Am J Physiol Cell Physiol 295: C931–C943, 2008.
First published July 23, 2008; doi:10.1152/ajpcell.00266.2008.

In vitro and in vivo wound healing-promoting activities of β-lapachone

Hsiu-Ni Kung,[1] Mei-Jun Yang,[1] Chi-Fen Chang,[1] Yat-Pang Chau,[2]* and Kuo-Shyan Lu[1]*

[1]*Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University;*
and [2]*Institute of Anatomy and Cell Biology, School of Medicine, National Yang-Ming University, Taipei, Taiwan*

Submitted 21 May 2008; accepted in final form 17 July 2008

Kung H-N, Yang M-J, Chang C-F, Chau Y-P, Lu K-S. In vitro and in vivo wound healing-promoting activities of β-lapachone. *Am J Physiol Cell Physiol* 295: C931–C943, 2008. First published July 23, 2008; doi:10.1152/ajpcell.00266.2008.—Impaired wound healing is a serious problem for diabetic patients. Wound healing is a complex process that requires the cooperation of many cell types, including keratinocytes, fibroblasts, endothelial cells, and macrophages. β-Lapachone, a natural compound extracted from the bark of the lapacho tree (*Tabebuia avellanedae*), is well known for its antitumor, antiinflammatory, and antineoplastic effects at different concentrations and conditions, but its effects on wound healing have not been studied. The purpose of the present study was to investigate the effects of β-lapachone on wound healing and its underlying mechanism. In the present study, we demonstrated that a low dose of β-lapachone enhanced the proliferation in several cells, facilitated the migration of mouse 3T3 fibroblasts and human endothelial EAhy926 cells through different MAPK signaling pathways, and accelerated scrape-wound healing in vitro. Application of ointment with or without β-lapachone to a punched wound in normal and diabetic (*db*/*db*) mice showed that the healing process was faster in β-lapachone-treated animals than in those treated with vehicle only. In addition, β-lapachone induced macrophages to release VEGF and EGF, which are beneficial for growth of many cells. Our results showed that β-lapachone can increase cell proliferation, including keratinocytes, fibroblasts, and endothelial cells, and migration of fibroblasts and endothelial cells and thus accelerate wound healing. Therefore, we suggest that β-lapachone may have potential for therapeutic use for wound healing.

cell proliferation; mitogen-activated protein kinase signaling pathways

THE HEALING OF CUTANEOUS WOUNDS is a dynamic, complex, and well-organized process and requires the orchestration of many different cell types and cellular processes (29). Immediately after skin is injured, various factors are released by different types of inflammatory cells. Then, starting several hours after injury, inflammatory cells, such as neutrophils, monocytes, and lymphocytes, initiate the proliferative phase of wound repair. At 3–7 days after injury, new capillaries endow the neostroma with its granular appearance. Leukocytes, fibroblasts, and blood vessels move into the wound area, and each contributes to the wound healing process. Macrophages provide a continuing source of cytokines to stimulate fibroplasia and angiogenesis. During angiogenesis, the vascular basement membrane and the fibrin or interstitial matrix are degraded by endothelial cells. The endothelial cells then start to migrate into the matrix and form new capillary-like tubes by proliferation (46). Migration and proliferation of keratinocytes at the wound edge then commence and are followed by proliferation of fibroblasts in the proximal area of the wound (45, 53).

Keratinocytes, fibroblasts, and endothelial cells are very important in skin wound repair. Wounding the epidermis generates cytokines and growth factors and initiates the synthesis of extracellular matrix components, all of which can regulate the processes of keratinocyte migration and proliferation, essential for reepithelialization (29). Fibroblasts predominantly appear in the wound after the inflammatory phase, then proliferate and synthesize new extracellular matrix, which is necessary to support the additional cell ingrowth (56), form granulation tissue (6, 16, 56), and subsequently generate mechanical forces within the wound to initiate wound contraction (14). Wound contraction is beneficial to overall wound healing by decreasing the wound area and forming a mechanically strong reparative scar (19, 38). Blood vessels play an important role in sustaining cell metabolism by providing oxygen and nutrients (19). The integrity of the granulation tissue depends on the presence of biological modifiers (e.g., growth factors), the activity of target cells, and the environment of the extracellular matrix (18). Macrophages, in the wound area, secrete numerous enzymes and cytokines, including collagenases, which debride the wound, and growth factors, which promote angiogenesis, stimulate fibroblasts to produce collagen, and stimulate the growth of keratinocytes (29). An understanding of the mechanisms that regulate the cell migration, proliferation, and wound contraction of the keratinocytes, dermal fibroblasts, and endothelial cells could be beneficial in devising novel therapies to regulate fibrosis and wound contraction to ultimately improve the wound healing process.

β-Lapachone is a natural *o*-naphthoquinone compound obtained from the bark of the lapacho tree (*Tabebuia avellanedae*). Its inner bark is often used as an analgesic, antiinflammatory, antineoplasic, antimicrobial, and diuretic in the northeast of Brazil (8, 51). β-Lapachone has a good antitumor effect on several carcinoma cells (23), including hepatoma (22), osteosarcoma (27), breast cancer (24), prostate cancer (9), and human leukemia (4, 43). However, whether it facilitates the process of wound healing and has a beneficial effect on the proliferation and migration of cells remains to be explored.

Diabetic patients frequently suffer serious problems with impaired wound healing, and the etiology of this impaired healing process is poorly understood. In mutant diabetic (*db*/*db*) mice, the gene encoding the leptin receptor (ObR) is inactivated and the mice develop obesity, insulin resistance, and severe diabetes with marked hyperglycemia, resembling adult-onset diabetes mellitus (5). As in diabetic humans, wound healing in *db*/*db* mice is markedly delayed (15, 54).

* Y.-P. Chau and K.-S. Lu contributed equally to this work.

Addresses for reprint requests and other correspondence: K.-S. Lu, Dept. of Anatomy and Cell Biology, College of Medicine, National Taiwan Univ., No. 1, Sec. 1, Jen Ai Rd., Taipei, 100, Taiwan; Y.-P. Chau, Inst. of Anatomy and Cell Biology, School of Medicine, National Yang-Ming Univ., 155, Sec. 2, Li-Nung St., Shih-Pai, Taipei, 112, Taiwan (e-mail: lks@ntu.edu.tw; leonchau@ym.edu.tw).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

In the present study, we first demonstrated that β-lapachone promoted the proliferation and migration of human keratinocytes, fibroblasts, and endothelial cells through different MAPK signals, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun NH_2-terminal kinase (JNK), and p38. Moreover, we showed that β-lapachone accelerated wound healing in both normal and mutant diabetic (*db*/*db*) mice, suggesting a potential application of this compound as an accelerator of wound repair.

MATERIALS AND METHODS

Chemicals. β-Lapachone, prepared as described by Schaffner-Sabba et al. (41), was dissolved as a 20 mM stock solution in DMSO and stored at −20°C. Antibodies against ERK, phosphorylated ERK, p38, phosphorylated p38, JNK, and phosphorylated JNK were purchased from Cell Signaling Technology (Danvers, MA). Macrophage marker antibody (MCA1849) was purchased from AbD Serotec (Kidlington, UK). Anti-mouse proliferating cell nuclear antigen (PCNA) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK inhibitor (catalog no. 328006), p38 inhibitor (SB-203580; catalog no. 559389), and JNK inhibitor (SP-600125, catalog no. 420119) were purchased from Calbiochem (Merck, Darmstadt, Germany). All fluorescent secondary antibodies were purchased from PerkinElmer (Boston, MA). Other chemicals or antibodies were purchased from Sigma (St. Louis, MO).

Cell culture. Human epidermal keratinocyte (HEKn) cells isolated from neonatal foreskin were cultured in 5% CO_2 at 37°C in Epilife medium (cells and medium were purchased from Cascade Biologics). The mouse keratinocyte XB-2 cells were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, 2 mM glutamine, and 100 μg/ml each of penicillin/streptomycin, with 3T3 cells as feeder layer. The mouse fibroblast 3T3 cells, human foreskin fibroblast HS68 cells, and mouse macrophage RAW264.7 cells were cultured in 5% CO_2 at 37°C in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 100 μg/ml each of penicillin/streptomycin (all from GIBCO-BRL, Rockville, MD). Human endothelial cells, EAhy926, derived from fusion of human umbilical vein endothelial cells with the lung adenocarcinoma A549 cell line, were cultured under the same conditions, but with 100 μM sodium hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine included in the medium. Human umbilical vascular endothelial cells (HUVEC) were harvested from the umbilical cord, incubated for 10 min at 37°C with 1% collagenase IV (Sigma), and grown at 37°C in 5% CO_2 in M199 medium containing 20% FCS, 100 μg/ml each of heparin and penicillin/streptomycin (100 U/ml), and 15 mg/ml of endothelial cell growth supplement (all from Upstate Biotechnology, Lake

