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# Nuclear Localization of Fibroblast Growth Factor Receptor 1 in Breast Cancer Cells Interacting with Cancer Associated Fibroblasts

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Cancer-associated fibroblasts (CAFs) represent a major component of the tumor microenvironment and interplay with cancer cells by secreting cytokines, growth factors and extracellular matrix proteins. When estrogen receptor-negative breast cancer MDA-MB-231 cells were treated with the CAF-conditioned medium (CAF-CM), Akt and STAT3 involved in cell proliferation and survival were activated through phosphorylation. CAFs secrete fibroblast growth factor 2 (FGF2), thereby stimulating breast cancer cell progression. Akt activation induced by CAF-CM in MDA-MB-231 cells was abolished when FGF2-neutralizing antibody was added. Treatment of MDA-MB-231 cells directly with FGF2 enhanced the phosphorylation of Akt and the FGF receptor (FGFR) substrate, FRS2$\alpha$. These events were abrogated by siRNA-mediated silencing of FGFR1. In a xenograft mouse model, co-injection of MDA-MB-231 cells with activated fibroblasts expressing FGF2 dramatically enhanced activation of Akt. Stable knockdown of FGFR1 blunted Akt phosphorylation in xenograft tumors. MDA-MB-231 cells co-cultured with CAFs or directly stimulated with FGF2 exhibited enhanced nuclear localization of FGFR1. Notably, FGF2 stimulation produced reactive oxygen species (ROS) accumulation in MDA-MB-231 cells, and FGF2-induced nuclear accumulation of FGFR1 was abrogated by the ROS scavenging agent, *N*-acetylcysteine.

**Key Words** Breast cancer, Cancer-associated fibroblasts, Fibroblast growth factor 2, Fibroblast growth factor receptor 1, Tumor microenvironment

## INTRODUCTION

Tumor is composed of both cancer cells and surrounding normal cells in the stroma which cooperate with each other. Fibroblasts, major stromal cells in the tumor microenvironment, play a pivotal role in tumor growth and metastasis [1]. Firoblasts associated with tumor stroma have been called cancer-associated fibroblasts (CAFs) [2]. Fibroblast growth factors (FGFs) secreted by CAFs are known to promote cancer cell proliferation. FGFs mediate their effects through binding to the corresponding membrane-bound fibroblast growth factor receptors (FGFRs) [2,3]. FGF-FGFR axis accounts for acquired resistance to estrogen receptor-directed therapy that can be overcome by FGFR inhibitors [4].

FGFR comprises highly conserved transmembrane receptor tyrosine kinases (RTKs), FGFR1-4, and another membrane-associated receptor that lacks the intracellular domain (FGFR5/FGFRL1) [5]. Like the majority of other transmembrane RTKs, FGFRs undergo dimerization and phosphorylation of C-terminal tyrosines upon ligand binding [6,7]. The resulting phosphorylated tyrosines dock a distinct set of adaptor proteins, leading to transcriptional activation of downstream oncogenic targets including PI3K/Akt and STATs [8-10]. Our previous study has demonstrated that FGF2 predominantly produced by CAF stimulates breast cancer cell proliferation, migration and progression [11]. Of the FGF receptors,

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FGFR1 exhibits high affinity for FGF2 [12]. Here, we report a non-canonical function of FGFR1 in the FGF2-induced target gene expression in the context of breast cancer cell growth and survival.

## MATERIALS AND METHODS

### Reagents and antibodies

Human recombinant FGF2 protein was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Primary antibodies for FGFR1, P-FRS2α, P-Akt, P-STAT3, Akt, STAT3 and cylin D1 were products of Cell Signaling Technology (Danvers, MA, USA). Antibodies against Nrf2, CREB-binding protein (CBP), α-tubulin, lamin B and β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The bicinchoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL, USA). MTT and 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) were products of Sigma-Aldrich (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (IgG) secondary antibody, 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were purchased from Invitrogen (Waltham, MA, USA).

### Cell culture

Human breast cancer cell lines were maintained as described elsewhere [11]. Primary normal fibroblasts (NFs) and CAFs supplied from Asterand Bioscience (Detroit, MI, USA) were incubated in Dulbecco's modified Eagle's medium supplemented with 10% fatal bovine serum (Gibco BRL, Grand Island, NY, USA) and 100 ng/mL penicillin/streptomycin/fungizone mixtureand other supplements. CCD-1068sk breast NFs ATCC, (Gaithersburg, MD, USA) were maintained in Minimum Essential Medium supplemented with 10% fetal bovine serum and 100 ng/mL penicillin/streptomycin/fungizone mixture. The cells were plated at an appropriate density according to each experimental scale.

### Collection of conditioned media (CM)

CAFs were seeded on 100 mm dishes at 1 × $10^6$ cells/mL. Culture medium was removed 24 hours after cell seeding, and cells were washed with PBS, followed by addition of 8 mL of serum-free medium. After 48 hours of incubation, the CM was collected and passed through 0.2 μm membrane syringe filter to remove residual cells and debris. For neutralization of FGF2 in the CM of CAFs, CM was pre-incubated with 25 μg/mL of human FGF2 antibody or its IgG control (R&D Systems, Inc., Minneapolis, MN, USA) for 1 hour at room temperature prior to use.

