

## RESEARCH COMMUNICATION

# Fucosyltransferase IV Enhances Expression of MMP-12 Stimulated by EGF via the ERK1/2, p38 and NF- $\kappa$ B Pathways in A431 Cells

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### Abstract

Fucosyltransferase IV (FUT4) has been implicated in cell adhesion, motility, and tumor progression in human epidermoid carcinoma A431 cells. We previously reported that it promotes cell proliferation through the ERK/MAPK and PI3K/Akt signaling pathways; however, the molecular mechanisms underlying FUT4-induced cell invasion remain unknown. In this study we determined the effect of FUT4 on expression of matrix metalloproteinase (MMP)-12 induced by EGF in A431 cells. Treatment with EGF resulted in an alteration of cell morphology and induced an increase in the expression of MMP-12. EGF induced nuclear translocation of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and resulted in phosphorylation of I $\kappa$ B $\alpha$  in a time-dependent manner. In addition, ERK1/2 and p38 MAPK were shown to play a crucial role in mediating EGF-induced NF- $\kappa$ B translocation and phosphorylation of I $\kappa$ B $\alpha$  when treated with the MAPK inhibitors, PD98059 and SB203580, which resulted in increased MMP-12 expression. Importantly, we showed that FUT4 up-regulated EGF-induced MMP-12 expression by promoting the phosphorylation of ERK1/2 and p38 MAPK, thereby inducing phosphorylation/degradation of I $\kappa$ B $\alpha$ , NF- $\kappa$ B activation. Base on our data, we propose that FUT4 up-regulates expression of MMP-12 via a MAPK-NF- $\kappa$ B-dependent mechanism.

**Keywords:** Fucosyltransferase IV - MMP-12 - MAPK signaling pathway - NF- $\kappa$ B

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### Introduction

Endometrial carcinoma is the most common malignancy in the female genital tract. The invasion of endometrial adenocarcinoma into endometrial tissue and metastasis to the nearby pelvic or distal tissues and organs are crucial factors affecting the prognosis and mortality of women. The synthesis and secretion of several proteases are up-regulated in endometrial carcinoma, including matrix metalloproteinase (MMP) (Shaco-Levy et al., 2008; Oh et al., 2009).

Matrix metalloproteinases (MMPs), a family of more than 20 extracellular, zinc-dependant proteases that degrade the multiple structural components of the extracellular matrix (ECM), have been shown to engage in tumor invasion. MMPs play essential roles in many aspects of biology, including cell proliferation, differentiation, apoptosis, and migration. These processing enzymes have clear links to cancer and tumor progression, in which proteolysis of the ECM is required to accommodate increased growth, migration, and invasion of tumor cells (Korpos et al., 2009; Thrailkill et al., 2009; Tonti et al., 2009).

In a previous study we showed that increased expression of MMP-12 was associated with the extent

of adenocarcinoma invasion accompanied by marked macrophage infiltration (Yang et al., 2007a). MMP-12, another elastase also called macrophage metalloelastase, is a member of the MMPs. MMP-12 is a 54-kDa proenzyme that is secreted extracellularly, and then processed into 45- and 22-kDa active forms. Some studies have reported that epithelial-derived cells secrete MMP-12. Cultured bronchial epithelial cells have been shown to secrete MMP-12 (Lavigne et al., 2004); MMP-12 is overexpressed in lung epithelial cells (Qu et al., 2009); Wnt-7a regulates MMP-12 and can promote cell proliferation in corneal epithelial cells during wound healing (Lyu & Joo, 2005). Nuclear factor  $\kappa$ B (NF- $\kappa$ B) transcription factor is a heterodimer consisting of p50/p105/NF- $\kappa$ B1, p52/p100/NF- $\kappa$ B2, or p65/RelA and c-Rel or RelB. NF- $\kappa$ B participates in the induction of a wide variety of cellular genes encoding cell adhesion molecules, cellular survival, transformation, and oncogenesis. NF- $\kappa$ B is present in the cytoplasm of non-stimulated cells, where NF- $\kappa$ B associates with I $\kappa$ B family inhibitors.