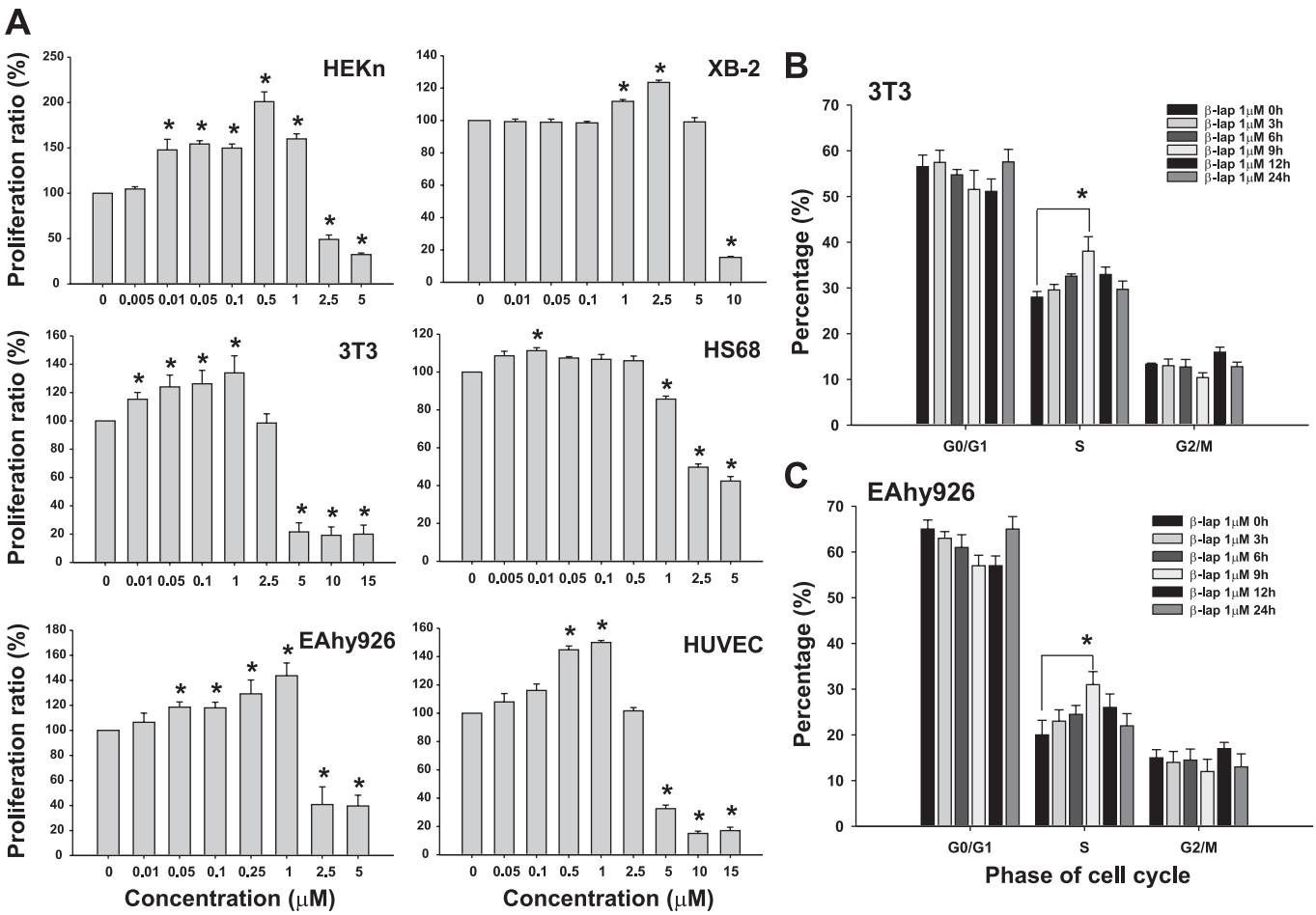


Fig. 1. Low concentrations of β-lapachone (β-lap) increase proliferation in various cell types and raise the percentage of cells in S phase. *A*: effect of various concentrations of β-lapachone on the proliferation of human epidermal keratinocyte cells (HEKn), mouse keratinocyte cells (XB-2), mouse fibroblast cells (3T3), human fibroblast cells (HS68), human endothelial cells (EAhy926), and human umbilical vein endothelial cells (HUVEC), respectively. *Significant difference ($P < 0.05$) from vehicle treatment (0 μM β-lapachone; $n = 3$). *B* and *C*: flow cytometric analyses showing an increase in the percentage of S-phase cells in 3T3 cells (*B*) and EAhy926 cells (*C*) treated for 3–9 h with 1 μM β-lapachone. *Significant difference ($P < 0.05$) from the beginning (0 h) of the treatment ($n = 3$).

Placid, NY). HUVEC cultures were passaged using 0.05% trypsin-0.2% EDTA in phosphate-buffered saline (PBS); *passages 3* to *7* were used for tests.

Cell treatment and cell viability assays. HS68 cells (10^3), 3T3 cells (10^3), or EAhy926 cells (10^4) in 100 μl medium were seeded for 24 h at 37°C in a 96-well culture plate in a humidified 5% CO_2 atmosphere. HEKn cells (10^4), XB-2 cells (10^4), and HUVEC (10^4) were seeded for 48 h because of the lower growth rate. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, various concentrations of β-lapachone were added to the medium 24 h before the cell viability assay. In brief, 10 μl MTT (0.5 mg/ml) were added to each well and the plates were incubated at 37°C for 4 h. The

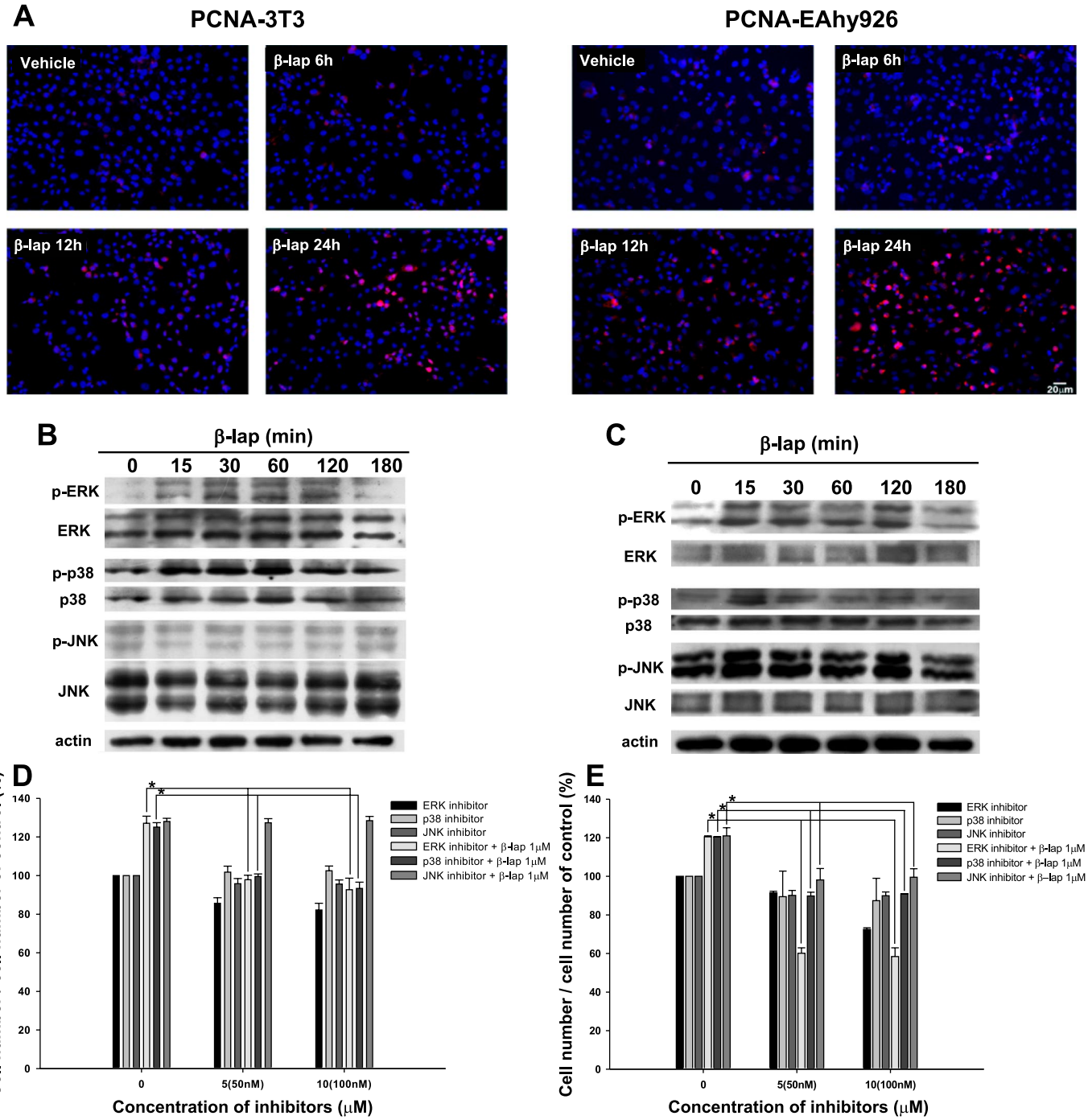
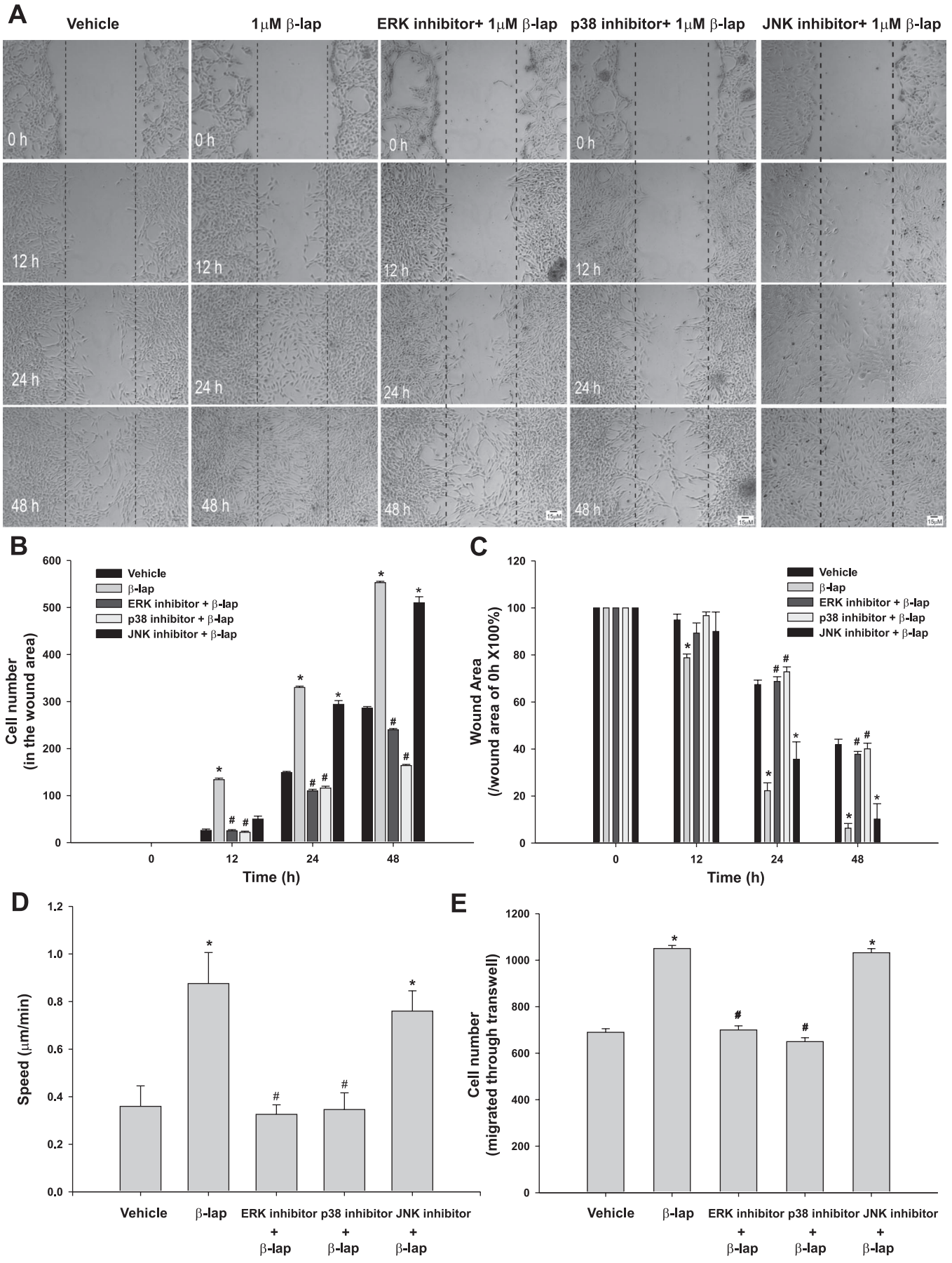