### Measurement of cell viability

MCF-7 cells, MDA-MB-231 cells and MDA-MB-468 cells were plated at a density of 1 × $10^5$ cells/mL in 48-well plates, and the cell viability was determined by the conventional MTT reduction assay. After 24-hour incubation with CAF-CM, cells were treated with the MTT solution (final concentration 0.5 mg/mL) for 3 hours at 37ºC. The dark blue formazan crystals that formed in intact cells were solubilized by dimethy sulfoxide (DMSO), and absorbance at 570 nm was measured with a microplate reader (Molecular devices, Sunnyvale, CA, USA). Results were expressed at the percentage of MTT reduction obtained in the treated cells, assuming that the absorbance of control cells was 100%.

### Xenograft assay

Female Balb/c (nu/nu) mice, 5 weeks of age, were purchased from Orientbio (Seoul, Korea). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of Seoul National University. After 1 week of adaptation, MDA-MB-231 cells with control or TGF-β-activated CCD-1068sk fibroblasts were co-injected subcutaneously into the flank of mice as described previously [11].

### Western blot analysis

Cells were lysed in radio-immunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 0.5% Triton X-100, 50 mM Tri-HCl [pH 7.4], 25 mM NaF, 20 mM EGTA, 1 mM dithiothreitiol [DTT], 1 mM $Na_3VO_4$, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], protease inhibitor cocktail tablets) for 15 minutes on ice followed by centrifugation at 13,000 *g* for 20 minutes. The protein concentration of the supernatant was measured by using the BCA reagent (Thermo Scientific, Rockford, IL, USA). Protein (30 μg) was separated by running through 8% to 12% SDS-PAGE gel and transferred to the polyvinylidene fluoride (PVDF) membrane (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% nonfat dry milk/TBST (Tris-buffered saline buffer containing 0.1% Tween-20) for 1 hour at room temperature. The membranes were incubated for 4 hours at room temperature with 1:1,000 dilution of polyclonal antibody of FGFR1, P-FRS2α, P-Akt, P-STAT3, and β-actin. The blots were rinsed three times with TBST buffer for 10 minutes each. Washed blots were incubated with 1:5,000 dilutions of horseradish peroxidase-conjugated secondary antibody (Thermo Scientific) for 1 hour and washed again three times with TBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

### Reverse transcription (RT)-PCR

The RT-PCR assay was performed as described previously [13] using the below primers. *CCND1*, 5'-ACCTGGATGCTGGAGGTCT-3' and 5'-GCTCCATTTGCAGCAGCTC-3', 241 bp; *NFE2L2*, 5'-ACT GGT TGG GGT CTT CTG TG-3' and 5'-CGG TAT GCA ACA GGA CAT TG-3', 263 bp; *GAPDH*, 5'-AAGGTCGGAGTCAACGGATTT-3' and 5'-GCAGTGAG-

GGTCTCTCTCT-3', 1,053 bp.

### Small interfering RNA (siRNA) transfection

siRNA oligonucleotide targeting *FGFR1* was purchased from Genolution Pharmaceuticals (Seoul, Korea). The sense and antisense strands of *FGFR1* siRNA were as follows; 5'-AUUCAAACCUGACCACAGA-3' (forward) and 5'-UCUGUGGUCAGGUUUGAAU-3' (reverse). MDA-MB-231 cells were transfected with 25 nM of specific or scrambled siRNA oligonucleotides using Lipofectamine RNAiMAX according to manufacturer's instruction (Invitrogen).

### Immunocytochemistry

Cells (MDA-MB 231 with CAF or NF and MDA-MB-231 alone) were seeded at 1 x $10^4$ cells per well in an 8 chamber plate. The cells were incubated for 1 hour in the absence or presence of FGF2 and then fixed with fixation solution containing 95% methanol and 5% acetic acid. The cells were then washed in PBS, permeabilized with 0.2% triton X-100, washed in PBS and blocked with 5% bovine serum albumin (BSA) in PBS. Polyclonal rabbit anti-FGFR1, diluted 1:100 in 1% BSA in PBS, was applied overnight at 4ºC. This was followed by washing cells in PBS and then incubation for 1 hour at room temperature with FITC-conjugated anti-rabbit IgG secondary antibody diluted at 1:1,000 in 1% BSA-PBS. After washing, cells were treated with DAPI or PI. The expression of FGFR1 was detected using a confocal microscope (Nikon, Tokyo, Japan).

### Immunoprecipitation

Immunoprecipitation assay of CBP and Nrf2 was performed as described previously [14]. Cells were washed with ice-cold PBS and lysed in RIPA buffer for 1 hour on ice, followed by centrifugation for 15 minutes at 12,000 *g*. Three hundred μg of pre-cleared lysates were incubated with 10 μL of anti-CBP antibodies for overnight. Forty μL of protein A/G-agarose beads (Santa Cruz Biotech) was then added to the mixture and rotated for 4 hours at 4ºC. The beads were washed with ice-cold PBS prior to Western blot analysis.