I $\kappa$ B is phosphorylated and degraded following stimulation by growth factors, cytokines, hormones, or other agents, resulting in dissociation from NF- $\kappa$ B and nuclear translocation of NF- $\kappa$ B. Constitutive activation of NF- $\kappa$ B via persistent nuclear NF- $\kappa$ B localization, and

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thus NF- $\kappa$ B-dependent transcription, has been detected in many cancers, including endometrial cancer (Oh et al., 2009). In addition, NF- $\kappa$ B also regulates the expression and activation of MMPs, which play a significant role in ECM degradation and in facilitating cell motility, tumor growth, and metastasis (Karin et al., 2002). Thus, we predict that compounds that block NF- $\kappa$ B activity will be useful for inhibiting MMP-dependent tumor growth and invasion.

LeY is a difucosylated oligosaccharide, which is overexpressed in the majority of carcinomas (Cao et al., 2001; Escrevente et al., 2006) and has the following chemical structure: [Fuc $\alpha$ 1 $\rightarrow$ 2Gal $\beta$ 1 $\rightarrow$ 4(Fuc $\alpha$ 1 $\rightarrow$ 3)GlcNAc $\beta$ 1 $\rightarrow$ R]. Arai (Arai & Nishida, 2003), and Skovlund (1997) showed that LeY is highly expressed in endometrial cancer and related to tumor grade. The  $\alpha$ 1,3 fucosylation of LeY is catalyzed by fucosyltransferase IV (FUT4). FUT4 is a critical enzyme that controls LeY oligosaccharide synthesis (Taniguchi et al., 2000; Wang et al., 2001). In a previous study, we reported that MMP-12 is an important oncogene in high-stage and high-grade endometrial adenocarcinoma; however, the relationship between FUT4 and MMP-12 is not clear.

We investigated the possible signaling pathways involved in EGF-induced, NF- $\kappa$ B-mediated MMP-12 expression in A431 cells and tested the effect of FUT4 during the process. Our results suggest significantly increased EGF-induced MMP-12 expression via ERK1/2 and p38 MAPK-mediated phosphorylation and degradation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B activation in A431 cells.

## Materials and Methods

### Materials

A431 cell line was obtained from American Type Culture Collection (Manassas, VA). DMEM/F12 (1:1), fetal bovine serum (FBS), Lipofectamine TM Reagent and Plus TM Reagent were purchased from Invitrogen. Enhanced chemiluminescence (ECL) assay kit was purchased from Amersham. G418, PD98059, SB203580 were obtained from Sigma Chemical. The anti-p-ERK1/2 and ERK were purchased from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and anti-rabbit secondary antibody were purchased from Santa Cruz. FUT4 interfered plasmid (FUT4-RNAi) was constructed in our own lab (Zhang et al., 2008).

### Cell Culture

A431 cells were cultured in DMEM/F12 (1:1) supplemented with 10% FBS, 100 U/ml penicillin and 50  $\mu$ g/ $\mu$ l streptomycin at 37°C under 5% CO<sub>2</sub> in humidified air. A431 cells were transfected with pEGFP-N1-FUT4 and screened by G418 for FUT4 stably transfected cells, which has been described elsewhere (Yang et al., 2007b).

### Preparation of Nuclear and Cytosolic Fractions

The cells ( $6 \times 10^5$ ) were cultured on a 100 mm culture dish in 10% FBS DMEM/F12 for 24 hr. These cells were incubated in serum-free DMEM/F12 for another 24 hr and treated with EGF at designated times. Cells were

washed twice with cold PBS and then scraped to an eppendorf in 1 ml of PBS, centrifuged at 4 °C, 12,000 rpm for 5 min. Discarding the supernatant, the pellet was homogenized in 300  $\mu$ l of hypotonic lysis buffer (HEPES (pH 7.6) 10 mM, EDTA 0.1 mM, dithiothreitol (DTT) 1 mM, PMSF 0.5 mM). After repeated homogenization, the homogenate was centrifuged at 4 °C, 12,000 rpm for 10 min. The supernatant was the cytosolic fraction and was kept at -70 °C overnight. The pellet was washed twice in 20  $\mu$ l of hypotonic lysis buffer to remove residual cytosolic proteins. After washing, the pellet was dissolved in 30  $\mu$ l of hypertonic lysis buffer (HEPES (pH 7.6) 20 mM, EDTA 1 mM, DTT 1 mM PMSF 0.5 mM, 25% glycerol, 0.4 M NaCl), pipetted to homogeneity, fiercely vortexed, and then stored at -70 °C overnight. Next day, the solution was centrifuged to collect the supernatant, which was the nuclear fraction. The cytosolic and nuclear fractions were quantified, and equal amounts of protein were subjected to Western blot.

