EGF-induced Corneal Epithelial Wound Healing Though NF-κB Subtype-Regulated CTCF Activation

Ling Wang, Xiaolin Wu, Ting Shi and Luo Lu*
Department of Medicine, David Geffen School of Medicine
University of California Los Angeles,
Torrance, CA 90503

Running Title: Wound healing regulated by NFκB-p50-activated CTCF

*Address correspondence to: Luo Lu, Department of Medicine, David Geffen School of Medicine at UCLA, 1124 W. Carson St., H-H Bldg., Torrance, CA 90502; Tel. (310) 781-1404; Fax. (310) 781-9065; E-mail: lluou@ucla.edu

Capsule
Background: To study NF-κB-activated CTCF in EGF-induced wound healing.
Results: Corneal epithelial wound healing was significantly impaired because of lacking CTCF activity in NFκB-p50−/− mice. EGF-induced NFκB activation regulated CTCF by interacting with CTCF-promoter to increases motility, migration and wound healing.
Conclusion: CTCF is a NFκB-p50-interactive target in EGF-induced corneal epithelial wound healing.
Significance: NF-κB-controlled CTCF activation plays important roles in EGF-regulated wound healing.

Epidermal growth factor (EGF) plays an important role in corneal epithelial migration and proliferation to improve the wound healing process. The study aimed to understand the role of NFκB in EGF-induced corneal epithelial wound healing through regulation of CTCF activity that plays important roles in cell motility and migration to promote wound healing. The effect of NFκB p50 on corneal epithelial wound healing was investigated by comparing eyes of wildtype and p50-knockout mice and found that there was a significant retardation in corneal epithelial wound healing in the corneas of p50-knockout mice. Wound closure rates were measured in human corneal epithelial cells transfected with a NFκB activation-sensitive CTCF expression construct to demonstrate the effect of human CTCF expression under control of EGF-induced NFκB activation on the wound healing. EGF stimulation activated NFκB that directly triggered expression of the exogenous human CTCF in transfected cells, and subsequently promoted human corneal epithelial cell motility, migration and wound healing. Overexpression of CTCF in corneal epithelial cells and mouse corneas significantly enhanced the wound healing process. Furthermore, the effect of over-expressing NFκB p50 in corneal epithelial cells on promotion of wound healing was abolished by knockdown of CTCF with CTCF-specific shRNA. Thus, the directly regulatory relationship between EGF-induced NFκB p50 and CTCF activation to regulate corneal epithelial wound healing has been
revealed, indicating that CTCF is indeed a NFκB p50-targeted and effective gene product in the core transcriptional network downstream from the growth factor-induced NFκB signaling pathway.

Corneal epithelial layer protects the eye structures behind from environmental insults and infections to maintain the intact function of the vision system (1). Corneal epithelial cells undergo a self-renewal process to replace the surface layer cells, and repair corneal surface wounds dependent on stimulation of growth factors. Epidermal growth factor (EGF) is one of the growth factors that play important roles in corneal epithelial self-renewal and wound healing (2-9). EGF stimulates intracellular signaling pathways including NF-κB, phosphatidylinositol 3-kinase (PI3K/AKT) and mitogen-activated protein kinase (MAPK/Erk) cascades to regulate cell cycle progression and to activate transcription factors that control the genetic responses (10-14) Earlier studies demonstrate that the mitogenic effect of EGF on proliferation of corneal epithelial cells requires suppression of the eye-specific Pax6 expression (15). The effect of EGF on suppressing Pax6 expression is through activation of CTCF, an epigenetic CCCTC binding factor and zinc finger protein (16). In corneal epithelial cells, we found that EGF induces NF-κB subtype-specific signaling cascades to regulate CTCF activity and to promote cell proliferation (14). Our recent study demonstrates that CTCF is required for EGF-induced alteration of focal adhesion, and increases in cell motility and migration (17). However, the direct evidence to understand how EGF-induced activation of NF-κB and its subtypes regulates transcription activity of CTCF to affect corneal epithelial wound healing in the eye is still under investigation.

EGF activates transcription factors, such as NF-κB, CTCF and other immediate early genes upon exposure of mammalian cells to the growth factor (12,18-23). NF-κB is an important gene regulator in the Rel transcription factor family involving inflammatory responses, developmental processes, cellular growth, and apoptosis (24,25). CTCF is another gene regulator that plays important roles in epigenetic regulation of genes. It functions as an insulator sensitive to DNA methylation to epigenetically control DNA imprinting, and X chromosome inactivation during development (26-28). Many studies demonstrate that CTCF also plays roles as transcription activator and repressor. Recent studies indicate that CTCF involves in regulation of cell migration in cancer cell proliferation, tumor suppression and apoptosis (29-31). In corneal epithelial cells, EGF-induced activation of NF-κB pathway regulates cell fate in a subtype specific fashion through interactions with CTCF that functions as a downstream component in the core transcriptional network (14,32). We found in corneal epithelial cells that CTCF is a targeted gene of the growth factor-induced pathways including Erk, AKT and NF-κB signaling cascades. Activations of these signaling pathways by stimulation of EGF, insulin and other stresses subsequently regulate expression levels of CTCF to determine corneal epithelial fate in the process of wound healing (12-14,32).