### Preparation of nuclear extracts

After treatment with FGF2, cells were washed with ice-cold PBS, scraped in 1 mL PBS and centrifuge at 7,000 *g* for 15 minutes at 4ºC. Pellets were suspended in 50 μL of hypotonic buffer A (10 mM HEPES [pH 7.8], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) for 15 minutes on ice, and 1 μL of 10% Nonidet P-40 solution was added for 5 minutes. The mixture was centrifuged at 12,000 *g* for 7 minutes. The pellets were washed with hypotonic buffer and were resuspended in hypertonic buffer C (20 mM HEPES [pH 7.8], 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) for 30 minutes on ice and centrifuged at 12,000 *g* for 7 minutes. The supernatant containing nuclear proteins was collected and stored at –70ºC after determination of the protein concentration by using the Bradford method using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

### Measurement of intracellular reactive oxygen species (ROS)

DCF-DA was used as a fluorescent probe to monitor the net intracellular accumulation of ROS. After treatment with FGF2 or CAF-CM, MDA-MB-231 cells were rinsed with PBS and were loaded with 10 μM of DCF-DA. After 30-minute incubation, the intracellular ROS accumulation was determined by fluorescent microscopy set at 488 nm for excitation and 530 nm for emission or by flow cytometry.

### Human cancer genome expression analyses

RNA-seq data set of the Cancer Genome Atlas (TCGA) breast invasive carcinoma was download from XenaBrower (https://xenabrowser.net). The mRNA expression levels of total 1,097 samples (Illumina HiSeq log [normalized counts + 1]) were prepared by quantile normalization. Pearson correlation coefficient was used to predict the relationship between two genes. Gene set enrichment analysis (GSEA) for FGFR1 expression in primary breast cancer samples was performed as described previously [15]. The Kaplan–Meier plotter, an online biomarker validation tool (kmplot.com/analysis), was used to estimate survival probabilities for 255 triple negative breast cancer (TNBC) patients based on FGFR1 and Nrf2 gene expression.

To determine the relationship between FGFR1 and FGF2 or Akt3 proteins in breast cancers, we selected "Breast Invasive Carcinoma (TCGA, Pan-Cancer Atlas)" data set publicly available from TCGA data portal (www.cbioportal.org). The clinical significance of association between FGFR1 and FGF2 or AKT3 protein expression was evaluated by calculating the Spearman's correlation coefficient.

### Statistical analysis

When necessary, data were represented as means of ± standard deviation at least three independent experiments, and statistical analysis between groups was performed using the Student's *t*-test (Sigma plot 7.0; Systat Software, Inc., Chicago, IL, USA): $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

## RESULTS

To determine whether CAFs are involved in cancer cell proliferation, breast cancer cells were treated with CM from CAFs (CAF-CM) or that from NFs (NF-CM). As illustrated in Figure 1A, estrogen receptor-negative MDA-MB-231 and MDA-MB-468 cells treated with CAF-CM were more proliferative than estrogen receptor-positive MCF-7 cells. In addition, MDA-MB-231 cells stimulated with CAF-CM exhibited elevated activation of oncogenic signaling molecules such as Akt and STAT3 through phosphorylation (Fig. 1B).

