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# An *in vitro* analysis of mechanical wounding-induced ligand-independent KGFR activation☆

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SUMMARY

*Background:* KGFR (keratinocyte growth factor receptor), exclusively expressed in epithelial cells, plays an important role in wound healing. However, mechanisms of KGFR activation and signaling in wound healing are not clearly understood.
*Objectives:* We utilized an *in vitro* mechanical wounding model to examine ligand-independent KGFR activation, its regulation by reactive oxygen species (ROS) and the functional significance of this activation mechanism.
*Methods:* Confluent HaCaT cell line cultures were mechanically wounded and KGFR internalization and phosphorylation were examined using immunostaining with confocal microscopy and immunoprecipitation with Western blotting. Wounding-induced generation of reactive oxygen species and ligand-independent activation of KGFR were examined. In addition, phosphorylation of its associated molecules FRS2 and c-Src were examined in the presence and absence of the ROS and pathway specific inhibitors. The importance of this activation process on cell migration was also examined in the presence and absence of these inhibitors.
*Results:* Mechanical wounding induced ligand-independent KGFR activation and internalization. KGFR internalization and phosphorylation was associated with ROS generation along the wound edge and scavenging of ROS with NAC inhibited KGFR phosphorylation. Intracellularly, c-Src was phosphorylated by wounding but its inhibitor, PP1, significantly inhibited KGFR activation and associated FRS2 phosphorylation. Mechanical wounding induced wound edge migration, which was significantly reduced by the selective receptor and pathway inhibitors PP1 (82.7%), KGFR inhibitor SU5402 (70%) and MAPK inhibitor PD98059 (57%).
*Conclusion:* Mechanical wounding induces significant ROS generation at the wound edge which, in turn, induced ligand-independent KGFR and FRS2 activation via c-Src kinase signaling. Functionally, downstream MAPK signaling induced wound edge cell migration.


## 1. Introduction

KGFR (keratinocyte growth factor receptor), also described as FGFR2IIIb is exclusively expressed on epithelial cells and classically responds in a paracrine manner to growth factors expressed by cells associated with the adjacent connective tissue [1,2]. This ligand–receptor complex regulates epithelial cell behavior during wound healing and in chronic inflammation [3–5]. Disregulation of this signaling complex in transgenic mice expressing a dominant-negative FGFR2IIIb mutant in basal keratinocytes caused delayed wound re-epithelialization [6]. Therefore signaling through the KGFR is critical for normal wound healing. This signaling may be mediated in either a ligand-dependent or ligand-independent manner. Ligand-dependent signaling induces downstream phosphorylation of FGFR-associated molecule FRS2 that in turn activates MAPK, PLCγ, and P13K. Collectively these signaling pathways regulate proliferation, differentiation, migration and cell survival [7]. Ligand-independent signaling has been described for a number of tyrosine kinase receptors as well. However, the ability of mechanical wounding to activate KGFR signaling in a ligand-independent manner has not yet been described.

ROS (reactive oxygen species) generation can induce tyrosine kinase ligand-independent receptor signaling. Generation of ROS can act as a secondary messenger involved in receptor activation.

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ROS, such as superoxide radicals and hydroxyl radical, and their derivative, hydrogen peroxide ($H_2O_2$), are generated in response to a variety of external stimuli such as heat, ultraviolet (UV) light, infection, and mechanical wounding [8,9]. Specifically, the IGF receptor [10], PDGF receptor [11], EGFR [12–14], and Src kinase, a non-receptor tyrosine kinase [15,11] have all been shown to be activated by ROS. Intracellulary c-Src plays an intermediary signaling role in this ligand-independent receptor activation [16,17].

KGFR is also activated in the presence of ROS. Generation of ROS in UVB-treated keratinocytes or increased ROS in cells treated with the pro-oxidative agent cumene hydroperoxide all induced ligand-independent KGFR activation and internalization [18,19]. Conversely KGFR activation and internalization was abolished by pre-incubation with ROS inhibitor NAC (N-acetyl-L-cysteine) [19,20]. This ligand-independent KGFR activation induced by generation of ROS is signaled through similar downstream pathways used by ligand-dependent KGFR activation [18]. We have previously shown KGFR clustering at the wound edge in HaCaT epithelial cultures under ligand-independent conditions [22]. However, the mechanism by which wounding leads to KGFR-mediated signaling has yet to be elucidated.

The purpose of this study was three-fold. First we determined the functional activity of KGFR post-wounding using an *in vitro* microwounding cell culture model [21,22]. Second, we examined the mechanism by which ligand-independent KGFR phosphorylation was regulated and third we explored the downstream signaling pathways that were activated and the functional significance of this activation in a wound healing context.

