

## Protein engineering, expression, and activity of a novel fusion protein possessing keratinocyte growth factor 2 and fibronectin

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**Growth factor-induced proliferation and differentiation often require adhesion of cells to the extracellular matrix proteins such as fibronectin (FN). In this study, we aimed to investigate the effect of protein engineering of the keratinocyte growth factor 2 (KGF2) fused to the FN on the mitogenic activity of KGF2. The fusion protein (KGF2-FN10), which was expressed in *Escherichia coli*, showed significantly enhanced mitogenic activity of KGF2 on human keratinocytes. Moreover, KGF2-FN10 fusion protein showed significantly increased activity to differentiate keratinocytes from native KGF2. In conclusion, these results suggest that KGF2-FN10 fusion protein has certain advantages over native KGF2 and may offer a novel strategy to potentiate the therapeutic effect of KGF2.**

**Keywords** fusion protein; growth factor; fibronectin; keratinocyte; keratinocyte growth factor 2

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### Introduction

Keratinocyte growth factor 2 (KGF2), originally isolated from the rat embryo cDNA as determined by homology-based polymerase chain reaction (PCR), is a member of the fibroblast growth factor superfamily [1]. KGF2 is mainly synthesized by mesenchymal cells and acts predominantly on epithelial cells in a paracrine manner [2]. KGF2-mediated mesenchymal-epithelial communication has been shown to play a role in regeneration of epidermis during wound healing and appears to be critical for the establishment of normal skin architecture during development [3,4].

Growth factor-induced proliferation and differentiation have been shown to require adhesion of cells to the

extracellular matrix (ECM) components [5,6]. Fibronectin (FN) is a major ECM component used by many cell types as a substrate for adhesion [7]. In normal skin, FN is present at the dermal–epidermal junction and is increased in skin tissues in wound healing [8,9]. FN has a modular architecture with homologous repeating modules consisting of 40–90 amino acids [7]. The 10th type III domains of fibronectin (FN10) is the major cell-adhesive domain of FN and contains the Arg-Gly-Asp (RGD) sequence that is recognized by the members of the integrins [10,11].

In this study, we designed a novel KGF2-FN10 fusion protein for a cell-adhesive function, and described its effects on cellular adhesion, proliferation, and differentiation in primary human keratinocytes.

### Materials and Methods

#### Construction of expression plasmids and purification

To link FN fragment with KGF2, FN10, the key cell-binding domain of FN, was fused with KGF2, designated KGF2-FN10. The cDNA encoding FN10 was subcloned into a pBAD/HisA-KGF2 expression vector [12] at the *Xho*I restriction sites for bacterial expression. This vector contains an araBAD promoter for tightly regulated expression and an amino-terminal polyhistidine sequence for affinity purification.

PCR primers were designed to recognize FN10 as follows; 5'-TCGAGCAACAATCAACAGTTTC-3' (upstream), and 5'-CTCGAGTGGTTTGTCAATTTTC-3' (downstream). PCR was carried out in a 30  $\mu$ l reaction containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml gelatin, 0.2 mM deoxyribo nucleotide triphosphates, 1.25 U of *Taq* polymerase (iNtRON, Seoul, Korea), and 50 pmol each of the upstream and downstream primers. The thermocycling

parameters used in PCR were as follows: annealing for 1 min at 55°C; extension for 2 min at 72°C; and denaturation for 1 min at 94°C. After 30 cycles, PCR-amplified cDNA products were digested by *Xho*I. After digestion, PCR products were in-frame ligated into the multiple cloning sites of pBAD-His<sub>6</sub>-KGF2 [13].

For the expression of recombinant KGF2-FN10, *E. coli* TOP10 cells were grown overnight in LB-Amp medium at 37°C. When the cultures reached an  $A_{600}=0.6$ , induction was initiated with 0.02% (w/v) of L-arabinose as inducer. After 3 h, bacteria were pelleted by centrifugation, lysed, and sonicated. A soluble extract was prepared by centrifugation for 30 min at 14,000 g in refrigerated centrifuge and supernatant was transferred to a fresh tube.

The crude protein from the sonicated bacterial supernatant was purified through binding of the poly-His<sub>6</sub> tag (located at the amino-terminal end of the protein) to the nickel-nitrilotriacetic acid resin column according to the manufacturer's protocol (Invitrogen, Carlsbad, USA).