As mentioned that EGF is one of the important growth factors in corneal epithelial wound healing, it facilitates corneal epithelial wound repair by promoting migration and proliferation in both in vivo and in vitro model systems (1,15,33-35). The question remained to be answered is whether CTCF is one of the key factors that directly switch EGF-induced activation of NF-κB signaling to genetic responses that subsequently change corneal
epithelial cell stages resulting in acceleration of wound healing. In the corneal surface, corneal epithelial wound healing requires proper activities of cell migration that is essential for successful re-epithelialization in the process of corneal epithelial self-renewal (1). We demonstrate that EGF-induced CTCF activation accelerates corneal epithelial cell migration that is favored for wound healing and tissue repairing in the cornea (15,16). However, results obtained for EGF-induced NFkB subtype activation are sometimes contradictory and the role of CTCF in the corneal epithelial wound healing remains unclear. The present study aimed to advance our findings that EGF-induced NFkB subtype p50 directly activates CTCF to increase cell motility and migration in human corneal epithelial cells to promote corneal epithelial wound healing. We further revealed EGF-induced activation of the NFkB p50 subtype that interacts with CTCF in the promoter region, resulting in activation of CTCF and facilitating corneal epithelial wound healing.

EXPERIMENTAL PROCEDURES

Experimental animals and cell cultures. Transgenic mice, NF-kB p50 knockout transgenic mice (NF-κB 1/−) and wild type mice were obtained from Jackson Laboratory (Bar Harbor, ME), and genotypes of these mice were confirmed by PCR analysis from prepared tail DNA. All animal experiments were conducted in accordance with the institutional guidelines of the Animal Care and Use according to NIH guidelines. Cultures of human corneal epithelial cells. Human telomerase-immortalized corneal epithelial (HTCE) cells were cultured in a keratinocyte serum-free medium (KSFM) containing 120 μM calcium and supplemented with 0.4% bovine pituitary extract and 0.2 ng/mL EGF (Invitrogen, Carlsbad, CA). Human SV-40 large T-transformed corneal epithelial (HCE) cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (1:1) containing 10% fetal bovine serum and 5 μg/ml insulin. Cells were cultured in an incubator supplied with 95% air and 5% CO2 at 37°C. Culture media were replaced every 2 days, and cells were sub-cultured by the treatment with 0.05% trypsin-EDTA. For EGF-induced experiments, cells were synchronized in growth factor–deprived media for 24-48 h before EGF stimulation.

Over-expression and knockdown of NFκB p50: Full-length cDNA encoding human p50 was cloned into pcDNA4-to-A vector (Invitrogen, Carlsbad, CA), named as pcDNA4-p50. Both of the pcDNA4-p50 construct and pcDNA4-to-A vector (served as control) were transfected into HTCE cells by FuGENE HD Transfection Reagent (Roche) for wound healing and Western analysis. For experiments of knocking down cellular NF-kB p50, p50-specific siRNA (sense: GGGGCUAUAUCCUGG-ACU and antisense: AGUCCAGAUUAU-AGCCCC) and control siRNA (Santa Cruz) were transfected into HCE cells that were subjects to wound healing assays and Western analysis.

Infections of NF-κB and CTCF cDNAs to HCE cells and mouse corneas: Lentiviral particles, containing shRNA of CTCF tagged with a variant of green fluorescent protein (Turbo-GFP; Sigma-Aldrich, St. Louis, MO), were packaged in HEK-293T cells (17). The viral concentrations in the culture medium were titrated by PCR after co-transfection of HTCE cells with pCMV-VSV-G, psPAX2, and pGIPZshRNA-CTCF fused to the GFP for 72 h (Open Biosystems, Huntsville, AL). The culture medium containing the lentiviral particles secreted from HEK-293T cells was added to HTCE cells, and infected clones stably expressing shRNAs were selected in selective culture
with puromycin (2 µg/ml). HTCE cells infected with a pGIPZ-shRNA-control vector packed in the lentivirus were served as the controls. In addition, expression of GFP from the pGIPZ-TurboGFP vector allowed measuring efficiency of the viral infection and distinguishing green from non-green cells. The green cells integrated with shRNA were visualized by a fluorescence microscope (Nikon). For corneal wound healing studies, the cDNA encoding full-length CTCF gene were introduced into a linearized Adeno-x-vector by using InFusion HD Cloning System (Clontech). The recombinant adenovirus was packaged in HEK293 cells and amplified by transfecting PaCl-digested vectors. The viral titer of 10⁷ pfu is obtained from crude viral lysates. The recombinant adenoviruses Adeno-x-vector (for controls) or Adeno-x-CTCF were added to culture medium at 2.5x10⁸ pfu per ml. After 5 days incubation, the eyeballs were transferred to a new dish containing normal defined-KSF medium without virus, and incubated for additional 10 to 15 days. The medium was changed every other day and photographed with Nikon fluorescent microscope. The wound area was calculated with the Nikon Tis NIS-Elements software.