### Western Blot Analysis

The nuclear and cytoplasmic extracts (30  $\mu$ g) were resolved by SDS-PAGE and then electrotransferred to the nitrocellulose membrane. Western blot analysis was done as described elsewhere (Yang et al., 2007b).

### RT-PCR

For RT-PCR analysis, RNA of MMP-12 was isolated from cells using Trizol according to the manufacturer's instruction, followed by first-strand cDNA synthesis using TrueScript reverse transcriptase. cDNA was amplified by PCR using the specific primer for MMP-12 or  $\beta$ -actin as an internal control. The sequences of the upstream and downstream primers were as follows: 5'-TTGTTTCCTCACTGCTGTTCAC-3' (F) and 5'-GTCCATCATGTCTCCTTTC-3' (R) for MMP-12; 5'-ATCTGGCACCACACCTTCACAATGAGCTGCG-3' (F) and 5'-CGTCATACTCCTGCTTGCTGATCCACA TCTGC-3' (R) for  $\beta$ -actin, respectively. PCR analysis was performed under the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation for 40 s at 94°C, annealing for 30 s at 63°C for MMP-12, 58°C for  $\beta$ -actin, and extension for 40 s at 72°C. The amplified products were analyzed by 1.0% agarose gel electrophoresis, followed by ethidium bromide staining. Band intensities were measured using BioImaging systems (UVP, labworks<sup>TM</sup>, ver 4.6).

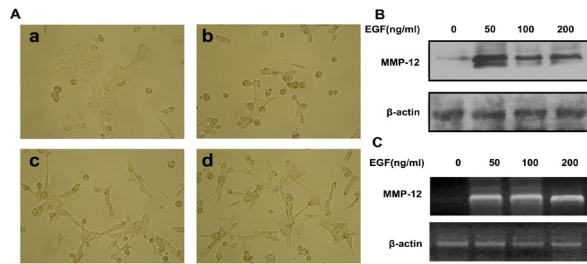
### Statistics

Results are expressed as the mean  $\pm$  the standard error of the mean (SEM) of at least three independent experiments. Statistical significance of difference between test groups was assessed by one-way ANOVA followed by Scheffe's test (post hoc). Statistical significance was defined at  $P < 0.05$ .

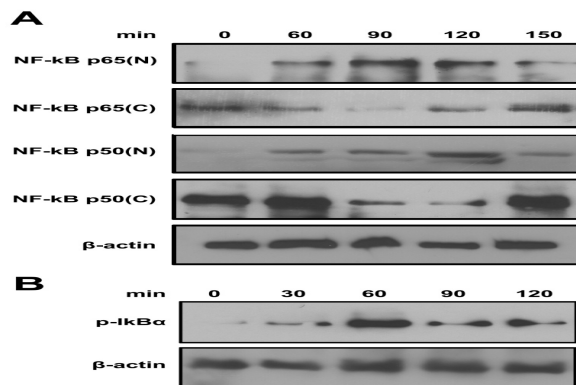
## Results

### EGF increased A431 cell migration

To assess the effect of EGF on cell morphology, A431 cells were grown on 6-well plates and serum-



**Figure 1. Effect of EGF on Invasion of A431 Cells.** (A) A431 cells were treated with various concentrations of EGF (0, 50, 100, or 200 ng/ml) for 24 hr. In vitro invasion assay was performed according to the manufacturer's instructions and photographed under a microscope at 100 $\times$ . (B) (C) The effect of various concentrations of EGF on the expression of MMP-12. A431 cells were treated with various concentrations of EGF (0, 50, 100, or 200 ng/ml) for 24 hr. Then, the proteins were collected and analyzed by Western blot (B) with antibody against MMP-12 and RT-PCR analysis (C)

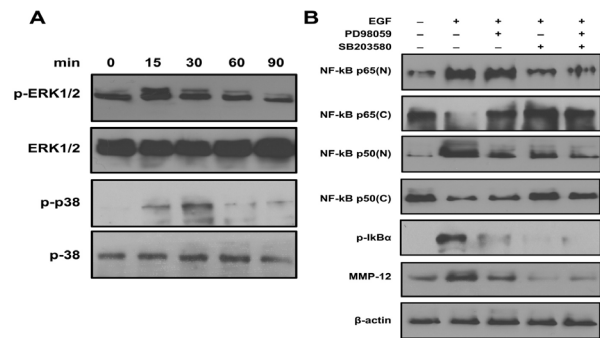


**Figure 2. Western Blotting Results.** (A) Effect of EGF on the cellular localization of the NF- $\kappa$ B p65 and p50 subunits in A431 cells. Cytoplasmic (C) and nuclear (N) extracts from untreated cells and cells treated with 100 ng/ml of EGF for 60, 90, 120, or 150 min were immunoblotted with p65 and p50 antibodies. (B) EGF stimulated phosphorylation of I $\kappa$ B $\alpha$  in A431 cells. Cells were treated with 100 ng/ml of EGF for 30, 60, 90, or 120 min. Western blotting was performed on cell lysates using an anti-phospho-I $\kappa$ B $\alpha$  antibody