#### Cell culture

Experiments were carried out with primary human keratinocytes (Invitrogen). The cells were grown in a keratinocyte-serum free medium (SFM), supplemented with recombinant epidermal growth factor (0.1–0.2 ng/ml) and bovine pituitary extract (20–30 µg/ml) according to the manufacturer's protocol (Invitrogen). Cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. The medium was changed three times a week, and cultures were split by treating the cells with 0.05% (w/v) trypsin-EDTA for 5 min at 37°C prior to confluency. In the cell growth assay, cells were incubated in unsupplemented keratinocyte-SFM medium.

#### Cell adhesion assay

Primary human keratinocytes were harvested by 0.05% (w/v) trypsin-EDTA, resuspended in keratinocyte-SFM, washed three times with growth medium containing 500 µg/ml soybean trypsin inhibitor, and plated at  $5 \times 10^4$  cells per well in keratinocyte-SFM. Twenty-four well plates were coated with KGF2, FN10, or KGF2-FN10 (0.5–1.0 µM) overnight at 4°C at the indicated concentrations and then blocked with 1% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, and rinsed with PBS.

Non-specific adherence was blocked by incubation with 0.5% (w/v) BSA in PBS for 1 h at 37°C. After 45 min incubation at 37°C, adherent cells were washed

twice with PBS, fixed with 3% (w/v) paraformaldehyde (Sigma, St Louis, USA), and stained with 0.25% (w/v) crystal violet (Sigma) in 2% (v/v) ethanol/water. After extensive washings with distilled water, plates were allowed to dry. The absorbance was measured at 570 nm and non-specific adhesion was determined in wells coated with 1% (w/v) BSA as negative control.

#### Cell growth assay

Cell growth was assessed by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (Celltiter96™ AQ non-radioactive cell proliferation assay; Promega, Madison, USA), which measures the number of viable cells as described by the manufacturer. This assay measures the conversion of a methyl terazol sulfate into aqueous soluble formazan product that absorbs at 490 nm.

Cells were grown in 24-well plates at a density of 5000 cells per well, with FN10, KGF2, or KGF2-FN10 added at concentrations of 0.5 µM. After incubation at 37°C for 5 days, MTS (40 µl) was added, followed by an additional incubation at 37°C for 4 h. The optical density (OD) was measured at 490 nm using an ELISA reader (Tecan, Männedorf, Switzerland).

#### Quantitative real-time reverse transcription polymerase chain reaction

First strand cDNA was synthesized from total RNA (2 µg) using the SuperScript first strand synthesis system for reverse transcription polymerase chain reaction (Invitrogen) according to the manufacturer's instructions. Volume of the reaction mixture was made up to 50 µl. Quantitative real-time PCR was carried out using SYBR GreenER qPCR SuperMix reagents (Invitrogen) in a Bio-Rad iCycler real-time PCR detection system (Bio-Rad, Hercules, USA). The PCR conditions were 1 cycle at 95°C for 5 min followed by 40 cycles at 95°C for 20 s, 56°C for 20 s, and 75°C for 30 s, terminating with 80 cycles at 55°C for 10 s for melting curve analysis. Relative transcript quantities were calculated using the  $\Delta\Delta C_t$  method with both  $\beta$ -actin and GAPDH as the endogenous reference genes amplified from the samples. Primer sequences were as follows: human cytokeratin K10, 5'-GAACCA CGAGGAGGAAATGA-3' (forward) and 5'-TGCACACA GTAGCGACCTTC-3' (reverse); involucrin, 5'-CAACTG AAGCATCTGGAGCA-3' (forward) and 5'-AGGGCTGG TTGAATGTCTTG-3' (reverse);  $\beta$ -actin, 5'-TTGCCGAC AGGATGCAGAA-3' (forward) and 5'-GCCGATCCACA CGGAGTACTT-3' (reverse); GAPDH, 5'-TGGAAGGAC

TCATGACCACA-3' (forward) and 5'-TTCAGCTCAGGG ATGACCTT-3' (reverse).

**Statistical analysis**

Experiments were repeated three to four times. Data were analysed using Student's *t*-test or one-way analysis of variance with Tukey's multiple comparison test to a confidence level of  $P < 0.05$ .