**Constructions of NF-κB TRE-Control and TRE-CTCF.** NF-κB transcription response elements (TRE) sties were inserted in 5 repeats upstream from mini-CMV (mCMV) promoter followed by GFP or GFP plus full-length human CTCF cDNAs. The constructs were termed TRE-Control and TRE-CTCF, respectively. GFP served as a reporter of NF-κB activity. Lentiviral particles that contained NF-κB TRE-Control or TRE-CTCF, were co-transfected with pCMV-VSV-G, psPAX2 into HEK-293T cells for packaging. The culture medium containing high titer lentivirus secreted from HEK-293T cells was collected and freshly added to HTCE cells. All clones that were integrated with TRE-Control or TRE-CTCF were selected for 4 weeks by adding puromycin (2 µg/ml) into culture medium to establish stable expression cell lines.

**Wound healing assays.** Two wound healing assays were performed including corneal wound healing in cultured whole-eye organ and scratch-induced directional wound-healing assay. **Corneal wound healing assays:** The corneas in cultured whole-eye organs were used for experiments of corneal epithelial wound healing. Under a dissecting microscope, the surface layer of the mouse corneas was debrided without damaging the basement membrane of the corneal epithelia using a corneal rust ring remover with a 0.5-mm burr (Algerbrush; The Alger Company, Inc., Lago Vista, TX). The whole eye ball was dissected and placed in culture wells (the corneas facing up) with the medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic solution at 37°C and 5% CO₂ in a humidified incubator. The rate of epithelial healing in whole-eye organ culture was measured immediately after wounding. Eyeballs were taken from wildtype or NF-κB p50-knockout mice and allowed to heal in the culture conditions. Lesions of the corneas were topically stained with fluorescein (fluorescein sodium 1.0% wt/vol) and photographed with an inverted microscope (Nikon). The corneal epithelial layer was removed in an area of 1.5 mm in diameter (or 2 mm in diameter for Ad-x-CTCF transduction assays) near the central cornea. Wounded corneas were incubated for 1-2 days in the normal and EGF induced conditions and up to 15 day in the absence of FBS and EGF. All animals used in our experiments were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the LAbioMed Animal Care Committee at University of California Los Angeles School of Medicine. **Scratch-induced directional**
wound-healing assay: Corneal epithelial cells were seeded at 3 x 10^5 cells/well in 12-well plates and grown to 100% confluence. A cross-stripe scratch wound was made on the cell surface with a yellow micropipette tip. The wound area was measured by calculating the average values at multiple points (at least 10 points per wound along the edges) by using the commercial software (NIS-Element; Nikon, Tokyo, Japan) and was photographed with an inverted microscope (Eclipse Ti; Nikon) during the healing period. The microscope was able to record exactly the same area at each time point, by memorizing the x–y directions through a computer-controlled and motorized head stage. The width of wounded areas was measured, and the rate of wound closure was calculated in a scale of micrometers per hour.

Cell migration assays. The cell migration assay was performed following the instructions of the manufacturer (Transwell; Corning Inc., Corning NY). The migration chamber culture insert contains a polyethylene terephthalate membrane 6.5 mm in diameter with an 8 µm pore size. HTCE cells expressing NF-κB TRE-CTCF or TRE-Control (5 x 10^4) were seeded in the culture insert (upper chamber) with plain medium and incubated for 24 h. EGF (20 ng/ml) or the sham was added to the culture insert and the cells were incubated for 48 hours. Migrated cells that grew on the culture well (bottom chamber) were counted and photographed with an inverted fluorescence microscope (Nikon). The cells were fixed in 4% paraformaldehyde, stained with 0.3% crystal violet (CV), and photographed. The dye in the cells was then dissolved in 10% acetic acid, and the absorbance of the dissolved dye was measured at a wavelength of 600 nm.

Live cell imaging and cell motility analysis. The Motility of HTCE cells expressing NF-κB TRE-CTCF and TRE-Control was measured by utilization of the inverted microscope (Eclipse Ti; Nikon) with the following functions: (1) time-lapse videos of the phase-contrast/fluorescent live images; (2) built-in TIRF (total internal reflection fluorescence) and FRET; (3) perfect focus system (PFS); and (4) a digital CCD camera in a time interval of 2 minutes for each photo. The system was equipped with a heated chamber at 37°C and flushed with mixed 5% CO_2 that kept cells in a normal culture condition. Live cells were recorded for a period of 0.5 to 3 h. Cell motility was examined by tracking cell movements and distances (mm/h) using an inverted microscope with motorized head-stage and software (Tis NIS-Elements; Nikon).