Fig. 2. β-Lapachone increases the level of proliferating cell nuclear antigen (PCNA) protein, and MAPK signaling pathways are involved in the β-lapachone-induced proliferation of fibroblasts and endothelial cells. *A*: immunostaining for PCNA, a protein synthesized in early G1 and S phase, showing a greater number of PCNA-positive cells in β-lapachone-treated group than in the untreated group. *B*: Western blots showing an increase in phosphorylated (p)-ERK and p-p38, but not p-JNK, within 15 min in 3T3 cells treated with β-lapachone. *C*: Western blots of EAhy926 cells treated with β-lapachone showing that levels of p-ERK, p-p38, and p-JNK dramatically increase in 30 min. *D*: ERK inhibitor or p38 inhibitor, but not JNK inhibitor, inhibits β-lapachone-induced 3T3 cell proliferation. *E*: all three inhibitors inhibit β-lapachone-induced EAhy926 cell proliferation. *Significant difference ($P < 0.05$) from the group treated with 1 μM β-lapachone but without inhibitors (n = 3; *D* and *E*).



formazan product was then dissolved in 100 μl DMSO at 37°C for 30 min, and absorbance at 570 nm was measured with a microplate reader.

To test the effects of MAPK inhibitors, 3T3 cells or EAhy926 cells (10^3 cells in 100 μl medium/well) were incubated for 1 h with 0, 5, or 10 μM ERK inhibitor or p38 inhibitor (SB-203580) or 0, 50, or 100 nM JNK inhibitor (SP-600125); the cells were then changed to medium containing the same MAPK inhibitor with or without 1 μM β-lapachone. The number of viable cells after treatment was measured using the MTT assay. For all studies, at least three sets of independent experiments were carried out, each in triplicate.

Cell cycle analysis. Cells were treated with 1 μM β-lapachone for 3, 6, 9, 12, or 24 h, harvested with 0.5% trypsin-EDTA, and fixed with cold 80% ethanol. After three washes with PBS, the cells were incubated for 1 h at 37°C with RNase A (1 μg/ml) and then for 15 min at 37°C with propidium iodide (50 μg/ml). Stained cells were detected by flow cytometry (Becton-Dickinson) using the FL-2 parameter, and the data were analyzed using Cell Quest Pro software (Becton-Dickinson).

Immunofluorescence staining. Cells were incubated with 1 μM β-lapachone for 0 to 24 h and were then fixed in 4% paraformaldehyde for 15 min. After being blocked for 1 h at room temperature with 10% normal goat serum (NGS), the cells were stained overnight at 4°C with monoclonal antibody against PCNA (1:1,000), incubated with rhodamine-conjugated secondary antibody and Hoechst dye for 1 h at room temperature, and examined and photographed using a Leica fluorescence microscope.

Western blot analyses. Cells treated with 1 μM β-lapachone for 0–24 h were lysed with lysis buffer (0.25 mM HEPES, pH 7.4, 14.9 mM NaCl, 10 mM NaF, 2 mM $MgCl_2$, 0.5% NP-40, 0.1 mM PMSF, 20 μM pepstatin A, and 20 μM leupeptin). The lysates were then centrifuged at 1,000 *g* for 15 min at 4°C, and the supernatants were collected for immunoblotting. The amount of protein in the samples was measured by the Bradford assay (Bio-Rad, Hercules, CA) using an ELISA reader. Approximately 25–50 μg protein from each sample were separated by 10–12% SDS-PAGE and then transferred to Immobilon-P membranes (Millipore, Bedford, MA) in an electrophoretic transfer cell (2 h at 200 V). All subsequent steps were at room temperature. The membranes were blocked for 1 h with 5% skim milk in PBS containing 0.05% Tween 20 (PBST), incubated for 2 h with anti-phosphorylated-ERK, anti-ERK, anti-phosphorylated-JNK, anti-JNK, anti-phosphorylated-p38, anti-p38, or anti-actin antibodies (1:1,000 dilution) in 1% BSA, washed with PBST for 30 min, and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (PerkinElmer; 1:5,000 dilution in PBST). Bound antibody was detected with ECL Western blotting reagent (PerkinElmer), and the chemiluminescence was detected with Fuji Medical X-ray film (Tokyo, Japan). The amount of each protein was quantified using Scion software.

Scrape-wound healing assay. Cells were grown to confluence on a 24-well dish, the medium was aspirated, and new medium with or without 1 μM β-lapachone alone or together with ERK inhibitor, p38 inhibitor, or JNK inhibitor was added. A single stripe (~150 μm wide) was scraped on the cell-coated surface with a 200-μl disposable plastic pipette tip, and the wound was allowed to heal for 24 h (endothelial cells) or 48 h (fibroblast cells) at 37°C. The average extent of wound closure was evaluated by measuring the width of the wound. The migration speed of cells into wounded areas was examined and photographed by time-lapse microscopy (Laica, Wetzlar, Germany) and was measured with Metamorph software (Molecular Devices, Toronto, Ontario, Canada).

Transwell migration assay. Cell migration was assessed using a modified Millicell chamber (8-μm pores; Transwell, Millipore, Billerica, MA). Cells seeded into the upper chamber at 1×10^4 cells/well in 0.2 ml medium were treated with β-lapachone alone or with β-lapachone plus ERK inhibitor, p38 inhibitor, or JNK inhibitor, and 0.6 ml of the medium was added to the bottom chamber. After 24 h at 37°C, the cells on the upper surface of the membrane were mechanically removed, and the migrated cells on the lower surface of the membrane were fixed and stained with Coomassie brilliant blue (Sigma). The total number of migrated cells on the lower surface of the membrane was counted. Each experiment was performed in triplicate.

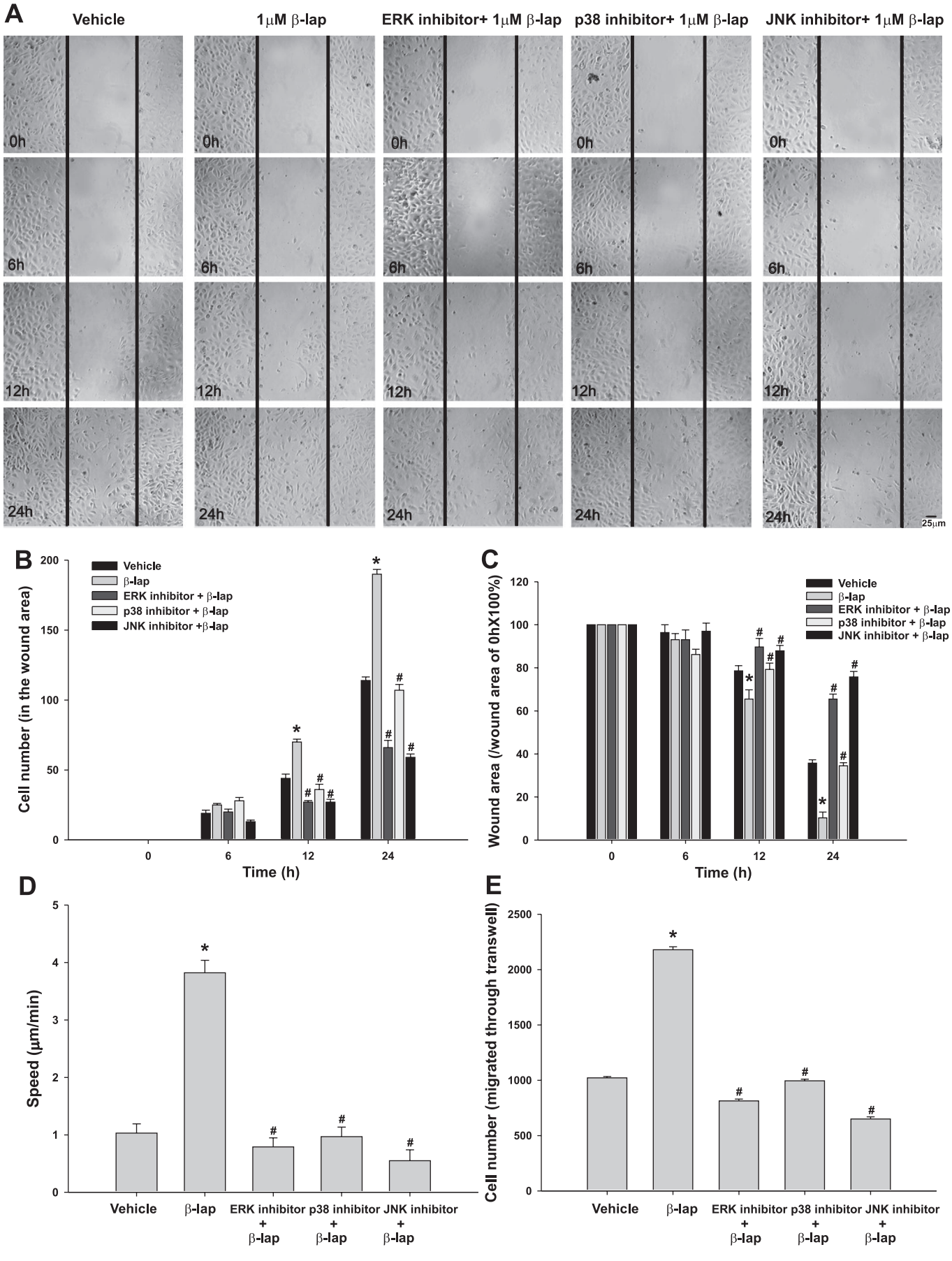
Animals. Adult C57BL/6 or *db*/*db* male mice (10 wk old) were purchased from the National Taiwan University Animal Center and housed in individual cages in a temperature- and humidity-controlled room (12:12-h light-dark cycle) with free access to tap water and diet. All of the animal experiments were performed according to National Institutes of Health guidelines and were approved by the Laboratory Animal Committee of the College of Medicine, National Taiwan University.