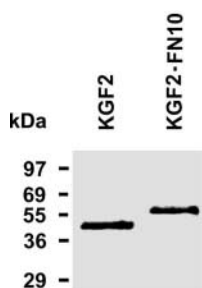
**Results**

**Expression and purification of the recombinant fusion protein in *E. coli***

The constructed KGF2-FN10 fusion proteins were expressed in *E. coli* TOP10 with poly-His<sub>6</sub> tag for affinity purification. Through the use of this system, KGF2-FN10 fusion proteins were purified in one step with nickel-nitrilotriacetic acid resin column from the soluble fraction. The degree of purification of the recombinant protein was examined under denaturing conditions as determined by Coomassie blue staining of 10% (v/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Upon induction with L-arabinose, *E. coli* Top 10 produced a protein of Mr = 550,000 Da (as estimated by SDS–PAGE), the size expected for a fusion protein consisting of KGF2-FN10 and the amino-terminal His<sub>6</sub> tag (Fig. 1).

**Cell adhesion activity of KGF2-FN10 fusion protein**

To analyse the ability of KGF2-FN10 fusion protein to promote cell adhesion, primary human keratinocytes were

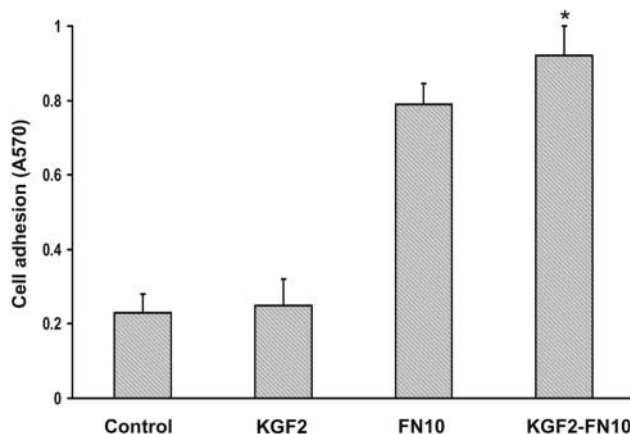


**Fig. 1 Characterization of purified recombinant KGF2-FN10 fusion protein by sodium dodecyl sulfate–polyacrylamide gel electrophoresis** Recombinant KGF2-FN10 was expressed as histidine (His<sub>6</sub>) tag fusion protein and purified using Ni-NTA column. Eluted materials was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing condition and visualized by Coomassie blue staining. About 5 µg of protein was applied on 10% (w/v) sodium dodecyl sulfat–polyacrylamide gel electrophoresis. Molecular mass markers are indicated in kilodalton shown on the left.

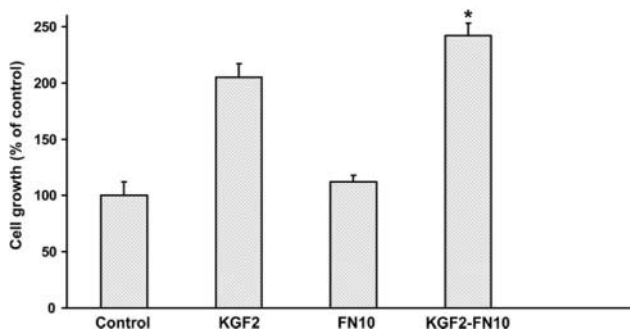
seeded on plastic tissue-culture plates coated with FN10, KGF2, and KGF2-FN10. Cells attached to the wells in both the FN10-coated plates and KGF2-FN10-coated plates. However the cell attachment was barely observed on KGF2-coated plates or non-coated plates (Fig. 2).

**Effect of KGF2-FN10 on cell growth**

Next, we evaluated the mitogenic activity of KGF2-FN10 on human keratinocytes in the culture. Assays were carried out in the absence of supplements to exclude addition of the other stimuli such as brain extract. As shown in Fig. 3, KGF2-FN10 significantly stimulated the proliferation of primary human keratinocytes when compared with KGF2 or FN10 ( $P < 0.05$ ). These results suggest that KGF2 fused with FN10



**Fig. 2 Adhesion of primary human keratinocytes to plates coated with KGF2, FN10, and KGF2-FN10** Adhesion assays were carried out on keratinocytes seeded on the various substrates as described. Non-coated substrate was used as a control. Results are calculated from OD values of crystal violet-stained cells and expressed as mean ± SE ( $*P < 0.05$ ;  $n = 4$ ).