Immunocytochemistry and Western blots. Immunocytochemistry experiments were performed following a protocol as described previously (36). Briefly, mouse eyeballs were fixed with 4% paraformaldehyde and sectioned with 8 µm. The tissue section perforated with 0.3% triton-100 in PBS (PBS-T). After blocked with 2% BSA and 5% normal serum in PBS-T, the sections were incubated with primary antibody against CTCF (Millipore) in 1% BSA-0.1% triton 100-PBS for 16 h at 4 °C. Cy3-conjugated secondary antibody was applied in 1% BSA-0.1% triton 100-PBS for 1 h at room temperature (RT). Stained tissues were mounted with shield mounting medium (Vector Laboratories Inc.) and photographed using the Nikon Eclipse Ti inverted microscope with 60x oil TIRF lens. Western blots were performed by lysing corneal epithelial cells (2 x 10^5) in sodium dodecyl sulfate-polyacrylamide (SDS) sample buffer that contains 62.5 mM Tris-HCl (pH 6.8), 2% (W/V) SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue. Proteins in the lysates were denatured by boiling for 5 min, and size-fractionated in 8-10% PAGE gels. Proteins in PAGE gels
RESULTS

Retarded corneal epithelial wound healing in NF-κB p50−/− mice. Recent reports demonstrate that corneal epithelial wound healing is affected by deficiency of IκB (in the NF-κB pathway) in the eye of IκB-knockout mice (37). We predicted that the NF-κB subtype p50 deficiency would result in a delay of corneal epithelial cell wound healing. In the study, we utilized eyes from NF-κB p50 knockout mouse to test the effect of NF-κB p50 on corneal epithelial wound healing. The corneas from the same litter of wildtype (WT), heterozygous (p50+/−) and p50 knockout (p50−/−) mice were characterized by specific PCR genotyping (Fig. 1A). The corneas from eyes of these mice were subjects to the defined surface injuries by using an Algerbrush corneal rust ring. The eyeballs containing the corneas with wounds in the epithelial layer (shown in green of fluorescein staining) were incubated in normal KSF medium during tests of wound healing. We found that the rate of corneal epithelial wound healing was significantly slower in NF-κB p50−/− mice than that was observed in wild-type mice after 16 h incubation (Figs. 1B and 1C), while there was no obvious changes found in the eye of their p50+/− heterozygous siblings (data not shown). The result reveals for the first time that NF-κB p50-induced signaling pathway activation plays a functional role in growth factor-stimulated corneal epithelial wound healing.

Expression of NF-κB subtype p50 promoted wound healing. To answer the first question whether NF-κB subtype p50 plays a role in wound healing in corneal epithelial cells, we applied two opposite approaches in HCE cells including: 1) knocking down NF-κB p50 using specific siRNA, and 2) over-expressing NF-κB p50 cDNA. We found that knockdown of NF-κB p50 markedly delayed wound closure of human corneal epithelial cells (Figs. 2A and 2B). Western analysis verified the decrease of NF-κB p50 expression in NF-κB p50 knocked down HCE cells (Fig. 2C). Conversely, over-expression of NF-κB p50 by transfecting NF-κB p50 cDNA in HCE cells resulted in accelerated corneal epithelial wound closure when it was compared with vector-transfected control cells (Figs. 2D and 2E). The protein level of NF-κB p50 was also verified that the p50 level was increased in p50 over-expressed cells (Fig. 2F). These results indicate that increased and decreased cellular activities of NF-κB subtype p50 promoted and suppressed wound healing of HCE cells, respectively.

Suppression of NF-κB p50 overexpression-induced acceleration of wound healing by knocking down CTCF. We reported that expression of CTCF was regulated by NF-κB subtype p50 in human corneal epithelial cells (14). In the present study, NF-κB p50-specific cDNA and shRNA were used to overexpress and knock down the p50 in these cells, respectively. Overexpression of CTCF markedly increased rate of HCE cell wound healing following a 24 h period (Fig. 3A&3B). In contrast, knockdown of CTCF...
mRNA with CTCF-specific shRNA significantly suppressed HTCE cell wound healing rate within a 30 h (Fig. 3C&3D). Knockdown of p50 effectively suppressed expression of CTCF in HCE cells (Fig. 4A, left panel). In contrast, over-expression of NF-κB p50 markedly increased CTCF expression (Fig. 4A, right panel). The correlation of NF-κB p50 protein level and CTCF expression was demonstrated in these cells. The effect of EGF-induced activation of NF-κB p50 on CTCF expression was next examined in the corneas of wild-type and NF-κB p50−/− mice. Immunostaining experiments revealed that EGF failed to stimulate CTCF expression in the basal layer in the NF-κB p50-deficient corneal epithelium of p50−/− mice compared with the cornea of wild-type mice (Fig. 4B). Control experiment was done by performing staining in PBS without antibodies. Based on the observation that EGF failed to stimulate expression of CTCF expression in the p50−/− mouse cornea, we predicted that blockage of CTCF should prevent NF-κB p50- accelerated wound healing. Full-length cDNA encoding the NF-κB p50 was introduced into HCE cells in which the CTCF was already suppressed by CTCF-specific shRNA. Western analysis revealed that knocking down CTCF mRNA markedly diminished the effect of NF-κB p50 over-expression on CTCF activation (Fig. 4C). We found that over-expression of NF-κB p50 in cells in which CTCF was knocked down yielded very different results in the process of wound healing. The wound closure process in these cells was effectively accelerated by over-expression of NF-κB p50. However, knockdown of CTCF mRNA by CTCF shRNA abolished or delayed effects of NF-κB p50 over-expression and EGF-induced wound closure rate in HTCE cells (Fig. 4D). Statistical analysis further verified that knockdown of CTCF significantly suppressed the NF-κB p50 over-expression and EGF-induced effects on acceleration of corneal epithelial wound closure (Figs. 4E). The results indicate that CTCF plays a role in mediating NF-κB p50-induced acceleration of wound healing.