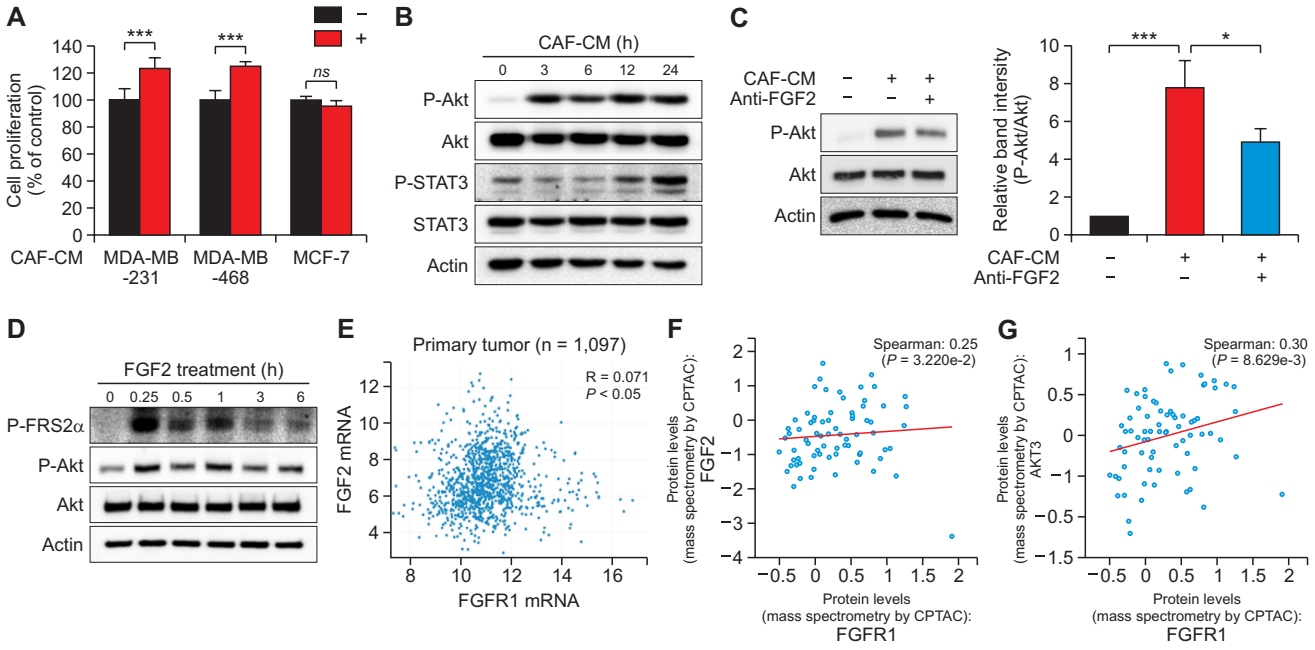


**Figure 1. Involvement of FGF2-FGFR1 axisin Akt activation.** (A) The effect of CAF-CM on proliferation of breast cancer (MCF-7, MDA-MB-231, and MDA-MB-468) cells was determined by the MTT assay. Cells were incubated with or without CAF-CM for 72 hours. ***Significantly different between the groups compared ($P < 0.001$). (B) MDA-MB-231 cells were incubated with CAF-CM for the indicated time periods. Phosphorylation of Akt and STAT3 were detected by Western blot analysis. (C) MDA-MB-231 cells were exposed to CAF-CM with or without FGF-2-neutralizing antibody for 3 hours. Phosphorylation of Akt was detected by Western blot analysis. *,***Significantly different between the groups compared (*$P < 0.05$; ***$P < 0.001$). (D) MDA-MB-231 cells were treated with 20 ng/mL of FGF2 for the indicated time periods. The phosphorylation of FRS2α as well as Akt was analyzed by Western blot. (E) RNA-seq data set of TCGA breast invasive carcinoma was downloaded from XenaBrower (https://xenabrowser.net). mRNA expression levels of total 1,097 samples (Illumina HiSeq log [normalized counts + 1]) were prepared by quantile normalization. Pearson correlation coefficient was calculated to assess the relationship between *FGF2* and *FGFR1*. (F, G) Correlation of FGFR1 protein expression with FGF2 (F) and Akt (G), based on 105 breast invasive carcinoma protein specimens (TCGA, Pan-Cancer Atlas) from the cBioportal database (www.cbioportal.org). FGF2, fibroblast growth factor 2; FGFR1, FGF receptor 1; CAFs, cancer-associated fibroblasts; NFs, normal fibroblasts; CM, conditioned medium; ns, not significantly different; FRS2, FGFR substrate 2; TCGA, The Cancer Genome Atlas; CPTAC, the Clinical Proteomic Tumor Analysis Consortium.

Our previous study demonstrated the secretion of FGF2 from CAFs derived from human breast cancer [11]. As described in the previous study [11], TNBC cells do express FGFR1 in a basal status, whilst expression of its ligand FGF2 was not detected. Thus, we used MDA-MB-231 and MDA-MB-468 TNBC cells to examine further cellular events under the paracrine condition. Notably, CAF-CM-induced Akt phosphorylation was significantly decreased in the presence of FGF2-neutralizing antibody in MDA-MB-231 cells (Fig. 1C). As shown in Figure 1D, treatment of MDA-MB-231 cells with human recombinant FGF2 induced phosphorylation of Akt and the FGFR substrate 2 (FRS2) required for the FGF-mediated activation of the PI3K-Akt signaling [16]. We then analyzed the transcriptome of 1,097 primary breast cancer tissue samples from TCGA datasets by RNA-seq. *FGFR1* and *FGF2* expression levels showed positive correlation in primary breast cancer samples (Fig. 1E). There was also statistically significant correlation between protein expression of FGFR1 and FGF2 (Fig. 1F) or AKT3 (Fig. 1G) in 97 invasive breast carcinoma specimens.

To address the involvement of FGFR1 in FGF2-induced oncogenic events, FGFR1 was silenced in MDA-MB-231 cells by use of specific si-RNA. As a result, FGF2-induced phosphorylation of FRS2 was abolished (Fig. 2A). Furthermore, siRNA silencing of FGFR1 suppressed the migration of MDA-MB-231 cells [11] and MDA-MB-468 cells as well (Fig. S1).

TGF-β is a representative inducer of fibroblast activation [17]. Tumors derived from MDA-MB-231 cells co-injected with TGF-β-activated fibroblasts (CCD-1068sk-TGF-β) showed enlarged stromal compartment as evidenced by increased Masson's trichrome staining (Fig. 2B) which measures collagen network, indicative of the presence of fibroblasts. Furthermore, there was increased alpha-smooth muscle actin (α-SMA) staining in tumors of mice co-injected with MDA-MB-231 and CCD-1068sk-TGF-β (Fig. 2B). These tumors also displaed significantly elevated Akt phosphorylation (Fig. 2C). Gene set enrichment analysis (GSEA) revealed that epithelial-mesenchymal transition (EMT), angiogenesis, TGF-β signaling, hedgehog signaling, and myogenesis gene sets were significantly enriched in breast cancers highly expressing FGFR1 (Fig. 2D). These results further support the onco-