deprived for 24 hr, followed by stimulation with different concentrations of EGF (0, 50, 100, or 200 ng/ml) for 24 hr. Microscopy revealed that addition of EGF resulted in an alteration of cell morphology. As shown in Figure 1A, EGF induced an elongated and fibroblastoid phenotype. The increase in concentration of EGF led to a more rapid and pronounced alteration in cell morphology (Figure 1A-b, c, d), suggesting that EGF stimulation was associated with a change of cell motility.

To confirm the transformation from the epithelial-to-fibroblastoid phenotype, we examined the expression of MMP-12. MMP-12 is a member of the family of MMPs, which are zinc-dependent endopeptidases. To delineate if MMP-12 was implicated in the increase of cell migration, A431 cells were stimulated with EGF (100 ng/ml) for 24 hr and the expression of MMP-12 was analyzed. The effect of EGF on MMP-12 expression was determined by RT-PCR and Western blot analysis. The data demonstrated that EGF stimulation can increase the expression of MMP-12, and there were no differences at different concentrations (Figure 1B, 1C).

*EGF induced nuclear translocation of NF- $\kappa$ B subunits p65*



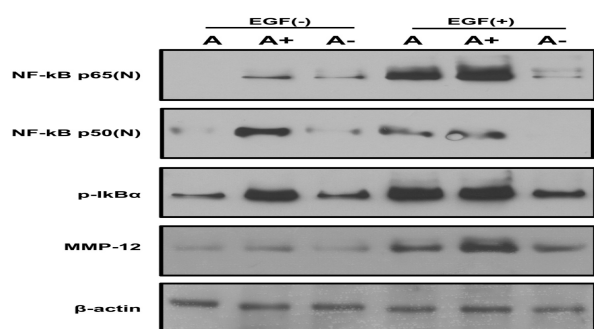
**Figure 3. MAPKs Activation is Required for MMP-12 Expression in A431 Cells.** (A) EGF-induced phosphorylation of ERK1/2 and p38. A431 cells were treated with 100 ng/ml of EGF stimulation for 15, 30, 60, or 90 min. Levels of p-ERK1/2, ERK1/2, p-p38, and p38 were analyzed by Western blot. (B) ERK1/2 and p38 were the mediators of EGF-induced, NF- $\kappa$ B-dependent MMP-12 expression in A431 cells. A431 cells were pre-treated with PD98059 (ERK1/2 inhibitor) and SB203580 (p38 inhibitor) for 1 hr before adding 100 ng/ml of EGF for 120 min (NF- $\kappa$ B p65 and p50), 60 min (p-I $\kappa$ B $\alpha$ ), and 24 hr (MMP-12)

*and p50, resulting in phosphorylation of I $\kappa$ B $\alpha$*

Transcription factor NF- $\kappa$ B has been reported to control MMP-12 gene expression (Churg et al., 2001). Thus, we examined the localization of NF- $\kappa$ B in nuclear and cytosolic fractions from A431 cells to determine whether or not NF- $\kappa$ B was involved in regulating MMP-12 gene expression. A431 cells were treated with 100 ng/ml of EGF for 24 hr. Next, nuclear and cytoplasmic fractions were prepared from the cells. As shown in Figure 2, we showed that EGF induced NF- $\kappa$ B nuclear translocation from the cytosol in a time-dependent manner. In the non-treated control cells, p65 and p50 were primarily localized to the cytoplasm. Following EGF treatment, p65 and p50 translocated to the nucleus in a time-dependent manner. Ninety and 120 min after EGF treatment p65 and p50 were predominantly localized to the nucleus, respectively (Figure 2A). Next, it was determined whether or not EGF-induced NF- $\kappa$ B activation was due to phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$ . The results showed that EGF treatment increased the amount of phospho-I $\kappa$ B $\alpha$  after 30 min and approached to the maximum at 60 min, then decreased (Figure 2B). Taken together, these results showed that EGF treatment resulted in nuclear translocation of NF- $\kappa$ B in A431 cells.