**EGF-induced over-expression of NF-κB-controlled CTCF.** To further study whether the effect of NF-κB on EGF-induced wound healing is mediated by CTCF, we generated a NF-κB-regulated exogenous CTCF over-expression constructs. As shown in upper panel of Fig. 5A, 5 repeats of NF-κB transcription factor response elements (TRE) were inserted into a specific location in an expression vector upstream from the mini-CMV promoter (mCMV). Green fluorescent protein (GFP, TRE-control) and GFP linked CTCF (TRE-CTCF) cDNAs were individually inserted in the vector immediately downstream from the mCMV promoter to allow that cellular NF-κB directly regulate the expression of CTCF in stably transfected cells. TRE-control and TRE-CTCF constructs were individually introduced into HTCE cells by the lentivirus-mediated gene transferring. We found that EGF and TNFα stimulated expression of GFP in both TRE-control and TRE-CTCF expressing HTCE cells (Figs. 5B, lower panel). Activation of the mCMV promoter was upon the NF-κB binding to NF-κB TRE regions resulting in transcription of GFP and CTCF simultaneously. As expected, TRE-CTCF expressing cells showed green color under a fluorescent microscope and produced exogenous CTCF proteins. Thus, expression of GFP and CTCF controlled by NF-κB activation in response to EGF or TNFα stimulation could be visualized in these cells. Western analysis showed that expression of CTCF was significantly increased upon stimulation of EGF in TRE-CTCF expressing HTCE cells compared with TRE-control expressing HTCE cells (Fig. 5B). The results demonstrate that EGF- and TNF-
α-induced activation of NF-κB promoted expression of exogenous CTCF through a NF-κB-controlled mechanism and verified the interaction of NF-κB with its downstream target CTCF in HTCE cells.

**Effect of NF-κB-controlled CTCF overexpression on EGF-induced wound healing.** Next, we examined the effect of EGF-induced exogenous CTCF expression controlled by NF-κB on motility, migration and wound closure of HTCE cells. We found that cell migration was significantly increased in TRE-CTCF expressing HTCE cells in the absence and presence of EGF stimulation compared with the TRE-control expressing counter part (Figs. 6A and 5B). In addition, we tracked the cells to measure cell motility changes under a computer-controlled fluorescent microscope with the motorized head-stage in an attached culture chamber for 50 min (Fig. 6C). Statistical analysis revealed that EGF stimulated an increase in cell motility up to 30% in TRE-CTCF expressing HTCE cells compared with TRE-control expressing cells (p<0.05, n=86; Fig. 6D). The rate of wound closure was also significantly accelerated in TRE-CTCF expressing HTCE cells compared with the cells that were expressing TRE-control (Figs. 6D and 6F). The results provide further evidence in three experiments that EGF-activated NF-κB upregulated expression of CTCF to promote increases in cell motility and migration resulting in acceleration of corneal epithelial cell wound healing.

**Effect of Ad-x-CTCF transduction on corneal epithelial wound healing.** To test whether CTCF overexpression would accelerate corneal epithelial wound healing in vivo in the absence and presence of NFκB p50, eyeballs obtained from wildtype and p50-knockout mice (p50−/−) were infected with adenoviral Ad-x-CTCF and Ad-x-vector (for controls). Both adenoviral Ad-x-CTCF and Ad-x-vector containing GFP expression marker were tested in HCE cells for efficiency of the gene transferring before they were used for eyeball infections. We found that transferring efficiencies for both constructs reached more than 95% within 2 days determined by Western blots (data not shown). Corneas transduced with Ad-x-CTCF and Ad-x-vector were wounded, and the healing process was monitored every day up to 15 days. There were significantly increased wound healing rate in 10 days in wildtype mouse corneas over-expressing CTCF compared with Ad-x-vector infected controls (Fig. 7A&7B). There were marked delays in wound healing rate in p50−/− corneas compared with wildtype corneas with/without over-expression of Ad-x-CTCF (Fig. 7C&7D). Acceleration of corneal epithelial wound healing transduced by Ad-x-CTCF was observed in corneas of wildtype mice and transduction of CTCF had a less effect on the wound healing in corneas from p50 knockout mice, indicating that the effect of CTCF on corneal epithelial wound healing is associated with the NFκB-p50 subtype pathway.

