Erdr1, erythroid differentiation regulator 1; EGF, epidermal growth factor; HDFs, human dermal fibroblasts; CCL2, C-C motif chemokine ligand 2.

In the present study, topical administration of Erdr1-HG enhanced the healing of skin wounds and induced complete wound closure after 10 days of treatment *in vivo*. It was observer that tissue repair was more rapid in the wounds that were treated with Erdr1 compared with that in the wounds treated with saline, and the wound healing effects of Erdr1 were comparable to those of EGF-HG *in vivo*. Notably, in the present study, the skin wounds *in vivo* were treated with saline, Erdr1 or EGF in HG at a final concentration of 20% (w/v); HG was used in these experiments as it has been used in several studies for delivering therapeutic agents to wound sites (40-42). However, HG was unable to penetrate the wound site owing to the formation of a fibrin clot, which inhibited the penetration of the compounds when the skin wounds were treated with saline, Erdr1 or EGF after 4 days of inflicting the wounds. Therefore, HG was not used for delivering the compounds to the wound sites after 4 days. It is necessary to modify the composition of the HG to improve the penetrative efficiency and targeted delivery of the compounds to the wound site in future studies.

It has been demonstrated that the role of the MAP kinase pathway in inflammatory skin diseases is mediated by inflammatory cytokines, such as IL-22 and IL-23, and several cellular functions, such as fibroblast and keratinocyte migration (5,31). The results of the present study demonstrated that the ERK, p38 and JNK pathways served an important role in promoting the migration and proliferation of HDFs, and were consistent with the results of several previous studies (5,28,31). Therefore, the