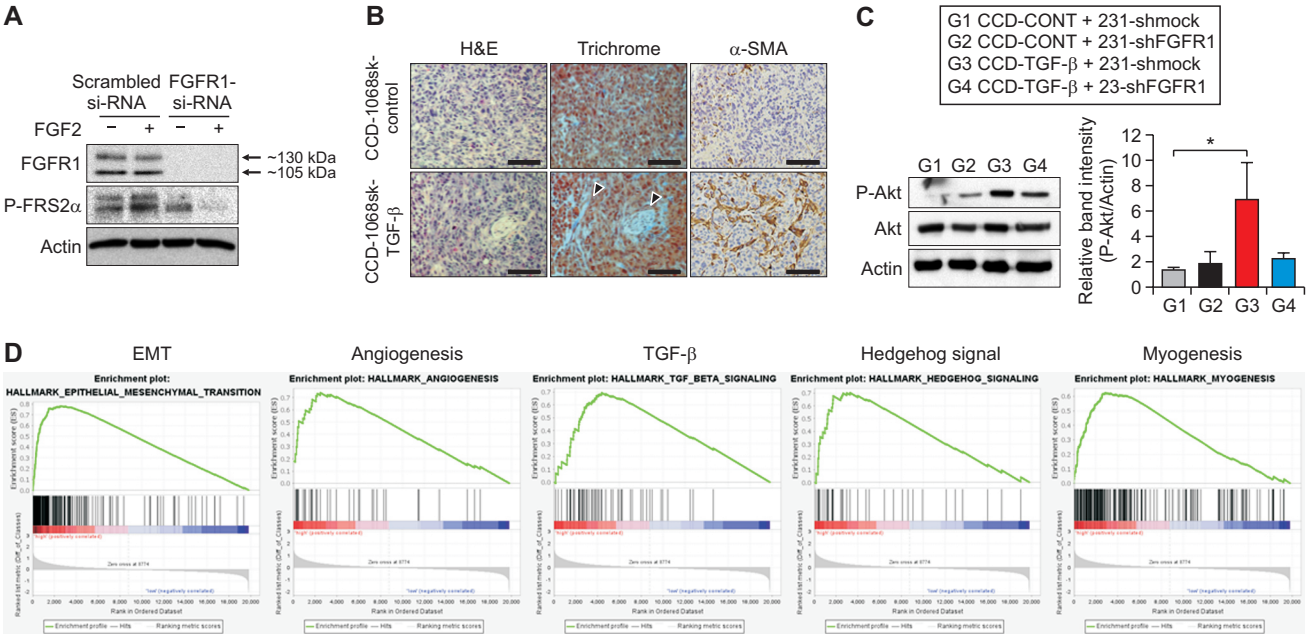


**Figure 2. Role of FGFR1 in Akt phosphorylation and breast cancer cell growth and progression.** (A) MDA-MB-231 cells were transfected with scrambled or FGFR1 si-RNA for 24 hours. Cells were then incubated with 20 ng/mL of FGF2 for 15 minutes to measure phosphorylated FRS2α. (B) Mice were subjected to xenograft co-injecting with fibroblasts and MDA-MB-231 breast cancer cells. A complex collagen network was detected in H&E-stained tumors by an intense pink and in Masson's trichrome stain by a blue stain (arrows). Stromal compartment was also detected by α-SMA immunostaining. Magnification, x100. Bars, 100 μm. (C) Phosphorylated Akt in the xenograft tumors was determined by Western blot analysis. *Significantly different between the groups compared ($P < 0.05$). (D) Enrichment plots of hallmark gene sets in the high FGFR1-expressing group. FGF2, fibroblast growth factor 2; FGFR1, FGF receptor 1; FRS2, FGFR substrate 2; α-SMA, alpha-smooth muscle actin; CONT, control; EMT, epithelial-mesenchymal transition.

genic function of FGFR1 in tumor microenvironment.

Besides their classical function as membrane-bound receptors in signal transduction, some RTKs are reported to be trafficked from the cell surface to the nucleus in response to ligand binding or heterologous agonist exposure [18]. Epidermal growth factor receptor (EGFR), for instance, associates with several gene promoters, suggesting its potential role as a transcription factor [19]. This prompted us to examine the possibility of nuclear localization of FGFR1 and its involvement in transcriptional regulation of FGF2 target genes.

When MDA-MB-231 cells were co-cultured with CAFs, FGFR1 in cancer cells underwent nuclear translocation (Fig. 3A). Additionally, direct treatment of MDA-MB-231 cells with FGF2 enhanced FGFR1 accumulation in the nucleus (Fig. 3B). The nuclear localization of FGFR1 in response of FGF2 stimulation was verified by Western blot analysis (Fig. 3C). Notably, MDA-MB-231 cells stimulated with FGF2 or CAF-CM exhibited markedly elevated levels of intracellular ROS as measured by using the fluorescent probe, DCF-DA (Fig. 3D and 3E).

It has been reported that the redox-sensitive transcription factor Nrf2, activated upon stimulation with FGFs, subsequently accumulates in the nuclear compartment [20-22]. Of note, an ROS scavenging agent *N*-acetylcysteine (NAC) attenuated FGF2-induced nuclear localization of FGFR1 as well as Nrf2 (Fig. 3F). As illustrated in Figure 3G, there was a direct physical interaction between FGFR1 and the transcriptional co-activator, p300/CBP as well as Nrf2 in MDA-MB-231 cells incubated with FGF2. We speculate that FGFR1 binding to p300/CBP may facilitate the recruitment of Nrf2.