Wound biopsy and measurement of wound closure. Mice (C57BL/6 or *db*/*db*) were anesthetized with 2% Rompun solution (0.1 ml/20 g body wt; Bayer, Leverkusen, Germany). The back of the mouse was shaved and then sterilized using an alcohol swab. A sterile biopsy punch (6-mm diameter) was used to punch through the full thickness of the back skin below the shoulder blades. A wound placed in this area cannot be reached by the mouse and therefore prevents self-licking. Ointment [100 mg pure white petrolatum jelly (Vaseline)] alone (control ointment) or containing 29.8 μg/g β-lapachone was applied to the wound and changed every 2 days. Wounds from individual mice were digitally photographed every 5 days, beginning on the day of wounding. For all measurements, the wound area was quantified using Scion software.

Histological examination. During the process of wound closure, skin samples (approximately 1×1 cm^2) containing the wound areas were collected at 3, 7, 14, or 21 days postwounding and fixed in 4% formaldehyde for histological study. The samples were frozen and transversely cut into 7-μm-thick sections from the middle part of the wounds, which were stained with hematoxylin and eosin before examination in a Zeiss Axiphot light microscope. The number of vessels in the wounding area was calculated using Imagescope software (Aperio, Vista, CA). The density of vessels (counts/mm^2 area) was measured and analyzed with Metamorph software.

Macrophage assay and measurement of VEGF and EGF. At 3, 7, 14, or 21 days postwounding, mice were euthanized with an overdose of 2% Rompun solution. The wound areas were removed by cutting out a square area containing the entire wound site, and the tissues were immediately placed in 4% paraformaldehyde for 1 wk. Cryosections (7 μm) from the middle part of the wound areas were cut for immunofluorescence for general histological observation. For immunofluorescence, the sections were blocked with 10% NGS in PBS for 2 h at room temperature and then incubated overnight at 4°C with rabbit anti-mouse VEGF or EGF antibodies

Fig. 3. β-Lapachone facilitates wound closure in 3T3 cell monolayers through the ERK and p38 signaling pathways. *A*: a 150-μm-wide wound (area between the dotted vertical lines) made in confluent fibroblast cultures was left untreated (left column) or was treated with 1 μM β-lapachone alone (second column from left) or together with ERK inhibitor (third column from left), p38 inhibitor (fourth column from left), or JNK inhibitor (right column). *B*: microscopic observation showing that the number of cells after cotreatment with ERK inhibitor or p38 inhibitor and β-lapachone is less than after treatment with β-lapachone alone. *C*: wound area is reduced rapidly from 0 to 48 h after β-lapachone treatment, but not after cotreatment with β-lapachone and ERK inhibitor or p38 inhibitor. *Significant difference ($P < 0.05$) among the same group (0, 12, 24, or 48 h) as compared with vehicle treatment (*B* and *C*). #Significant difference as compared with the β-lapachone treatment (second column of each group; *B* and *C*). *D*: migration speed of cells after cotreatment with ERK inhibitor or p38 inhibitor and β-lapachone is slower than that of cells treated with β-lapachone alone. *E*: Transwell migration assay showing that more β-lapachone-treated cells migrate through the pores than untreated cells and those cells cotreated with β-lapachone and ERK inhibitor or p38 inhibitor, but not JNK inhibitor. In *D* and *E*, similar comparison as in *B* or *C* was made between the treatments with vehicle (*) or β-lapachone (#).



and polyclonal rat anti-mouse macrophage marker MCA1849 (1:300). After several washes with PBS, the sections were incubated for 1 h at room temperature with fluorescence-conjugated secondary antibody, washed, and mounted with 50% glycerol in PBS. The number of macrophages in the wound area was counted, and the sections were photographed with a Leica fluorescence microscope.

For measurement of VEGF or EGF levels, 1×10^4 macrophages were seeded in 96-well plates and treated with 1 μM β-lapachone for 0, 30, 60, and 180 min, respectively. The medium was collected, and the levels of VEGF and EGF were measured with the RayBio VEGF and EGF ELISA kits using tetramethyl benzidine (TMB) as a chromogen. The level of VEGF or EGF was determined by the intensity of the absorbance at 450 nm on an ELISA reader (BioTek, Winooski, VT).

Statistical analysis. All data are presented as means ± SD, and differences between groups were examined using a one-way ANOVA with Scheffé's test. $P < 0.05$ was considered statistically significant.

RESULTS

Low concentrations of β-lapachone promote cell proliferation and increase the percentage of cells in S phase. We used the MTT assay to examine the effect of 24 h (HS68, 3T3, and EAhy926) or 48 h (HEKn, XB-2, and HUVEC) treatment with different concentrations of β-lapachone on various cells. After incubation with low concentrations (up to 1 μM) of β-lapachone, the cell proliferation rates of 3T3, EAhy926, and HUVEC increased in a dose-dependent manner and peaked at 1 μM at 133% for 3T3 cells, 145% for EAhy926 cells, and 145% of control levels for HUVEC cells, respectively. The cell survival also peaked at 0.5 μM at 196% for HEK cells, 2.5 μM at 125% for XB-2 cells, and 0.01 μM at 113% for HS68 cells incubating with β-lapachone (Fig. 1*A*). At higher concentrations, β-lapachone was cytotoxic to cells and the cell survival rates dropped to 20–40% in all cells (Fig. 1*A*). Similar observation was obtained from all six cell lines we tested in this experiment; therefore, we used two cell lines, 3T3 and EAhy926, in subsequent studies to explore the molecular mechanism underlying the proliferating activity of β-lapachone.

Flow cytometry showed that, in cells treated with β-lapachone, the percentage of cells in S phase increased in a time-dependent manner up to 9 h, but there was no significant change in the percentages of cells in G0/G1 and G2/M phase (Fig. 1, *B* and *C*). Cellular proliferation was also examined by immunostaining for PCNA, a protein synthesized in early G1 and S phase, and the number of PCNA-positive cells in 3T3 or EAhy926 cells treated with β-lapachone was found to increase in a time-dependent manner from 0 to 24 h (Fig. 2*A*).

MAPK signaling pathways are involved in β-lapachone-induced cell proliferation. Since MAPK signaling plays a very important role in cell growth and migration, phosphorylation of the MAPK proteins ERK1/2, JNK, and p38 was examined to determine whether β-lapachone-stimulated cell proliferation was mediated by a MAPK pathway. Western blot analysis revealed that, in 3T3 cells, levels of phosphorylated ERK 1/2 and p38 MAPK, but not of phosphorylated JNK, were significantly increased by treatment with β-lapachone for 15 to 60 min (Fig. 2*B*). In EAhy926 cells, levels of all three phosphorylated MAPK proteins increased at 15–30 min in β-lapachone-treated cells (Fig. 2*C*). To determine whether phosphorylation of MAPK proteins was crucial in β-lapachone-induced proliferation in 3T3 or EAhy926 cells, the effects of specific inhibitors of ERK1/2 (ERK inhibitor), p38 (SB-203580), and JNK (SP-600125) on β-lapachone-treated cells were assessed. For these inhibition experiments, the cells were pretreated with MAPK inhibitors for 1 h and were then incubated with fresh medium containing 1 μM β-lapachone plus the same inhibitor for 24 h. Treatment with the ERK inhibitor or p38 inhibitor, but not the JNK inhibitor, inhibited the β-lapachone-induced increase in proliferation (>20%) in 3T3 cells (Fig. 2*D*), whereas in EAhy926 cells, all three MAPK inhibitors, especially the ERK inhibitor, had an inhibitory effect (Fig. 2*E*).

β-Lapachone increases cell migration. The effect of β-lapachone on the migration of 3T3 and EAhy926 cells was tested in an in vitro wound healing model, in which scrape wounds were generated in confluent cell cultures. Cells with or without β-lapachone treatment were allowed to migrate into the denuded area for 12–48 h at 37°C. β-Lapachone-treated 3T3 cells started to migrate into the denuded area at 12 h after being scratched, and scratch closure was almost complete at 48 h (Fig. 3*A*, second column from left). In contrast, cells without β-lapachone treatment were less motile, as indicated by fewer cells in the denuded area at 12, 24, and 48 h after scratching (Fig. 3*A*, first column from left). The number of 3T3 cells in the wound area increased more quickly and the wound area decreased more rapidly in β-lapachone-treated cells than in other treatment cells (Fig. 3, *B* and *C*). The migration speed of 3T3 cells treated with β-lapachone was faster than others (2.25 times compared with nontreated cells; Fig. 3*D*). These observations indicated that 3T3 cells treated with β-lapachone migrated faster than control cells. Similar results were obtained using EAhy926 cells, which started to migrate at 6 h after scratching after incubation with β-lapachone, scratch closure being almost complete at 24 h (Fig. 4*A*, second column from left). More cells were seen in the denuded area at 6, 12, and 24 h after β-lapachone treatment than in the untreated controls (Fig. 4*A*, first column). In the wound area, number of EAhy926 cells increased more swiftly and the wound area decreased more promptly in β-lapachone-treated cells than in other treatment cells (Fig. 4, *B* and *C*), and the migration speed of EAhy926 cells treated with β-lapachone was faster than others (3.7 times compared with nontreated cells; Fig. 4*D*). These results show that 3T3

Fig. 4. β-Lapachone facilitates wound closure in EAhy926 cell monolayers through the ERK, p38, and JNK signaling pathways. *A*: cells treated with β-lapachone fill the wound area (area between the 2 solid dark vertical lines) faster than untreated cells at 12 to 24 h. Addition of ERK inhibitor (third column from left), p38 inhibitor (fourth column from left), or JNK inhibitor (right column) significantly reduced the effect of β-lapachone. *B*: cell numbers after cotreatment with β-lapachone and the ERK inhibitor, p38 inhibitor, or JNK inhibitor are less than after treatment with β-lapachone alone at 12 and 24 h. *C*: wound area is reduced more rapidly in cells treated with β-lapachone than in untreated cells, but is slower in cells cotreated with β-lapachone and ERK inhibitor, p38 inhibitor, or JNK inhibitor. *Significant difference ($P < 0.05$) among the same group (0, 6, 12, or 24 h) as compared with vehicle treatment (*B* and *C*). #Significant difference as compared with the β-lapachone treatment (second column of each group; *B* and *C*). *D*: migration speed of cells cotreated with β-lapachone and ERK inhibitor, p38 inhibitor, or JNK inhibitor is slower than that of cells treated with β-lapachone alone. *E*: Transwell migration assay showing that more β-lapachone-treated EAhy926 cells migrate through the pores than untreated cells or cells cotreated with β-lapachone and ERK inhibitor, p38 inhibitor, or JNK inhibitor. In *D* and *E*, similar comparison as in *B* or *C* was made between the treatments with vehicle (*) or β-lapachone (#).

fibroblasts and endothelial EAhy926 cells are more motile after treatment with 1 μM β-lapachone.