*Effect of EGF on MMP-12 activity through the MAPK/ NF- $\kappa$ B signaling pathway*

Several effector pathways for the EGF receptor have been reported. Among them, the mitogen-activated protein kinase (MAPK) cascade is considered to be most crucial (Seger & Krebs, 1995). To determine if the effector pathways were involved in the stimulation of MMP-12 activity by EGF, we first examined the kinetic profile of ERK activation upon stimulation by EGF using phospho-specific ERK1/2 and p38 antibodies. The results of Western blot analysis demonstrated that EGF (100  $\mu$ g/ml) induced a sharp and transient activation of the ERK and p38 signals, which was rapidly detected 15 or 30 min following the addition of EGF, then gradually declined (Figure 3A).



**Figure 4. FUT4 Up-regulated the Expression of EGF-Stimulated MMP-12 in A431 Cells via a MAPK-NF-κB-dependent Pathway.** Cells were treated with or without 100 ng/ml of EGF. Nuclear (N) extracts were for analysis of NF-κB p65 and p50. Cytoplasmic (C) extracts were for p-IκBα and MMP-12

To further confirm that MAPK was an intermediate in the pathway that links EGF exposure to NF-κB activation, endogenous ERK1/2 and p38 expression were silenced in A431 cells using an ERK inhibitor (PD98059) and/or a p38 inhibitor (SB203580). A431 cells were pretreated with an ERK inhibitor (10-6 μM PD98059) and/or a p38 inhibitor (10-6 μM SB203580) followed by EGF for 1 day. Inhibition of ERK1/2 and p38 by inhibitors resulted in reducing EGF-mediated NF-κB nuclear translocation compared with cells stimulated by EGF only (Figure 3B). NF-κB IκBα was inhibited by the same inhibitors. The results of Western blot analysis for MMP-12 revealed that PD98059 and SB203580 blocked the stimulatory effect of EGF on MMP-12 expression (Figure 3B). Taken together, these results indicated that MAPK is a critical mediator of EGF-induced, NF-κB-dependent MMP-12 expression in A431 cells.

#### *Effect of FUT4 on MMP-12 stimulated by EGF via the MAPK signaling pathway*

In previous studies we have shown that FUT4 promotes cell proliferation through the MAPK signaling pathway (Yang et al., 2010). FUT4 overexpression increased the activation of ERK1/2 and p38. To test if FUT4 could increase EGF-induced p65 and p50 translocation, A431 cells, FUT4 overexpression cells, and FUT4-RNAi cells were treated with or without EGF for 24 hr. Nuclear and cytoplasmic fractionation were prepared from the cells. The results showed that FUT4 promoted EGF-induced nuclear translocation of the NF-κB subunits, p65 and p50, in A431 cells (Figure 4). Next, we determined whether or not FUT4 increased EGF-induced MMP-12 expression. The results showed that FUT4 overexpression could increase MMP-12 expression induced by EGF (Figure 4). Taken together, the data showed that FUT4 effectively increased EGF-induced, NF-κB-dependent MMP-12 expression.

## Discussion

The delicate balance between MMPs and inhibitors is maintained in normal physiologic conditions. Excessive production of MMPs could increase the invasion and mobility of tumor cells. A recent study has shown that

MMP-12 is overexpressed in various human cancers, e.g., epithelial ovarian carcinoma (Li et al., 2009), prostate cancer (Nabha et al., 2008), endometrial adenocarcinoma (Yang et al., 2007a), glioma cell (Sarkar et al., 2006), non-small cell lung cancer (Hofmann et al., 2005), oral verrucous and squamous cell cancer (Impola et al., 2004) and squamous cell carcinoma (Kerkela et al., 2002). In addition, FUT4 is mainly expressed in leukocytes and some epithelial cells (Allahverdian et al., 2006). Increases in the expression of FUT4 level are seen in different cancers, e.g., gastric carcinoma (Petretti et al., 1999), colorectal cancer (Kudo et al., 1998), pancreatic cancer (Taniguchi et al., 2000) and lung adenocarcinoma (Martin-Satue et al., 1998). FUT4 has an effect on cell invasion and mobility (Yang et al., 2007b). However, little is known about FUT4-mediated regulation of MMP-12 expression in epithelial-derived A431 cells. In this study we characterized the mechanism of EGF-induced, NF-κB-mediated MMP-12 secretion in A431 cells. Our results showed that EGF induced phosphorylation of IκBα, which ultimately led to phosphorylation and degradation of IκBα and NF-κB nuclear translocation and transcriptional activation. Moreover, inhibition of MAPK decreased EGF-induced NF-κB activation and MMP-12 secretion. In addition, FUT4 activated NF-κB and stimulated MMP-12 secretion through the MAPK signaling pathway.