**DISCUSSION**

One of the major stimuli to stimulate corneal epithelial wound healing is epidermal growth factor (EGF) that promotes cell migration and proliferation (1). As it has been demonstrated in human corneal epithelial cells, EGF elicits complex responses at early times by inducing specific cellular signaling pathways that transfer the signals to the nucleus and to activate transcription factor at later times (3,14,15,33,38). In previous studies, we found: 1) EGF-induced formations of NFκB p65/p50 heterodimer and p50/50 homodimer activate CTCF transcription by binding to a κB site located in the promoter region of...
CTCF gene; and 2) increase in CTCF activity in corneal epithelial cells promotes cell motility and migration (14,17). These results indicate that NFκB subtypes directly interact with CTCF gene in the promoter region. However, it is unknown whether this interaction has functional significance in corneal epithelial wound healing. In the present study, we provided direct evidence for the first time to connect EGF-induced CTCF activation through NFκB subtype p50 and to subsequently regulate corneal epithelial wound healing in HTCE cells and in the corneas of mice. The effect of NFκB p50 on EGF-induced wound healing was examined in HTCE cells when the cellular level of the p50 was knocked down by NFκB p50 siRNA or enhanced by over-expression of the p50. Suppressed and enhanced expressions of NFκB p50 resulted in deceleration and acceleration of cell wound healing, respectively. This observation is consistent to the report indicating that NFκB p50 activation promotes migration in breast cancer cells (39). There was also a parallel relationship between knockdown/over-expression of NFκB p50 and decrease/increase in CTCF activities in HTCE cells. In addition, over-expression of NFκB p50 enhanced CTCF expression, but it failed to activate CTCF in the presence of CTCF shRNA transfection (Fig. 4). Further evidence that the effect of over-expression of NFκB p50 on promoting HTCE cell wound healing was retarded by knocking down CTCF mRNA indicate that CTCF is indeed a downstream target which plays a key role in EGF-induced NFκB signaling cascades to regulate corneal epithelial wound healing.

An in vivo model system containing TRE-control and TRE-CTCF expression constructs was engineered with multiple κB binding sites to sense EGF-induced changes of intracellular NFκB activity that controls expression levels of GFP and CTCF. Upon stimulation of EGF and TNF-α, exogenous GFP and CTCF proteins were expressed in transfected cells under controls of cellular NFκB p50 activities (Fig. 5). Thus, transfection of TRE-CTCF in to HTCE cells enable us to monitor both effects of EGF-induced NFκB activation and NFκB-controlled CTCF activity on corneal epithelial wound healing. It appeared that there were more green cells in TRE-control transfected cells compared with TRE-CTCF expressing cells. The different level of GFP expression may be resulted from the size difference due to linking the CTCF cDNA to GFP in the TRE-CTCF construct. In addition, TNF-α-induced cells displayed a stronger expression of GFP than those of EGF-induced cells, indicating that there were probably more NF-κB activations induced by TNF-α in these cells.

Corneal epithelial wound healing is largely dependent on the motility and migration capability of the basal layer cells. As the first step, the effect of EGF-induced NFκB activation to regulate exogenous CTCF on corneal epithelial cell migration and motility were studied by measuring transwell migrated cell population and continuous cell tracking. Increase in expression of exogenous CTCF in response to EGF-induced NFκB activation significantly increased cell migration and motility. The basal migration rate of TRE-CTCF expressing cells was also faster than TRE-control expressing HTCE cells even in the absence of EGF stimulation. As expected, we found that EGF induced a significant increase in wound healing rate in HTCE cells expressing TRE-CTCF compared with TRE-control expressing cells. The result of wound healing study is consistent with the finding that TRE-CTCF transfection and expression significantly increased migration and motility of TRE-CTCF expressing HTCE cells (Fig. 6). EGF stimulation
activated NFκB p50 activity in the cornea of wild-type mice, but it was not seen in NFκB p50-knockout mouse corneas. In the absence of EGF, corneal epithelial wound healing was much slower in eyeballs of both wildtype and p50−/− knockout mice (Fig. 7). Overexpression of CTCF by adenovirus-mediated CTCF transduction enhanced corneal epithelial wound healing in both wildtype and p50−/− knockout mice, which is consistent with the results observed in wound closure assays in HCE cells. The observation provides useful evidence to support that CTCF is a downstream component in the NFκB pathway. It also suggests that the effect of CTCF on wound healing is not exclusively regulated by NFκB p50. In fact, CTCF can be activated by other proliferative signaling pathways as well, such as the Erk pathway (15). In addition, at post-translational level, it has been shown that CTCF is also a target protein subjective to modifications of SOMOylation and phosphorylation (40).

Finally, the present study is for the first time to demonstrate that corneal epithelial wound healing was significantly delayed in the cornea of NFκB p50 knockout mice. There was more than 30% delayed in corneal epithelial wound healing in the cornea of NFκB p50 knockout mice, suggesting that the NFκB signaling pathway plays important roles in growth factor-promoted corneal epithelial self-renewal and corneal protective functions.

Acknowledgments: This work was supported by grants from NIH (R01-EY015281 & EY022364) to L.L..