Effect of MAPK inhibitors on the migration of β-lapachone-treated cells. When the same system was used to study the effects of MAPK inhibitors on β-lapachone-induced cell migration, pretreatment with the ERK or p38 inhibitor, but not the JNK inhibitor, inhibited the β-lapachone-dependent migration of 3T3 cells, especially at 24 and 48 h (Fig. 3*A*, right three columns), whereas all three significantly reduced β-lapachone-dependent EAhy926 cell migration at 12 and 24 h (Fig. 4*A*, right three columns). The number of cells in the wound area increased approximately twofold after β-lapachone treatment, whereas addition of the ERK or p38 inhibitor greatly reduced the number of 3T3 cells (Fig. 3*B*, 24 h and 48 h) or EAhy926 cells (Fig. 4*B*, 24 h) in the scratched area. The healing of the wound area in β-lapachone-treated 3T3 cell cultures was faster than that in the presence of the ERK or p38 inhibitor (Fig. 3*C*). The migration speed of cells treated with β-lapachone was also double compared with that of nontreated cells (Fig. 3*D*). In EAhy926 cell cultures, healing of the wound area in β-lapachone-treated cells was slower in the presence of ERK, p38, or JNK inhibitor (Fig. 4*C*) and the speed of migration in β-lapachone-treated cells was reduced by pretreatment with ERK, p38, or JNK inhibitor (Fig. 4*D*).

To exclude the proliferation effect of β-lapachone in the scrape-wound healing assay, the Transwell migration assay was used to investigate the migration effect of β-lapachone on 3T3 and EAhy926 cells. The Transwell migration assay also showed that more β-lapachone-treated 3T3 cells migrated through the Transwell pores than untreated cells or cells cotreated with β-lapachone and ERK inhibitor or p38 inhibitor (Fig. 3*E*). Similarly, all three inhibitors decreased the number of β-lapachone-treated EAhy926 cells migrating through the Transwell pores (Fig. 4*E*). In contrast, ERK (5 μM), p38 (5 μM), or JNK (50 nM) inhibitor did not affect cell migration either in 3T3 or EAhy926 cells without β-lapachone treatment in scrape-wound assays and Transwell migration assays (see Supplemental Figs. S1 and S2 in the online version of this article). Taken together, the data above indicate that the MAPK signaling pathways are involved in β-lapachone-induced fibroblast and endothelial cell migration.

Effects of β-lapachone on in vivo wound healing. To determine whether β-lapachone had a therapeutic effect on wound healing, ointment alone or containing 29.8 μg/g β-lapachone was applied to a wound on the back of C57BL/6 or *db*/*db* mice for 21 days, and the wounds were examined for healing every 5 days from wounding day (*day 0*) to *day 21* postwounding (Figs. 5 and 6). Skin tissue (approximately 1×1 cm^2) in the center of the wounds was cut out on *day 3*, *7*, *14*, or *21* postwounding and was processed for hematoxylin and eosin staining (Fig. 6). The density of vessels underlying the healing skin was measured using Imagescope software (Fig. 6*E*). Microscopic observation showed that the time required for wound healing on *db*/*db* mice was significantly longer than that on C57BL/6 mice (Fig. 5, *A* and *C*), and the wound area in C57BL/6 or *db*/*db* mice treated with ointment containing β-lapachone (Fig. 5, *B* and *D*) was markedly smaller than that in mice treated with control ointment (Fig. 5, *A* and *C*) in 5 to 20 days. Compared with mice treated with control ointment, the area of the β-lapachone-treated wounds was significantly reduced in both C57BL/6 and *db*/*db* mice (Fig. 5*E*). Compared with the wound treated with ointment without β-lapachone, the recovery process of wound healing by β-lapachone treatment was faster either in C57BL/6 or in *db*/*db* mice (Fig. 6, *A–D*). On *day 14*, in C57BL/6 mice, the scar tissue was thick and the dermis appeared disorderly in the wound treated with control ointment (Fig. 6*A3*); however, on the same day, the skin layers were completely rehabilitated in the β-lapachone-treated wound (Fig. 6*B3*). Similarly, the scar tissue was relatively thinner, and hair follicles appeared in the dermis in the β-lapachone-treated wound at 14 days (Fig. 6*D3*) in *db*/*db* mice, but hair follicles in the wound treated with the control ointment were only observed at 21 days.

Since MAPK inhibitor assays revealed that ERK inhibitor exerted its inhibitory effect dramatically on β-lapachone-mediated wound healing in both 3T3 (Fig. 2*D*) and EAhy926 cells (Fig. 2*E*), we next investigated the effect of ERK in β-lapachone-facilitated wound healing. ERK inhibitor with or without β-lapachone was used to treat the back wound in C57BL/6 and *db*/*db* mice. Compared with β-lapachone treatment alone, wound healing was markedly reduced after ERK inhibitor with or without β-lapachone treatment (Fig. 5*E*). However, ERK inhibitor did not affect normal wound healing but significantly reduced the healing effect of β-lapachone on wound healing (Supplemental Fig. S3).

Additionally, the density of vessels in β-lapachone-treated wounds was higher than that in control-ointment-treated wounds at *days 3* to *21* postwounding in both C57BL/6 and *db*/*db* mice (Fig. 6*E*). The number of macrophages that appeared in the wound area in β-lapachone-treated mice was more numerous than that treated with ointment without β-lapachone at *day 14* in C57BL/6 mice and at *day 7* in *db*/*db* mice (Fig. 7*A*). Double immunostaining with anti-MCA1849 (a macrophage marker, FITC) and anti-VEGF or -EGF antibodies (tetramethylrhodamine isothiocyanate) illustrated that macrophages secrete VEGF and EGF in β-lapachone-treated wounds in C57BL/6 (Fig. 7*B*) or *db*/*db* mice. We also demonstrated the release of VEGF and EGF by macrophages following 30–180 min of β-lapachone treatment (Fig. 7*C*) in vitro.

DISCUSSION

In the present study, we first reported that low concentrations of β-lapachone promoted the proliferation of keratinocytes (0.5 μM for HEKn; 1 and 2.5 μM for XB-2), fibroblasts (0.01 μM for HS68, and for 3T3), and endothelial cells (1 μM for EAhy926 and HUVEC cells) compared with untreated cells (Fig. 1*A*). The responses of cells in reaction to different concentrations of β-lapachone is due to cell specificity. Human endothelium-like EAhy926 cell line is used for study because it displays the characteristics of endothelial cells, such as the CD31 marker and tube formation (11, 52). Immunofluorescence staining with an anti-human Factor VIII antibody demonstrated that EAhy926 cells continue to synthesize Factor VIII similar to human umbilical vein endothelial cells (HUVEC) (17, 32, 47). Because EAhy926 cells express the characteristics of human vascular endothelial cells and undergo tubule-like formation in matrigel as do HUVEC cells, these facts extensively supported that EAhy926 cells can be used as a homogeneous experimental endothelial cell line compared with the HUVEC cells collected from different donors (1, 10, 11).

As we know, β-lapachone at high concentrations (>2 μM) is a novel antitumor agent with specific anticancer activity against human lung, prostate, and breast tumors (34). A previous study showed that β-lapachone has a unique mechanism of action, which relies on its