Up-regulation of MMP-12 expression by FUT4 in A431 cells may lead to cell mobility and invasion, which depend on both cellular properties and ECM composition. The expression and secretion of ECM-modifying MMPs is an important determinant of tumor invasion. MMP expression is regulated at the transcriptional level and the MMP promoters contain NF-κB (Chen et al., 2009; Oh et al., 2009), which have been shown to regulate MMP-12 promoter activity (Castrillo et al., 2003; Sampath et al., 2006).

The NF-κB transcription factor family regulates number of genes involved in diverse cellular processes, including inflammation, immune response, cell proliferation, and apoptosis. NF-κB activity is regulated by the endogenous inhibitor, IκBα, and interaction with IκBα blocks the nuclear localization of NF-κB, keeping NF-κB it sequestered in the cytoplasm. In the current study we showed that EGF induced phosphorylation of IκB, which led to degradation of IκBα and to NF-κB nuclear translocation and transcriptional activation in A431 cells (Kim et al., 2008).

Cell motility and invasiveness are still dependent on high doses of EGF (Rijken et al. 1991; Peppelenbosch et al., 1993). The early response of A431 cells to EGF induces rapid alterations in the organization of the actin microfilament system that result in extensive ruffling, lamellipodia formation, and cortical actin polymerization. EGF potentiates the signal-transduction pathway for cell proliferation, which differs from the pathway for migration. Mitogen-activated protein kinases (MAPKs) are rapidly activated in cells stimulated by a variety of mitogens or motogens, including EGF, which delivers essential signals via Ras to a protein kinase network involving the Raf-1 kinase, MEK, and MAPK (Klemke et al., 1997; Kawahara et al., 2002). A431 cells, human

squamous carcinomas cells, express EGF receptor (EGFR) highly. And our previous reports showed FUT4 had the effect on cell proliferation of A431 cells through the MAPK signaling pathway. In this study, we focused on MAPK signaling pathway.

Because MMP-12 expression is regulated by different signaling pathways according to the stimuli, we performed an experiment to analyze the activation of NF- $\kappa$ B and its signaling cascades. We showed that FUT4-induced increase in expression of MMP-12 in A431 cells is regulated at the transcriptional level (Figure 3). An increase of MMP-12 expression induced by EGF is accompanied by NF- $\kappa$ B nuclear translocation, suggesting up-regulation of MMP-12 expression was dependent on a NF- $\kappa$ B-dependent pathway. The signal transduction pathways mediating EGF-induced MMP-12 expression is largely unknown.

Subsequently, we explored the signal transduction pathways that were potentially involved in mediating EGF signal transduction. Recent studies have demonstrated that three major MAPK subfamilies (ERK 1/2, JNK, and p38) regulate NF- $\kappa$ B activation (Guma et al.; Kook et al.; Zhu et al.). MAPK inhibitors suppressed EGF-induced expression of MMP-12 (Figure 3). These results indicated that MAPK activity was required for EGF-induced MMP-12 expression in A431 cells.

As demonstrated in Figure 4, FUT4 overexpression can promote the nuclear translocation of NF- $\kappa$ B, phosphorylation of I $\kappa$ B $\alpha$ , and increase the MMP-12 expression, while FUT4 RNAi had an opposite effect. When A431 cells were treated with 100  $\mu$ g/ml of EGF for 24 hr, the effects were strengthened. All the data suggested that FUT4 induces MMP-12 expression at the transcriptional level by regulating NF- $\kappa$ B activation.

In summary, we have shown that EGF induces NF- $\kappa$ B activation by phosphorylation and degradation of I $\kappa$ B $\alpha$ . Additionally, we propose a critical role for MAPK in mediating EGF-induced NF- $\kappa$ B activation and increased expression of MMP-12. Importantly, FUT4 promoted MAPK activation, and phosphorylation and degradation of I $\kappa$ B $\alpha$ , and FUT4 was an effective stimulator of EGF-induced cellular change.

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