**Fig. 3 Growth-stimulating effect of KGF2-FN10 on human keratinocytes** Human keratinocytes were incubated with FN10, KGF2, and KGF2-FN10 for 5 days. Data are expressed as the percentage of non-treated controls and mean ± SE ( $*P < 0.05$ ;  $n = 4$ ).

potentiates the mitogenic activity of KGF2 on human keratinocytes.

### Effect of KGF2-FN10 on the keratinocyte differentiation

To assess the impact of KGF2-FN10 on keratinocyte differentiation, we examined the effect of KGF2-FN10 on the expression of differentiation markers by real-time PCR. As shown in **Fig. 4**, the addition of KGF2-FN10 significantly enhanced the expression of differentiation markers, cytokeratin K10, and involucrin mRNA (1.5-fold) when compared with KGF2 ( $P < 0.05$ ). Although the post-transcriptional regulation of cytokeratin K10 and involucrin is an intriguing issue remaining to be tested, these results suggest that the KGF2-FN10 fusion protein is more potent than native KGF2 in their ability to differentiate keratinocytes.

### Discussion

Cell adhesion to ECM such as FN triggers a number of intracellular signaling events including the increased autophosphorylation of focal adhesion kinase and also the activation of extracellular signal-regulated kinase (ERK)-type mitogen-activated protein (MAP) kinase [14,15]. This integrin-mediated signaling has been suggested to act with other mitogenic signaling pathway to coordinate cell proliferation and differentiation. In our

previous studies, we have identified that FN-mediated signaling synergizes with signals from fibroblast growth factor receptors through ERK-type MAP kinase pathway [10,12]. Given the fact that the KGF2-FN10 binds to both FGFR2 and integrin, it is likely that these two signaling pathways are involved in the synergistic effect of the KGF2-FN10. Although the detailed mechanism of the involvement of KGF2-FN10 in the enhanced biological activities in human keratinocytes remains to be elucidated, the synergistic effect of KGF2 and FN may play a role in the potentiating activity of KGF2-FN10.

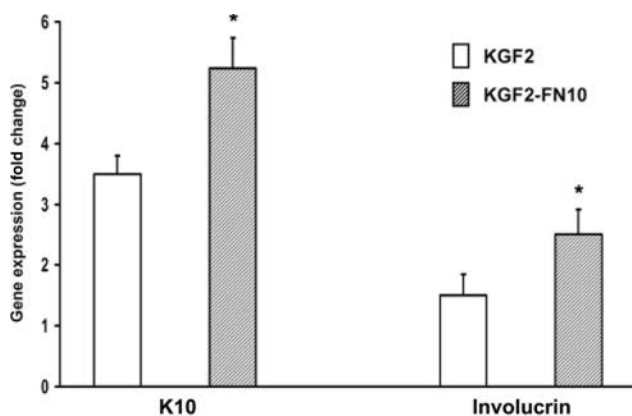
KGF-2 accelerates skin wound healing and re-epithelialization [16]. In a rabbit model of scar formation, KGF-2 was the most effective factor in wound healing and caused no obvious scarring when compared with other growth factors including KGF-1 and TGF- $\beta$  [17]. In addition, FN promotes keratinocytes adhesion and migration in wound healing [18]. In the present study, we successfully produced a fusion protein (KGF2-FN10) that retained the activity of both native KGF2 and FN. This fusion protein significantly enhanced the mitogenic activity of KGF2 on human keratinocytes. Moreover, KGF2-FN10 fusion protein exhibited significantly increased activity to differentiate keratinocytes from native KGF2. Thus, the present *in vitro* experiments clearly suggest that the KGF2-FN10 fusion protein has certain advantages over native KGF2 and may offer a novel strategy to potentiate the therapeutic effect of KGF2.

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**Fig. 4 Effect of KGF2-FN10 on endogenous cytokeratin K10 and involucrin mRNA level in human keratinocytes** Total RNA was obtained from KGF2 or KGF2-FN10-treated human keratinocytes and quantitative real-time polymerase chain reaction was carried out to quantify the endogenous levels of cytokeratin K10 and involucrin mRNA after culturing for 1 day. Data are indicated as fold expression, referring to the signal of the non-treated control as 1 U. The results are from at least three independent experiments and shown as mean  $\pm$  SE (\* $P < 0.05$ ).

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