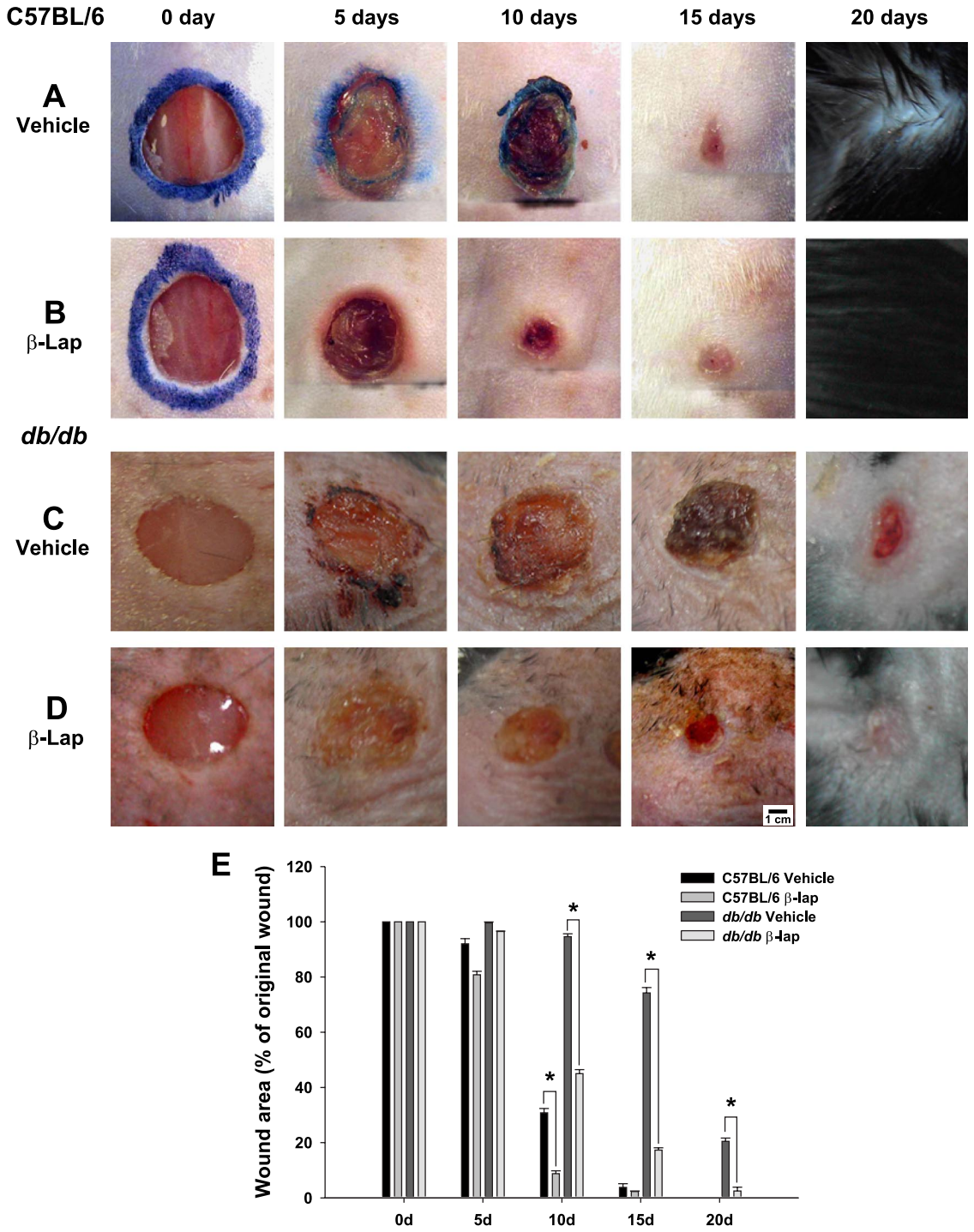


Fig. 5. β-Lapachone is effective in the in vivo wound healing. *A–D*: control ointment or ointment containing 29.8 μg/g β-lapachone was applied to a wound on the back of normal or *db*/*db* mice for 21 days, and photographs were taken every 5 days from wounding day (*day 0*) to *day 20* postwounding. The wound area on normal or *db*/*db* mice after treatment with ointment containing β-lapachone (*B* and *D*) is smaller than that on mice treated with control ointment (*A* and *C*). *E*: the area of the β-lapachone-treated wounds in both normal and *db*/*db* mice is significantly smaller than that of wounds treated with control ointment. At *day 10* after wounding, a significant improvement in wound healing was found in wild-type C57BL/6 mice treated with β-lapachone, whereas after 10 days, wound healing was significantly progressed in the *db*/*db* mice. *Significant difference ($P < 0.05$) as analyzed by Student's *t*-test.

bioactivation by the cytosolic enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) (37). The activity of this enzyme is higher in tumor cells (up to 20-fold) than in adjacent normal cells (44). Moreover, β-lapachone has distinct advantages over other chemotherapeutic agents in that it kills tumors by inducing a novel μ-calpain-mediated apoptotic response, which is independent of p53 status, cell cycle state, and caspase activation (37, 48).

In addition to its novel antitumor activity, β-lapachone has been shown to have a variety of pharmacological effects. Recent studies have shown that it has antiinflammatory effects,

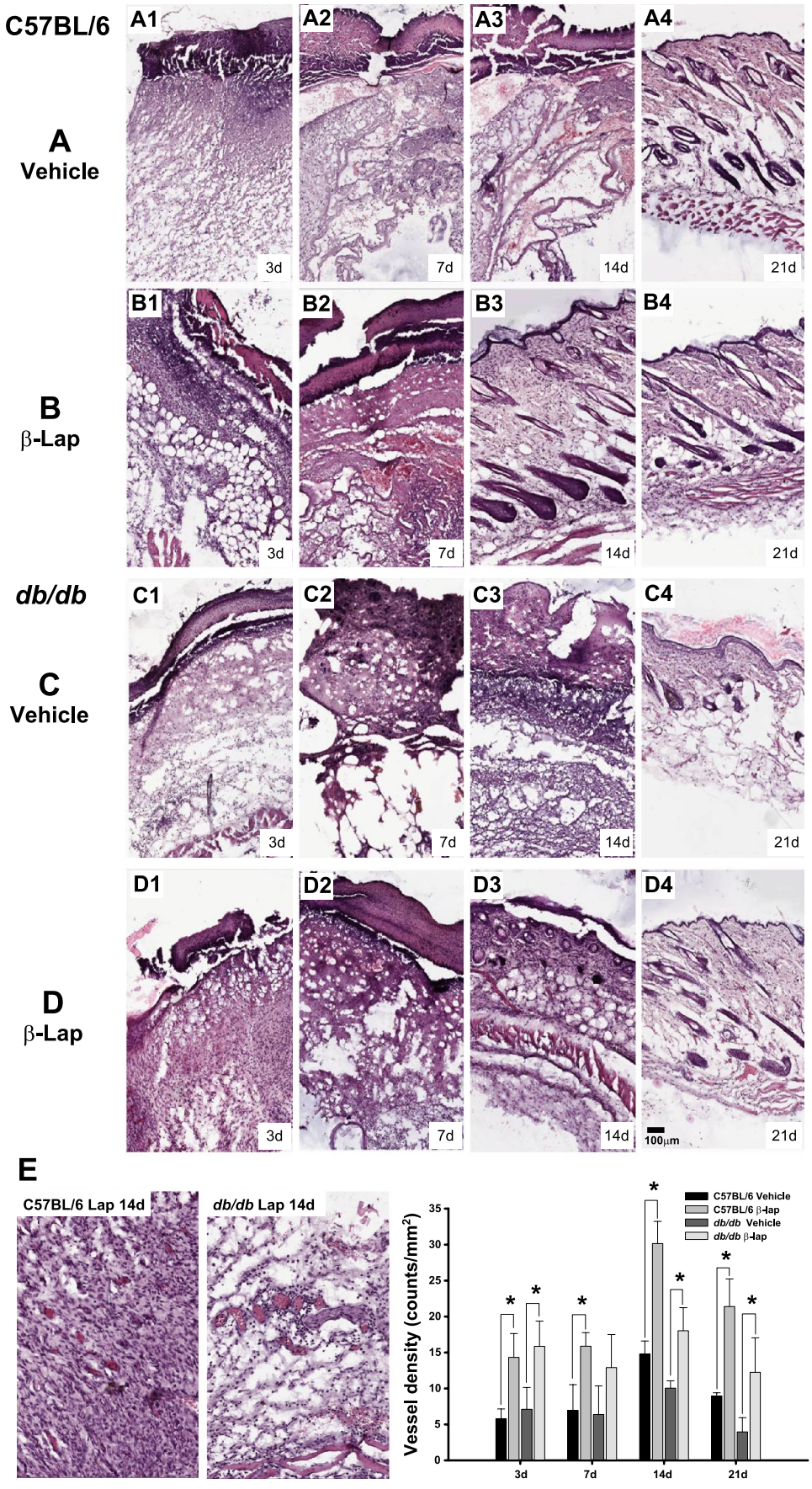


Fig. 6. A histological examination of skin and the underlying blood vessels in the wound area. *A–D*: hematoxylin and eosin-stained sections at *day 3*, *7*, *14*, or *21* post-wounding showed that the recovery of epidermis (cuticle or scar tissue) and dermis (connective tissue and hair follicle) in the wounds treated with β-lapachone (*B1–B4* and *D1–D4*) was faster than that treated with control ointment (*A1–A4* and *C1–C4*) either in C57BL/6 or *db*/*db* mice. *E*: photographs of vessels in tissue sections of C57BL/6 and *db*/*db* mice. Vascular density in β-lapachone-treated wounds was higher than in those treated with control ointment at *day 3* to *day 21* in C57BL/6 and *db*/*db* mice. The histogram is a quantitative analysis of vascular density in the dermis layer of C57BL/6 and *db/db* mice after wounding and treatment with vehicle or β-lapachone ointment. It is clear that vascular density after 3 days of β-lapachone treatment greatly increased both in C57BL/6 and *db/db* mice. *Significantly different from each paired group.

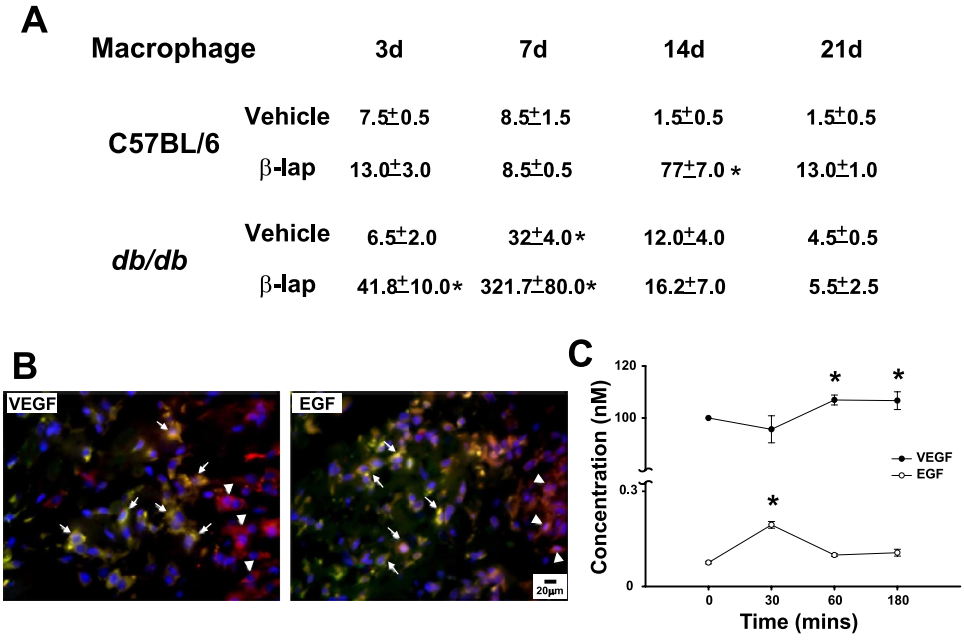


Fig. 7. Secretion of VEGF and EGF by macrophages after β-lapachone treatment promoting wound healing. *A*: number of macrophages (cells/mm^2) in the wound areas of C57BL/6 or *db/db* mice after treatment with or without β-lapachone. *Significant change ($P < 0.05$) in the number of macrophages as compared with vehicle treatment at *day 3* after wounding in C57BL/6 or *db/db* mice. *B*: double staining with macrophage marker, MCA1849 and VEGF or EGF, showed that macrophages contained VEGF and EGF in β-lapachone-treated wounds in C57BL/6 (data of *db/db* mice are not shown; the staining was the same with those presented in C57BL/6 mice). Arrows indicate the immunostaining of VEGF or EGF (red) in macrophage (green). Arrowheads indicated the staining of VEGF or EGF alone (red). *C*: in vitro assay showed that macrophages secrete VEGF and EGF at 30 to 180 min after β-lapachone administration. *Significant difference in VEGF or EGF secretion after β-lapachone treatment (for 30, 60, and 180 min) as compared with the beginning of the experiments (0 min).

because it can decrease inducible nitric oxide (NO) synthase expression, NO production (26), and NF-κB activation (31) in LPS-stimulated macrophages, thus protecting against LPS-induced lung edema and decreasing mortality in LPS-mediated sepsis (50). Most previous studies have reported that high doses (>2 μM) of β-lapachone induce either apoptotic or necrotic cell death in a variety of human carcinoma cells, but not in normal human cells. However, very little is known about the biological activity of low-dose β-lapachone on normal or primary cultured cells. In fact, normal human cells have usually been used as the negative control, without looking for any possible effects of β-lapachone on these cells. In the present study, we reported that low doses of β-lapachone (0.01–1 μM) enhanced the proliferation of various cells including different keratinocytes (HEKn and XB-2), fibroblasts (HS68 and 3T3), and endothelial cells (HUVEC and EAhy926) (Fig. 1*A*). Flow cytometric and immunostaining of PCNA showed that the percentage of cells in S phase in β-lapachone-treated cells was higher than that in untreated cells (Fig. 1, *B* and *C*, and Fig. 2*A*), suggesting that low-dose β-lapachone promotes DNA synthesis and stimulates cell proliferation. To explore the mechanism by which low-dose β-lapachone induces cell proliferation, we examined the proliferation-related signal transduction pathway, the MAPK pathway.