To examine the clinical importance of an interaction between FGFR1 and Nrf2 in breast cancer progression, we analyzed a TNBC patient survival rate upon cohort bifurcation based on the mean expression value of FGFR1 and Nrf2. Analysis of a cohort TNBC patients revealed that combination of FGFR1 and Nrf2 gene expression led to a decreased survival rate compared with FGFR1 or Nrf2 expression alone (Fig. 4A).

Cyclin D1, one of the key players in cell proliferation, has been reported to be a putative target of nuclear FGFR1 [23], and its expression is also associated with Nrf2 [24]. In line with this notion, the expression of cyclin D1 was increased in MDA-MB-231 cells stimulated with FGF2. FGF2-induced expression of cyclin D1 was abolished by the introduction of FGFR1-siRNA (Fig. 4B and 4C).

## DISCUSSION

In the current study, nuclear localization of FGFR1 was significantly elevated in response to FGF2. It is speculated that

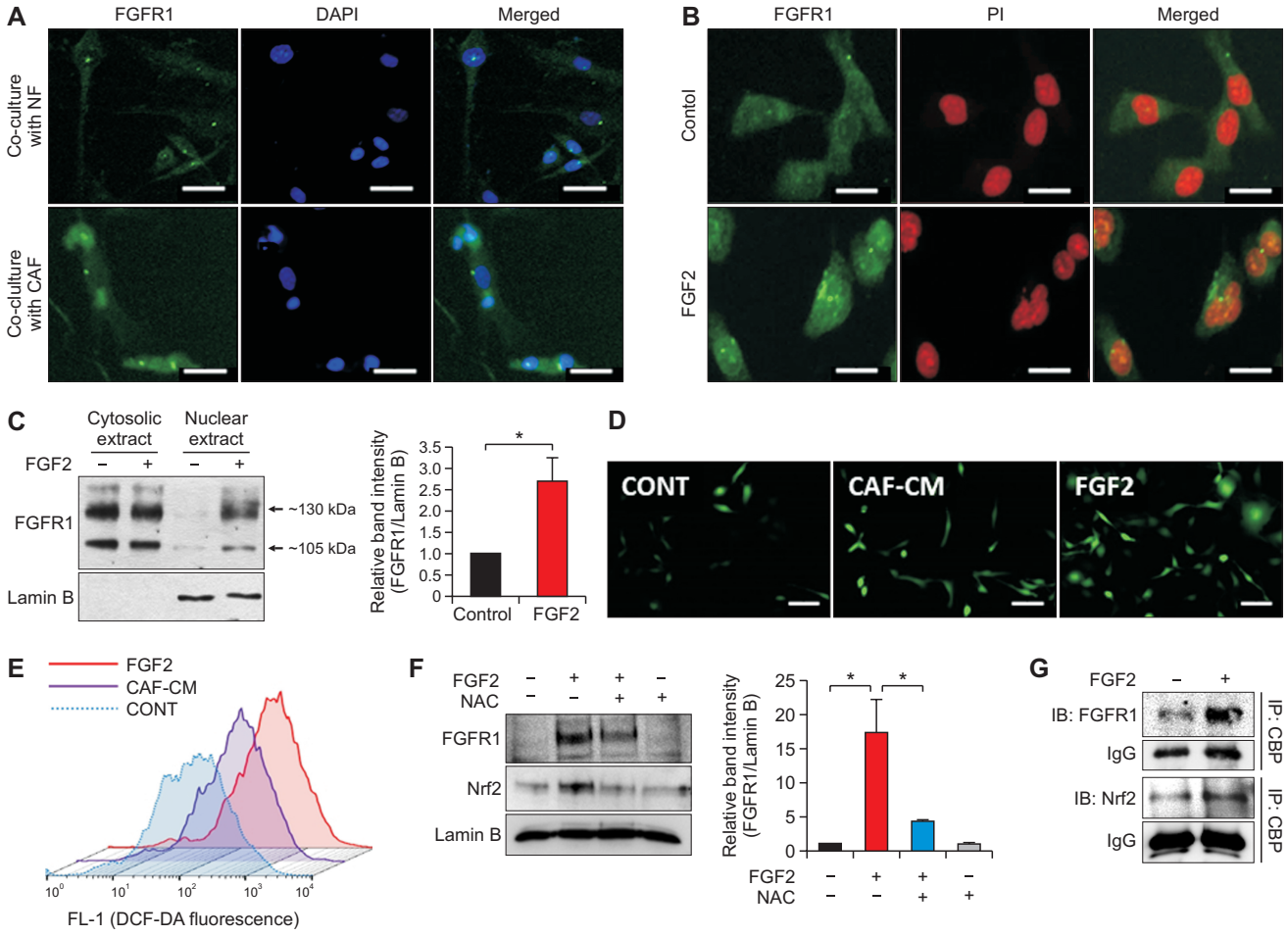


**Figure 3. The involvement of FGF2-induced ROS generation in nuclear localization of FGFR1.** (A) MDA-MB-231 cells were co-cultured with NFs or CAFs for 24 hours. MDA-MB-231 (5 x $10^3$ cells) and NFs or CAFs (5 x $10^3$ cells) were mixed prior to seeding and incubated for 24 hours. Immunocytochemical analysis was performed using anti-FGFR1 antibody. Cells were then stained with DAPI for detection of nuclei. Magnification, x100. Bars, 200 μm. (B) MDA-MB-231 cells were incubated with FGF2 for 1 hour. Immunocytochemical analysis was performed using anti-FGFR1 antibody. Cells were then stained with PI for detection of nuclei. Magnification, x100. Bars, 200 μm. (C) MDA-MB-231 cells were treated with 20 ng/mL of FGF2 for 1 hour, followed by Western blot analysis of FGFR1 in cytosolic and nuclear extracts. Lamin B was used as a nuclear marker. *Significantly different between the groups compared ($P < 0.05$). (D, E) MDA-MD-231 cells were incubated with CAF-CM or FGF2 for 3 hours and 1 hour, respectively. After staining with DCF-DA for 30 minutes, fluorescent microscopic (D) or flow cytometric (E) analysis was performed to