## 2. Materials and methods

### 2.1. Cell culture

HaCaT keratinocytes, a generous gift from Dr. Hubert Fusenig (German Cancer Center, Heidelberg, Germany), were cultured in a humidified 95% air, 5% $CO_2$ incubator at 37 °C and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD) supplemented with 10% fetal bovine serum (Gibco, Rockville, MD), 100 mg/ml streptomycin sulfate, 100 U/ml penicillin and 0.25 mg/ml amphotericin B. Subsequently a defined media made up of DMEM plus 100 mg/ml streptomycin sulfate, 100 U/ml penicillin, 0.25 mg/ml amphotericin B, 5.0 μg/ml insulin, 0.5 mM hydrocortisone, 0.1 mM ethanolamine and 0.1 mM phosphoethanolamine was used to quiesce the cultures and all post-wounding experiments [21,22].

### 2.2. Inhibitors and antibodies

SU5402 (Calbiochem, United Kingdom), wortmannin (Calbiochem, United Kingdom), Calphostin C and PD98059 (Sigma, St. Louis, USA), PP1 (BIOMOL Research Laboratories), N-acetyl-L-cysteine (NAC), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Sigma, St. Louis, USA), rabbit anti-KGFR (Bek, C-17), rabbit anti-FGFR1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-KGFR functional blocking antibody (Cat# MAB665, R&D System, Minneapolis, MN), mouse anti-phosphotyrosine (PY) (R&D, Minneapolis, MN), agarose-conjugated mouse monoclonal anti-phosphotyrosine antibody (R&D, Minneapolis, MN), rabbit polyclonal anti-FRS2 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit monoclonal anti-phospho-Src (Tyr416) (Upstate, Lake Placid, NY), rabbit anti-ERK1/2 polyclonal antibody (Chemicon International, Inc., Temecula, CA), rabbit anti-phospho-ERK1/2 polyclonal antibody (Chemicon International, Inc., Temecula, CA). KGF-1 (Upstate, Lake Placid, NY), secondary anti-rabbit (mouse) IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa Fluor 546 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse antibodies (Molecular Probes, Burlington, Canada), and rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

### 2.3. In vitro microwound models

#### 2.3.1. Single mechanical wound

$5 \times 10^4$ cells were plated on Lab-Tek 8-well glass chamber slides (Nalge Nunc International, Rochester, NY) and cultured to confluence prior to quiescing in defined medium for 8 h and wounded once across the maximum diameter with a pipette tip. At select time points cultures were fixed and prepared for immunohistochemical analysis.

#### 2.3.2. Multiple mechanical wound model

HaCaT cells were cultured to confluence in 100 mm cell culture plates and quiesced in defined medium for 8 h. Multiple parallel wounds were generated by a 2 cm portion of a standard plastic hair comb. Non-overlapping wounds across the diameter were made at 0°, 45°, 90° and 135° to the original scrapes. Cells were washed and incubated in defined medium with or without functional blocking antibodies or inhibitors from 0 to 24 h. Cell lysates were prepared for immunoprecipitation and Western blotting.

### 2.4. Immunostaining and confocal laser scanning microscope

Cultures on glass tissue culture chamber slides were fixed with 2% paraformaldehyde + 5% sucrose in PBS, permeabilized with 0.5% Triton X-100 in PBS for 4 min, washed 10 min × 5 times in PBS and blocked using 3% bovine serum albumin (BSA) + 1% glycine in PBS for 30 min at room temperature. Samples were incubated with either primary rabbit anti-KGFR (1:50) or anti-FGFR1 antibodies followed by mouse anti-phosphotyrosine (1:50) antibody in blocking solution for 1 h. In addition, samples were fixed and double stained with anti-KGFR and anti-phosphotyrosine. Slides were washed with 1 mg/ml BSA in PBS 3 × 5 min prior to incubation with anti-rabbit (Alexa Fluor-546, Molecular Probes, Eugene, Oregon) and anti-mouse (Alexa Fluor-488, Molecular Probes, Eugene, Oregon) secondary antibody for 1 h at room temperature in the dark. Slides were washed 5 × 3 min with PBS, mounted with Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL). All samples were analyzed using confocal laser scanning microscope (Nikon D-eclipse, Japan) with multiple *z*-axis sections taken every 2 μm. Sections representing the mid-cell region were used to demonstrate KGFR internalization.

### 2.5. Immunoprecipitation and Western blot analysis

For Western blot analysis multiple wounds were prepared in 100 mm dishes and at time points from 0 to 60 min post-wounding. The plates were washed 3 times with ice cold PBS and cells were lysed in RIPA buffer (1× TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide) (Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with the proteinase inhibitors (1 mM PMSF, protease inhibitor cocktail and 1 mM sodium orthovanadate (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C. Lysates were centrifuged for 20 min at 10,000 G at 4 °C and the supernatant collected. All samples were standardized to equal protein concentration (100 μg), fractioned under reducing condition on a 7.5% SDS-polyacrylamide gel and transferred to Immobilon PVDF Transfer Membrane (polyvinylidene difluoride) (Millipore Corporation, Bedford, MA). The membrane was blocked with 5% skim milk in PBS with 0.1% Tween-20 for 1 h at room temperature, washed with 0.1% Tween-20 PBS prior to incubation