The MAPK family consists of three major subfamilies with multiple members, including the extracellular signal-regulated kinases (ERK), p38 MAP-kinases (p38), and c-Jun NH_2-terminal kinases (JNK), which all play critical roles in the regulation of cell proliferation, differentiation, and apoptosis. Each MAPK subfamily is activated in response to diverse extracellular stimuli by phosphorylation within a conserved Thr-X-Tyr motif in its activation loop (7). Activation of MAPKs leads to the phosphorylation and activation of a variety of proteins, including a number of transcription factors involved in regulating the expression of genes controlling cellular proliferation and migration (2). Suppression of the ERK1/2 signaling pathway by 2-chloro-3-(4-hexylphenyl)-amino-1,4-naphthaquinone (NQ304), a synthetic 1,4-naphthoquinone derivative, has an antiproliferative effect on vascular smooth muscle cells (20). Moreover, the inhibition of endothelial cell proliferation by Notch-1 signaling is mediated by suppressing the MAPK and phosphatidylinositol 3-kinase/Akt pathways (20). As reported in studies on different cell lines, MAPKs trigger the downstream growth and migration signaling cascades after they are activated by cytokines and mitogens (12, 13). Angiogenic growth factors, such as vascular endothelial growth factor and basic fibroblast growth factor, which activate ERK, JNK, and p38, induce endothelial cell proliferation, migration, and survival (25, 39, 42). The JNK pathway is predominantly involved in the platelet-derived growth factor-stimulated migration and proliferation of osteoblastic cells, whereas the p38 pathway is involved in migration and the ERK pathway in proliferation (33, 40, 49). In corneal wound healing, both hepatocyte

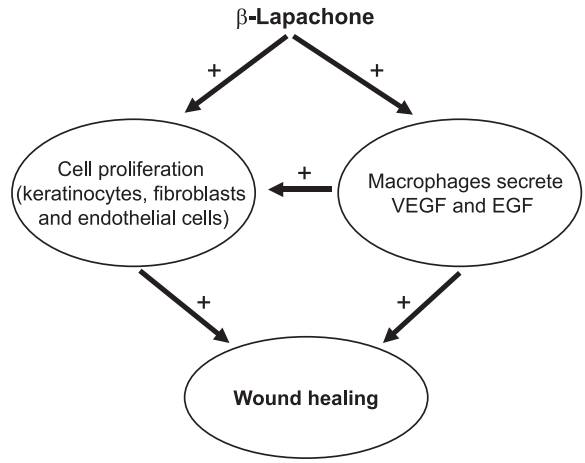


Fig. 8. A schematic representation of the mechanism of β-lapachone promoting the wound healing. β-Lapachone induces the proliferation of various cells, including keratinocytes, fibroblasts, and endothelial cells, and stimulates the macrophages to release the growth factors VEGF and EGF for promoting wound healing.

growth factor and keratinocyte growth factor have been demonstrated to activate the ERK1/2 pathway and facilitate wound closure (30, 42). We here report that stimulation of the MAPK signaling cascades is an important event in β-lapachone-mediated cell proliferation and migration. β-Lapachone was found to activate ERK, p38, and JNK pathways in EAhy926 cells, whereas only ERK and p38 signaling was activated in 3T3 fibroblasts (Fig. 2, *B–E*). This was confirmed by the use of specific MAPK inhibitors that decreased β-lapachone-mediated cell proliferation and migration (Figs. 3 and 4).

Some quinines, including embelin, 3-hydroxy-β-*N*-lapachone, and α-lapachone, have been reported to be helpful in wound healing, with a high rate of wound contraction (21), and can be used in topical preparations against wound infections (36). We therefore examined whether β-lapachone facilitated wound healing in normal mice (C57BL/6) or diabetic mice (*db*/*db*), in which wound healing is markedly delayed (36, 54). It is well known that the cells of diabetic patients or experimental animals (*db*/*db*) are longtime exposed in a microenvironment of high glucose concentration. To understand the in vitro effect of β-lapachone on the cells under high glucose condition, EAhy926 cells were incubated in the medium containing high concentrations of glucose (5 and 25 mM) with or without β-lapachone. The results indicated that high concentrations of glucose decrease the cell proliferative rate and cause cell death either in 5 or 25 mM glucose-treated cells (without β-lapachone). Moreover, for cells grown in the glucose-containing medium, the addition of 0.5 μM β-lapachone can increase the cell proliferative rate, whereas high concentrations of β-lapachone (5, 10, or 15 μM) cause a significant decrease of proliferative rate (Supplemental Fig. S4). According to studies of silver sulfadiazine, the most commonly used topical antibacterial agent for the treatment of burn wounds, the working concentration used on wounds of rabbit and human (1%) (28) is 100–1,600 times to that directly exposed to the microbial pathogens including *Staphylococcus aureus*, *Escherichia coli*, etc. (6.2–100 μg/ml) (3). β-Lapachone in the ointment is less effective than affecting cells directly in the medium and has to be diffused throughout the ointment to stimulate the cells around the wound area. Thus the β-lapachone concentration (29.8 μg/g ≅ 100 μM) was used in the in vivo wound healing assay, approximately 100 times of that in the in vitro scrape-wound healing assay (1 μM). To test the therapeutic effect of β-lapachone, a 6-mm-diameter wound was made on the back of normal and *db*/*db* mice, and ointment alone or containing 29.8 μg/g β-lapachone was then applied to the wounds for 0–21 days. These in vivo animal experiments confirmed that β-lapachone treatment significantly accelerated wound healing in C57BL/6 (Fig. 5*B*) and diabetic mice (Fig. 5*D*) compared with the control groups (Fig. 5, *A* and *C*). Microscopic examination showed that the recovery of the epidermis and dermis was faster (Fig. 6, *A–D*) and the vessel density was higher in the wound treated with β-lapachone-containing ointment than those treated with ointment containing no β-lapachone (Fig. 6*E*), suggesting that β-lapachone possesses a therapeutic effect in the wound healing process. On the other hand, we also demonstrated that ERK inhibitor decreased the recovery of wound healing by β-lapachone significantly (Supplemental Fig. S3). These data suggested that ERK may play an important role in the β-lapachone-mediated wound healing process.

During inflammation, macrophages are released from blood vessels in response to inflammatory stimuli, and an increased number of macrophages may facilitate more efficient cleaning of debris, dead cells, or extracellular matrix and help the wound healing process (35, 55). β-Lapachone dramatically increased the number of macrophages at 14 days postwounding in normal mice and at 7 days in *db*/*db* mice (Fig. 7*A*), and it induced macrophages to secrete VEGF and EGF in the process of wound healing (Fig. 7*B*); thus VEGF and EGF can attract other cells, such as fibroblasts, endothelial cells, and epidermal cells, to migrate into the wound area and aid in the recovery of skin injuries. In vitro ELISA assay demonstrated that macrophages secrete VEGF and EGF 30 to 180 min after β-lapachone administration (Fig. 7*C*).

From the in vitro assays, it is clear that β-lapachone promotes the proliferation in keratinocytes, fibroblasts, and endothelial cells, and accelerates the migration in 3T3 and EAhy926 cells. In in vivo assays, β-lapachone accelerates the process of wound healing either in normal or diabetic mice. In addition, β-lapachone induces macrophages to secret VEGF and EGF for facilitating the growth of other cells (Fig. 8). Taken together, we conclude that β-lapachone may have a potential as a healing promoting agent for wound healing.

ACKNOWLEDGMENTS

We thank Dr. T. Barkas for critical review of this manuscript.

GRANTS

This work was supported in part by grants from the National Science Council, Taiwan (nos. NSC 96-2320-B-002-052-MY2 to K.-S. Lu and NSC 96-2320-B-010-016 to Y.-P. Chau).

REFERENCES

1. **Ben-Yosef Y, Lahat N, Shapiro S, Bitterman H, Miller A.** Regulation of endothelial matrix metalloproteinase-2 by hypoxia/reoxygenation. *Circ Res* 90: 784–791, 2002.
2. **Bose C, Bhuvaneswaran C, Udupa KB.** Age-related alteration in hepatic acyl-CoA: cholesterol acyltransferase and its relation to LDL receptor and MAPK. *Mech Ageing Dev* 126: 740–751, 2005.
3. **Chang TW, Weinstein L.** Inactivation of *Treponema pallidum* by silver sulfadiazine. *Antimicrob Agents Chemother* 7: 538–539, 1975.
4. **Chau YP, Shiah SG, Don MJ, Kuo ML.** Involvement of hydrogen peroxide in topoisomerase inhibitor beta-lapachone-induced apoptosis and differentiation in human leukemia cells. *Free Radic Biol Med* 24: 660–670, 1998.
5. **Chen H, Charlat O, Tartaglia LA, Woolf EA, Weng X, Ellis SJ, Lakey ND, Culpepper J, Moore KJ, Breitbart RE, Duyk GM, Tepper RI, Morgenstern JP.** Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in *db*/*db* mice. *Cell* 84: 491–495, 1996.
6. **Davidson JM.** *Inflammation: Basic Principles and Clinical Correlates*. New York: Raven, 1992.
7. **Davis RJ.** The mitogen-activated protein kinase signal transduction pathway. *J Biol Chem* 268: 14553–14556, 1993.
8. **De Almeida ER, da Silva Filho AA, dos Santos ER, Lopes CA.** Antiinflammatory action of lapachol. *J Ethnopharmacol* 29: 239–241, 1990.
9. **Don MJ, Chang YH, Chen KK, Ho LK, Chau YP.** Induction of CDK inhibitors ($p21^{WAF1}$ and $p27^{Kip1}$) and Bak in the beta-lapachone-induced apoptosis of human prostate cancer cells. *Mol Pharmacol* 59: 784–794, 2001.
10. **Edgell CJ, Haizlip JE, Bagnell CR, Packenham JP, Harrison P, Wilbourn B, Madden VJ.** Endothelium specific Weibel-Palade bodies in a continuous human cell line, EA.hy926. In Vitro *Cell Dev Biol* 26: 1167–1172, 1990.
11. **Edgell CJ, McDonald CC, Graham JB.** Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* 80: 3734–3737, 1983.
12. **Frey MR, Dise RS, Edelblum KL, Polk DB.** p38 kinase regulates epidermal growth factor receptor downregulation and cellular migration. *EMBO J* 25: 5683–5692, 2006.