detect intracellular ROS accumulation. Magnification, x40. (F) After pretreatment with NAC for 3 hours, cells were exposed to FGF2 for additional 1 hour. Nuclear extracts were subjected to Western blot analysis to detect the presence of FGFR1 and Nrf2 in the nucleus. **Significantly different between the groups compared ($P < 0.01$). (G) MDA-MB-231 cells were exposed to FGF2 (20 ng/mL) for 1 hour. Cell lysates were subjected to immunoprecipitation using CBP antibody for 16 hours followed by immunoblotting with. FGFR1 or Nrf2 antibody. FGF2, fibroblast growth factor 2; FGFR1, FGF receptor 1; ROS, reactive oxygen species; CAFs, cancer-associated fibroblasts; CM, conditioned medium; NFs, normal fibroblasts; DAPI, 4′,6-diamidino-2-phenylindole; PI, propidium iodide; CONT, cotrol; DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; NAC, N-acetylcysteine; CBP, CREB-binding protein.

newly synthesized FGFR1, upon stimulation with its ligand FGF2, can enter the nucleus from endoplasmic reticulum [25]. Since FGFR1 does not have nuclear localization signal (NLS) [26], it may require cargo proteins which contain NLS. While surface FGFR1 can translocate to nucleus as a complex with FGF2 which has NLS [27], newly synthesized FGFR1 would require an alternative nuclear transport mechanism.

Nuclear localization of other RTKs including EGFR and ErbB-2 has been reported by several groups. The ligand-activated cell surface RTKs after internalization through endocytosis undergo retrograde trafficking from the Golgi apparatus to the endoplasmic reticulum and subsequently [28]. EGFR is translocated from endoplasmic reticulum to the inner nuclear membrane through the nuclear pore complexes, which is regulated by importin-β [29]. Importin-β is also considered to be involved in the nuclear transport of FGFR-1 [26].

Since importin-β requires NLS-containing protein in order to import its interacting protein such as FGFR1 to the nucleus, the nuclear import of FGFR1 may need another cargo protein. Sec61 can be a candidate of transport shuttle of FGFR1 [28] as it plays a key role in translocating the mem-