REFERENCES

**FIGURE LEGENDS**

*Figure 1. Effect of NFκB p50-dientent on corneal epithelial wound healing.* A. Knockout of NFκB p50 verified by genotyping in NFκB p50-/- mice obtained for the Jackson Laboratory. B. Retarded corneal epithelial wound healing in corneas of NFκB p50-/- knockout mice. C. Statistical analysis of corneal epithelial wound healing rates between the eyes of wild-type and NFκB p50-/- knockout mice. The corneal epithelial layer was debrided without damaging the basement membrane by using a corneal rust ring remover with a size of 0.5-mm burr. Lesions of the corneal surface were topically stained with fluorescein demonstrated by green colors in the
photos, and photographed with an inverted microscope (Nikon). Symbol ‘∗’ represents the statistical significance and data were collected from 27 independent experiments (n=27, p<0.05).

**Figure 2. Effect of altering NFκB p50 activity on HCE cell wound healing.** A. Effect of knocking down NFκB p50 on EGF-induced wound closure. B. Significant retardation of wound closure in NFκB p50 knocking down cells in the absence and presence of EGF induction. C. Western analysis of NFκB p50 expression in NFκB p50-knocked down cells. D. Effect of over-expressing NFκB p50 on EGF-induced wound closure. E. Significant acceleration of EGF-induced wound closure in NFκB p50 over-expressed cells. F. Western analysis of NFκB p50 expression in NFκB p50-over-expressed cells. Expression levels of NFκB p50 were altered to be suppressed and enhanced by knocking down HCE cells by knocking down NFκB p50 mRNA and over-expressing full-length cDNA encoding NFκB p50, respectively.

**Figure 3. Effects of altered CTCF activity on HCE and HTCE cell wound healing.** A. Effect of over-expression of CTCF on corneal epithelial cell wound closure. B. Significant acceleration of wound closure in CTCF over-expressing cells. C. Effect of knocking down CTCF on corneal epithelial cell wound closure. D. Significant retardation of wound closure in CTCF knocking down cells. HCE and HTCE cells were infected by lentiviral vector containing cDNA encoding full-length CTCF and CTCF-specific hsRNA to over-express and knock down CTCF, respectively. Symbol ‘∗∗’ represents the statistical significance and data were collected from 4 sets of independent experiments (n=4, p<0.05).

**Figure 4. Effect of suppressing CTCF on over-expression of NFκB p50-enhanced wound healing.** A. Effects of knocking down NFκB p50 mRNA and over-expression of p50 cDNA on CTCF activity. B. Lacks of EGF-induced CTCF expression in corneas of NFκB p50−/− knockout mice detected by immunostaining. The corneas obtained from wildtype and NFκB p50−/− knockout mice with/without stimulation of EGF were compared in immunostaining sections. C. Effect of knocking down CTCF on EGF-induced CTCF expression detected by Western analysis. D. Effects of over-expressing NFκB p50 on wound closure in CTCF-knocked down cells following a time course. E. Significant delay of NFκB p50 over-expression and EGF-induced wound closure by knockdown of CTCF. Symbol ‘∗∗’ represents the statistical significance and data were collected from 4 sets of independent experiments (n=4, p<0.05).

**Figure 5. Monitoring effect of EGF-induced NFκB activation on control of CTCF expression.** A. Effects of EGF- and TNF-α-induced changes of intracellular NFκB activity on exogenous CTCF expression. Upper panel illustrates the NFκB activity-controlled CTCF in vivo expression system containing TRE-control and TRE-CTCF constructs. Lower panel shows that EGF- and TNF-α-induced expressions of exogenous GFP and GFP+CTCF in lentiviral infected HTCE cells. B. EGF-induced significant increase in exogenous CTCF expression. Expression of exogenous CTCF in cells that were transfected with NFκB activity-controlled CTCF construct, was detected by Western analysis in the absence and presence of EGF or TNF-α. Data were collected from 4 independent experiments and symbol ‘∗∗’ represents the statistical significance (n=4, p<0.05).

**Figure 6. Effect of NFκB-controlled CTCF activity on EGF-induced wound healing.** A. Effect of NFκB-controlled CTCF activation on EGF-induced cell migration. B. Significant increase in
migration by EGF-induced and NFκB-controlled CTCF activation. HTCE cell migration was measured by transwell migration assays. Symbols “*” and “**” represent significant differences when compared cells between TRE-control and TRE-CTCF groups in the absence and presence of EGF, respectively (n=4, p<0.05). C. EGF-activated cell motility enhanced by NFκB-controlled CTCF activation. D. Statistical significance of motility increase by NFκB-controlled CTCF activation in EGF-induced cells. HCE cell motility was measured by using cell-tracking function of a Nikon fluorescent microscope. Symbol “*” represents significant increase in cell motility when compared cells between TRE-control and TRE-CTCF groups in response to EGF stimulation (n=86, p<0.05). E. EGF-activated wound closure enhanced by NFκB-controlled CTCF activation. F. Statistical significance of accelerated wound closure by NFκB-controlled CTCF activation in EGF-induced cells. HCE cell wound closure was measured by a scratch-induced directional wound-healing assay. Symbol “*” represents the significant increase in wound closure when compared cells between TRE-control and TRE-CTCF groups in response to EGF simulation (n=4, p<0.05).