13. **Frey MR, Golovin A, Polk DB.** Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling. *J Biol Chem* 279: 44513–44521, 2004.
14. **Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR, Majno G.** Granulation tissue as a contractile organ. A study of structure and function. *J Exp Med* 135: 719–734, 1972.
15. **Greenhalgh DG, Sprugel KH, Murray MJ, Ross R.** PDGF and FGF stimulate wound healing in the genetically diabetic mouse. *Am J Pathol* 136: 1235–1246, 1990.
16. **Grinnell F.** Fibroblasts, myofibroblasts, and wound contraction. *J Cell Biol* 124: 401–404, 1994.
17. **Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC, Bender JR.** Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ Res* 87: 677–682, 2000.
18. **Juliano RL, Haskill S.** Signal transduction from the extracellular matrix. *J Cell Biol* 120: 577–585, 1993.
19. **Katz MH, Alvarez AF, Kirsner RS, Eaglstein WH, Falanga V.** Human wound fluid from acute wounds stimulates fibroblast and endothelial cell growth. *J Am Acad Dermatol* 25: 1054–1058, 1991.
20. **Kim TJ, Yun YP.** Potent inhibition of serum-stimulated responses in vascular smooth muscle cell proliferation by 2-chloro-3-(4-hexylphenyl)-amino-1,4-naphthoquinone, a newly synthesized 1,4-naphthoquinone derivative. *Biol Pharm Bull* 30: 121–127, 2007.
21. **Kumara Swamy HM, Krishna V, Shankarmurthy K, Abdul RB, Mankani KL, Mahadevan KM, Harish BG, Raja NH.** Wound healing activity of embelin isolated from the ethanol extract of leaves of *Embelia ribes* Burm. *J Ethnopharmacol* 109: 529–534, 2007.
22. **Lai CC, Liu TJ, Ho LK, Don MJ, Chau YP.** beta-Lapachone induced cell death in human hepatoma (HepA2) cells. *Histol Histopathol* 13: 89–97, 1998.
23. **Li CJ, Li YZ, Pinto AV, Pardee AB.** Potent inhibition of tumor survival in vivo by beta-lapachone plus taxol: combining drugs imposes different artificial checkpoints. *Proc Natl Acad Sci USA* 96: 13369–13374, 1999.
24. **Lin MT, Chang CC, Chen ST, Chang HL, Su JL, Chau YP, Kuo ML.** Cyr61 expression confers resistance to apoptosis in breast cancer MCF-7 cells by a mechanism of NF-kappaB-dependent XIAP up-regulation. *J Biol Chem* 279: 24015–24023, 2004.
25. **Liu B, Ryer EJ, Kundi R, Kamiya K, Itoh H, Faries PL, Sakakibara K, Kent KC.** Protein kinase C-delta regulates migration and proliferation of vascular smooth muscle cells through the extracellular signal-regulated kinase 1/2. *J Vasc Surg* 45: 160–168, 2007.
26. **Liu SH, Tzeng HP, Kuo ML, Lin-Shiau SY.** Inhibition of inducible nitric oxide synthase by beta-lapachone in rat alveolar macrophages and aorta. *Br J Pharmacol* 126: 746–750, 1999.
27. **Liu TJ, Lin SY, Chau YP.** Inhibition of poly(ADP-ribose) polymerase activation attenuates beta-lapachone-induced necrotic cell death in human osteosarcoma cells. *Toxicol Appl Pharmacol* 182: 116–125, 2002.
28. **Lowbury EJ, Babb JR, Bridges K, Jackson DM.** Topical chemoprophylaxis with silver sulphadiazine and silver nitrate chlorhexidine creams: emergence of sulphonamide-resistant Gram-negative bacilli. *Br Med J* 1: 493–496, 1976.
29. **Martin P.** Wound healing—Aiming for perfect skin regeneration. *Science* 276: 75–81, 1997.
30. **Mehrotra M, Krane SM, Walters K, Pilbeam C.** Differential regulation of platelet-derived growth factor stimulated migration and proliferation in osteoblastic cells. *J Cell Biochem* 93: 741–752, 2004.
31. **Moon DO, Choi YH, Kim ND, Park YM, Kim GY.** Anti-inflammatory effects of beta-lapachone in lipopolysaccharide-stimulated BV2 microglia. *Int Immunopharmacol* 7: 506–514, 2007.
32. **Muller L, Valdenaire O, Barret A, Korth P, Pinet F, Corvol P, Tougard C.** Expression of the endothelin-converting enzyme-1 isoforms in endothelial cells. *J Cardiovasc Pharmacol* 36: S15–S18, 2000.
33. **Munoz-Chapuli R, Quesada AR, Angel MM.** Angiogenesis and signal transduction in endothelial cells. *Cell Mol Life Sci* 61: 2224–2243, 2004.
34. **Ough M, Lewis A, Bey EA, Gao J, Ritchie JM, Bornmann W, Boothman DA, Oberley LW, Cullen JJ.** Efficacy of beta-lapachone in pancreatic cancer treatment: exploiting the novel, therapeutic target NQO1. *Cancer Biol Ther* 4: 95–102, 2005.
35. **Pereira CF, Boven LA, Middel J, Verhoef J, Nottet HS.** Induction of cyclooxygenase-2 expression during HIV-1-infected monocyte-derived macrophage and human brain microvascular endothelial cell interactions. *J Leukoc Biol* 68: 423–428, 2000.
36. **Pereira EM, Machado TB, Leal IC, Jesus DM, Damaso CR, Pinto AV, Giambiagi-deMarval M, Kuster RM, Santos KR.** *Tabebuia avellanedae* naphthoquinones: activity against methicillin-resistant staphylococcal strains, cytotoxic activity and in vivo dermal irritability analysis. *Ann Clin Microbiol Antimicrob* 5: 5, 2006.
37. **Pink JJ, Planchon SM, Tagliarino C, Varnes ME, Siegel D, Boothman DA.** NAD(P)H:quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity. *J Biol Chem* 275: 5416–5424, 2000.
38. **Rudolph R, Berg J, Ehrlich HP.** Wound contraction and scar contracture. In: *Wound Healing: Biochemical and Clinical Aspects*, edited by Cohen IK, Diegelmann RF, and Lindblad WJ. Philadelphia, PA: Saunders, 1992, p. 114.
39. **Saika S.** TGF-beta signal transduction in corneal wound healing as a therapeutic target. *Cornea* 23: S25–S30, 2004.
40. **Salameh A, Galvagni F, Bardelli M, Bussolino F, Oliviero S.** Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of endothelial cells through the activation of ERK, AKT, and JNK pathways. *Blood* 106: 3423–3431, 2005.
41. **Schaffner-Sabba K, Schmidt-Ruppin KH, Wehrli W, Schuerch AR, Wasley JW.** beta-Lapachone: synthesis of derivatives and activities in tumor models. *J Med Chem* 27: 990–994, 1984.
42. **Sharma GD, He J, Bazan HE.** p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. *J Biol Chem* 278: 21989–21997, 2003.
43. **Shiah SG, Chuang SE, Chau YP, Shen SC, Kuo ML.** Activation of c-Jun NH_2-terminal kinase and subsequent CPP32/Yama during topoisomerase inhibitor beta-lapachone-induced apoptosis through an oxidation-dependent pathway. *Cancer Res* 59: 391–398, 1999.
44. **Siegel D, Ross D.** Immunodetection of NAD(P)H:quinone oxidoreductase 1 (NQO1) in human tissues. *Free Radic Biol Med* 29: 246–253, 2000.
45. **Singer AJ, Clark RA.** Cutaneous wound healing. *N Engl J Med* 341: 738–746, 1999.
46. **Staton CA, Brown NJ, Lewis CE.** The role of fibrinogen and related fragments in tumour angiogenesis and metastasis. *Expert Opin Biol Ther* 3: 1105–1120, 2003.
47. **Stula M, Orzechowski HD, Gschwend S, Vetter R, von HR, Dietz R, Paul M.** Influence of sustained mechanical stress on Egr-1 mRNA expression in cultured human endothelial cells. *Mol Cell Biochem* 210: 101–108, 2000.
48. **Tagliarino C, Pink JJ, Reinicke KE, Simmers SM, Wuerzberger-Davis SM, Boothman DA.** Mu-calpain activation in beta-lapachone-mediated apoptosis. *Cancer Biol Ther* 2: 141–152, 2003.
49. **Tanaka K, Abe M, Sato Y.** Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis. *Jpn J Cancer Res* 90: 647–654, 1999.
50. **Tzeng HP, Ho FM, Chao KF, Kuo ML, Lin-Shiau SY, Liu SH.** beta-Lapachone reduces endotoxin-induced macrophage activation and lung edema and mortality. *Am J Respir Crit Care Med* 168: 85–91, 2003.
51. **Ueda S, Umemura T, Dohguchi K, Matsuzaki T, Tokuda H, Nishino H, Iwashima A.** Production of anti-tumour-promoting furanonaphthoquinones in *Tabebuia avellanedae* cell cultures. *Phytochemistry* 36: 323–325, 1994.
52. **Van Oost BA, Edgell CJ, Hay CW, MacGillivray RT.** Isolation of a human von Willebrand factor cDNA from the hybrid endothelial cell line EA.hy926. *Biochem Cell Biol* 64: 699–705, 1986.
53. **Werner S, Grose R.** Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 83: 835–870, 2003.
54. **Wetzler C, Kampfer H, Stallmeyer B, Pfeilschifter J, Frank S.** Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: prolonged persistence of neutrophils and macrophages during the late phase of repair. *J Invest Dermatol* 115: 245–253, 2000.
55. **Winstanley EW.** The connective-tissue reaction in the healing of full-thickness excised skin wounds in the thoracic and metatarsal regions of the dog. *J Comp Pathol* 86: 211–219, 1976.
56. **Zavan B, Brun P, Vindigni V, Amadori A, Habeler W, Pontisso P, Montemurro D, Abatangelo G, Cortivo R.** Extracellular matrix-enriched polymeric scaffolds as a substrate for hepatocyte cultures: in vitro and in vivo studies. *Biomaterials* 26: 7038–7045, 2005.