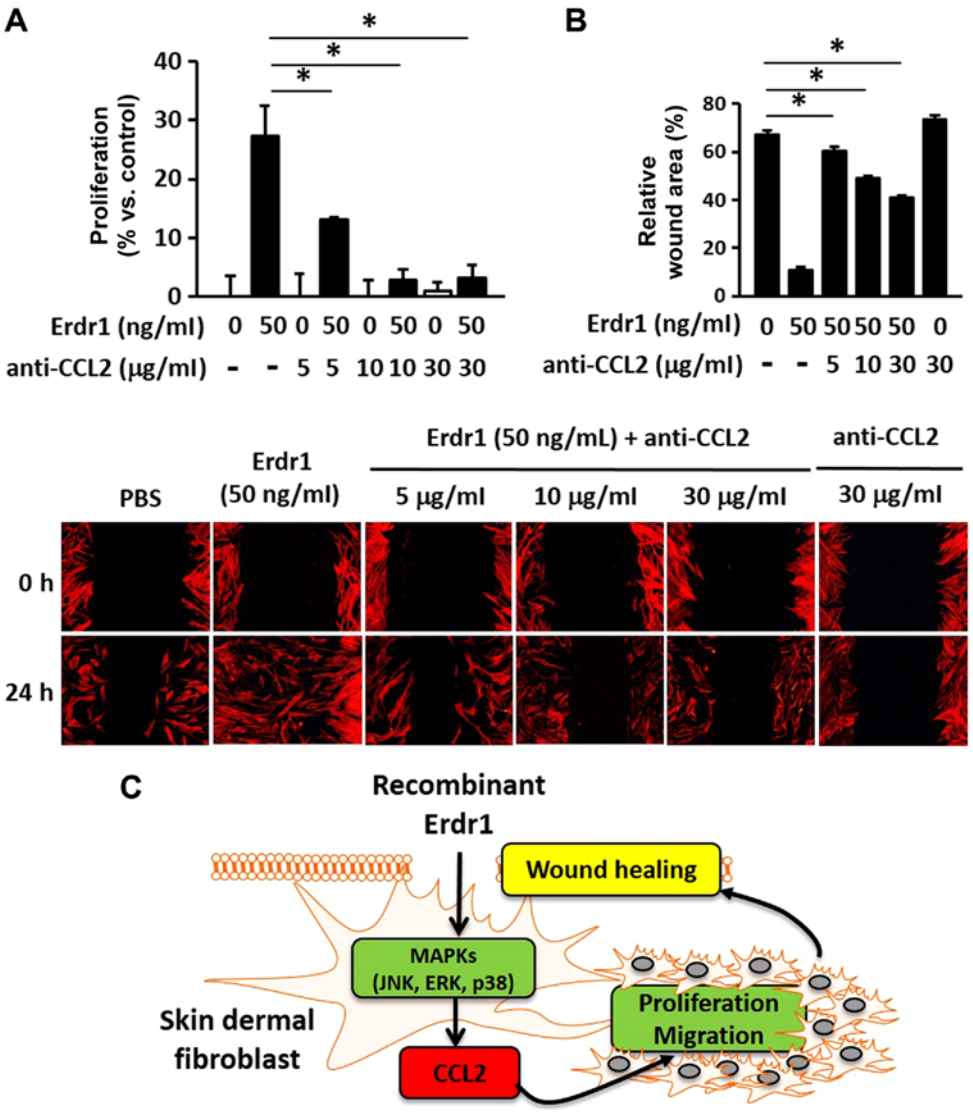

Figure 5. CCL2-neutralizing antibody inhibits the proliferation and migration of HDFs. (A) The proliferation of HDFs treated with 50 ng/ml Erdr1 or PBS at the indicated concentrations for 24 h following pretreatment with 1, 10 or 30 μg/ml CCL2-neutralizing antibody was determined using the MTT assay. n=6. (B) Wound healing assay in HDFs treated with 50 ng/ml Erdr1 or PBS for 24 h following pretreatment with 1, 10 or 30 μg/ml CCL2-neutralizing antibody. n=6. (C) The proposed scheme of the therapeutic action of Erdr1 in wound repair. *P<0.01 vs. Erdr1. Erdr1. Erdr1, erythroid differentiation regulator 1; EGF, epidermal growth factor; HDFs, human dermal fibroblasts; CCL2, C-C motif chemokine ligand 2.

role of the MAP kinase pathway in the migration, proliferation and CCL2 production was evaluated in the present study. The results demonstrated that Erdr1 served an important role in the migration and proliferation of HDFs via the activation of the ERK1/2, p38 and JNK1/2 signaling pathways. Although the levels of p-ERK1/2 and p-p38 began to decrease or disappeared after 5 min of EGF and Erdr1 treatments, this did not represent a reduction in CCL2 production. In addition, certain MAPK pathway inhibitors suppressed Erdr1-induced cell proliferation, CCL2 production and migration at 24 h.

Chemokines are chemoattractants that recruit monocytes, neutrophils and lymphocytes by binding to G-protein-coupled receptors (6,14,17). CCL2 is one of the key chemokines that regulate the migration and infiltration of monocytes and macrophages (43,44). Recent studies have demonstrated that several cytokines are expressed at the site of the wound and serve an essential role in the recruitment of inflammatory cells to the wound site (6,11,14). Furthermore, the expression of chemokine receptors on the resident cells suggests that chemokines contribute to re-epithelialization, tissue remodeling, and angiogenesis (10). In particular, MCP-1/CCL2 is a major chemoattractant that participates in the infiltration of inflammatory cells, including monocytes and macrophages, to the wound site (9-11,13,14,16,17,44). These studies suggest that CCL2 is directly involved in wound healing. The production of CCL2 is induced by oxidative stress, cytokines or growth factors in a variety of cell types, including endothelial, epithelial, smooth muscle and mesangial cells, astrocytes, monocytes, microglial cells and fibroblasts (45). It has been demonstrated that CCL2 is involved in wound healing and various diseases, including multiple sclerosis, rheumatoid arthritis, atherosclerosis, insulin-resistant diabetes (46-48).

A previous study has reported that treatment with a CCL-2-neutralizing antibody suppresses the number of macrophages at the wound site (15). In the present study, treatment with the CCL2-neutralizing antibody decreased in

the migration and proliferation of HDFs. Furthermore, Erdr1 significantly induced the production of CCL2 by activating ERK1/2, p38 and JNK1/2 in HDFs.

The results of the present study have several clinical limitations. First, Erdr1 is a novel factor for wound healing that significantly increased the CCL2 production in HDFs. Thus, we suggest that Erdr1 may be used as a therapeutic agent with other growth factors such as EGF for synergistic improvement of wound repair. Second, undefined receptors of Erdr1 should be investigated for increasing the therapeutic potential of Erdr1 to promote tissue repair. Third, it would be interesting to assess the effect of topical application of Erdr1 in chronic/diabetic wounds in future studies.

In conclusion, the results of the present study suggested that Erdr1 accelerated wound healing by increasing the production of CCL2, which induced the migration and proliferation of fibroblasts via the activation of the ERK1/2, p38 and JNK1/2 signaling pathways *in vitro*. Therefore, Erdr1 may serve as a potential therapeutic target for wound healing and for the development of therapeutic agents for topical treatment of wounds.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BCL conceived and designed the experiments. BCL, JS and AL performed the experiments. BCL and TSK wrote the manuscript. BCL, DC and TSK analyzed data. BCL and TSK were responsible for overall study design and supervised the project. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were performed following the ethical guidelines of the Korea University Institutional Animal Care and Use Committee (Seoul, Korea; approval no. KUIACUC-2018-0045).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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