Figure 7. Effect of adenovirus-mediated CTCF transduction on corneal epithelial wound healing. A. Effect of over-expressing Ad-x-CTCF on mouse corneal epithelial wound healing. B. Significant increase in wound healing rate in Ad-x-CTCF transduced corneas. C. Effects of over-expressing Ad-x-CTCF on corneal epithelial wound healing of p50⁻/⁻ knockout mice. D. Comparison of wound healing in corneas of wildtype and p50⁻/⁻ knockout mice with/without overexpression of CTCF. Green colors were cells expressing GFP after adenoviral transduction of the corneal surface. Arrows indicate nude regions of the corneal surface without epithelial coverage. The left eye of each mouse was subject to experiments and the right eye was served as the control. Symbol “*” represents the significant difference when compared with respective control group (n=4, p<0.05).
Figure 1

A

Mouse tail DNA

bp

190

100

p50  +/+  +/-  -/-

B

0 h 16 h

Wildtype

NFκB p50-/-

C

Wound closure (%)

Wildtype  NFκB p50-/-

*
Figure 2

A

<table>
<thead>
<tr>
<th>0h</th>
<th>18h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>EGF</td>
</tr>
</tbody>
</table>

B

- si-Ctrl
- si-p50

<table>
<thead>
<tr>
<th>Wound closure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>EGF</td>
</tr>
</tbody>
</table>

C

- p50
- β-actin

si-Ctrl | si-p50

D

- p50-OE
- Vector

<table>
<thead>
<tr>
<th>Wound closure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
</tr>
<tr>
<td>p50-OE</td>
</tr>
</tbody>
</table>

Figure 2
Figure 3

(A) Images showing wound closure at 0h, 6h, 12h, and 24h for Vector and CTCF-OE conditions.

(B) Graph showing wound closure percentage at 6h, 12h, and 24h for Vector and CTCF-OE conditions. CTCF-OE shows significantly higher wound closure compared to Vector.

(C) Images showing wound closure at 0h, 10h, 24h, and 30h for NS shRNA and CTCF shRNA conditions.

(D) Graph showing wound closure percentage at 10h, 24h, and 30h for NS shRNA and CTCF shRNA conditions. CTCF shRNA shows significantly lower wound closure compared to NS shRNA.

* indicates significant difference.
Figure 4

A. Graph showing arbitrary number of CTCF, p50, and β-actin in si-Ctrl, si-p50, Vector, and p50-OE conditions.

B. Western blot analysis of CTCF, p50, and β-actin in Wild-type and NFkB p50−/− conditions under Control and EGF treatment.

C. Graph showing arbitrary number of CTCF and β-actin in NS shRNA, CTCF shRNA, NS shRNA (Control), and CTCF shRNA (EGF) conditions.

D. Images of wound closure (% EGF Control, p50-OE, CTCF shRNA, NS shRNA) at 0h, 8h, 13h, and 19h.

E. Bar graph showing wound closure (% Control and EGF) at 8h, 13h, and 19h for NS shRNA, CTCF shRNA, NS shRNA (Control), and CTCF shRNA (EGF) conditions.
Figure 5

A

NFκB TRE

EGF

NFκB (EGF-activated NFκB binds to TRE sites)

TRE-CTCF

B

Arbitrary number

Control  EGF  TNF-α

Tre-Control

TRE-CTCF

CTCF

GFP

Downloaded from http://www.jbc.org/ by guest on September 3, 2017
Figure 6

Panel A: Images showing cell cultures with or without EGF treatment.

Panel B: Bar graph showing the number of arbitrary units comparing TRE-Control and TRE-CTCF in the presence of EGF.

Panel C: Microscope images comparing TRE-Control and TRE-CTCF at 0 min and 50 min.

Panel D: Bar graph showing cell motility and moving distance (μm/h) in the presence of EGF.

Panel E: Microscope images comparing TRE-Control and TRE-CTCF at 0h and 16h.

Panel F: Bar graph showing wound closure (%) in the presence of EGF.

Legend:
- Ctrl: Control
- EGF: Epidermal Growth Factor
- TRE-Control
- TRE-CTCF

Statistical significance indicated by:
- *: p < 0.05
- **: p < 0.01
Figure 7

Ad-x-Vector
Ad-x-CTCF
Wildtype
p50−/

A

B

Wound closure (%)

3 days 10 days 15 days

Ad-x-Vector Ad-x-CTCF

* * *

C

D

Wound closure (%)

Wildtype p50−/

Ad-x-Vector Ad-x-CTCF Ad-x-Vector Ad-x-CTCF

* *
EGF-induced Corneal Epithelial Wound Healing Though NF-κB Subtype-Regulated CTCF Activation
Ling Wang, Xiaolin Wu, Ting Shi and Luo Lu

J. Biol. Chem. published online July 10, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M113.458141

_alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2013/07/10/jbc.M113.458141.full.html#ref-list-1