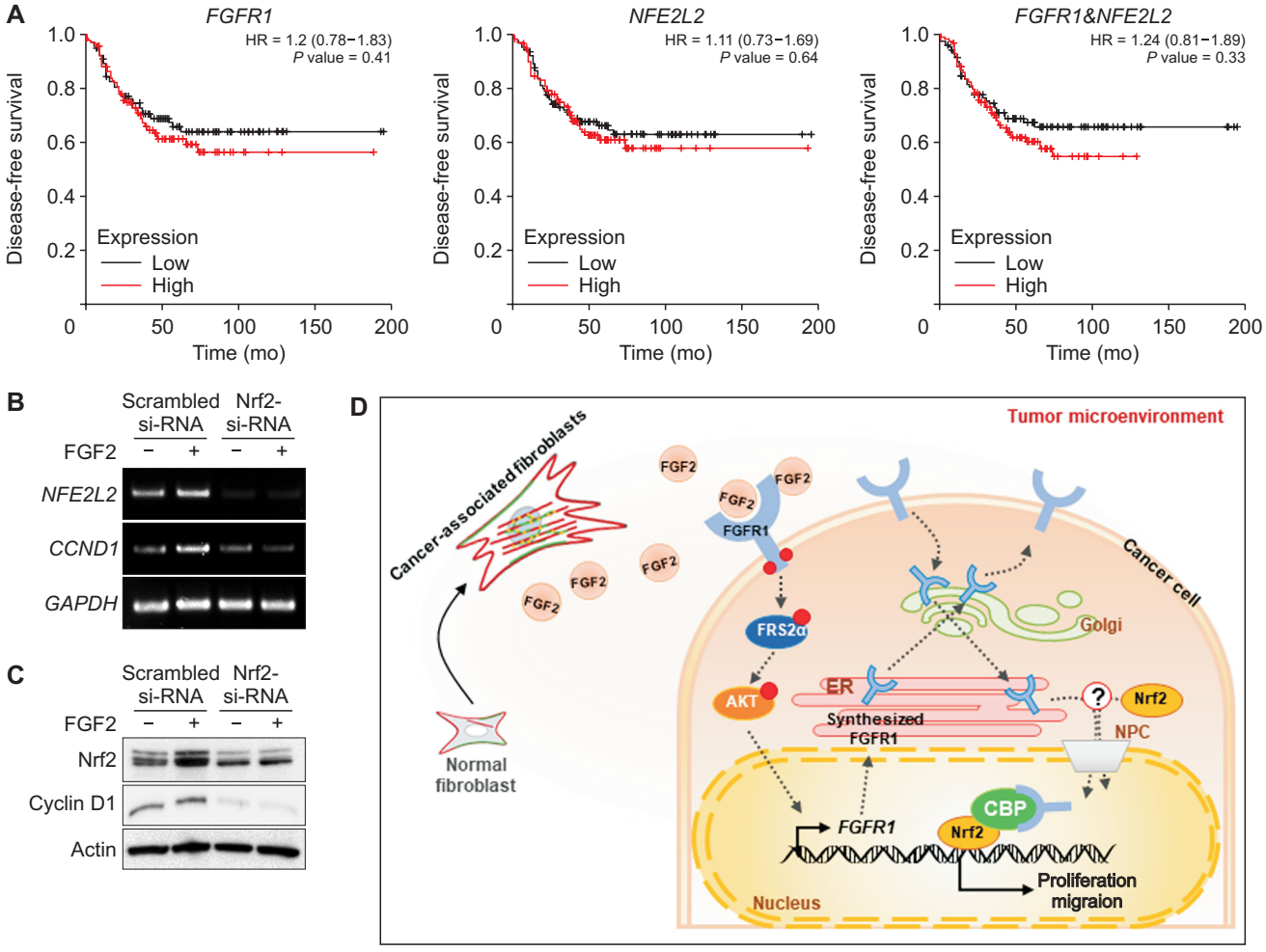


**Figure 4. Possible association between nuclear FGFR1 and Nrf2.** (A) TNBC patient cohorts were validated based on the mean expression value of the indicated single genes (*FGFR1* or *NFE2L2*) or as a signature of two genes together and patient survival was analyzed (n = 255). (B, C) MDA-MB-231 cells were transfected with scrambled or Nrf2 si-RNA for 24 hours. Cells were then incubated with 20 ng/mL of FGF2 for 3 hours. The mRNA (B) and protein (C) expression of cyclin D1 was assessed by RT-PCR and Western blot analyses, respectively. The expression of cyclin D1 was measured by RT-PCR (B) and Western blot (C) analyses. (D) In tumor microenvironment, fibroblasts are activated to form CAFs, which secrete FGF2. CAF-derived FGF2 could induces nuclear translocation as well as *de novo* synthesis of FGFR1, ultimately contributing to cancer cell proliferation, migration and tumor growth. While membrane bound FGFR1 may translocate to nucleus as a complex with FGF2 which has nuclear localization signal (NLS), the complex is likely rather to stimulate the intracellular signaling via FRS2α, which induces transcription of *FGFR-1* gene. On the other hand, newly synthesized FGFR-1 is speculated to enter the nucleus as a complex with a cargo protein harboring NLS. FGFR-1 is translocated to the inner nuclear membrane through the nuclear pore complexes (NPCs), which is regulated by importin β. FGF2, fibroblast growth factor 2; FGFR1, FGF receptor 1; TNBC, triple negative breast cancer; HR, hazard ratio; CAFs, cancer-associated fibroblasts; ER, endoplasmic reticulum; FRS2, FGFR substrate 2; CBP, CREB-binding protein.

brane bound EGFR complex by interacting with EGFR-bound importin-β [29]. FGFR1 does bind to Sec61 translocon [30], which is associated with inner nuclear membrane and hence may deliver FGFR1 from the endoplasmic reticulum to the nucleoplasm. However, the nuclear transport of FGFR-1 appears to be distinct from that of EGFR family proteins [31]; while the soluble importin-β regulates FGFR-1 nuclear transport, the endoplasmic reticulum membrane-associated importin-β mediates nuclear transport of EGFR through the membrane-bound INTERNET (integral trafficking from the endoplasmic reticulum to the nuclear envelope transport) pathway. The nuclear accumulation of FGFR1 is activated by changes in cell contacts and by stimulation of cells with growth factors, neurotransmitters and hormones as well as by a variety of different second messengers and thus was named integrative nuclear FGFR1 signaling [27].

An early study by Maher detected FGFR1 in the nuclear fraction of FGF2-treated cells, suggesting that the receptor may play a direct role in regulating gene transcription [32]. Nuclear FGFR1 has been reported to be involved in upregulation of invasive gene expression in breast cancer cells [33]. While our manuscript was being prepared, a report address-

ing an unconventional role for FGFR1 in gene transcription regulation in estrogen receptor-positive breast cancer has been published [34]. Of note, nuclear FGFR1 induces a gene expression profile which confers resistance to antiestrogens. Moreover, the nuclear localization of FGFR1 in pancreatic stellate cells has been shown to facilitate invasion of pancreatic cancer cells [35].

One of nuclear FGFR1's functions is to act as a transcriptional co-activator as proposed for EGFR [36]. If FGFR1 does not harbor a DNA binding domain [23], it may interact with transcription factors and indirectly regulates downstream gene expression (Fig. 4D). In line with this speculation, nuclear FGFR2 has been shown to bind to hypoxia inducible transcription factor-1 and -2 and negatively modulate their transcriptional activities [37]. We found that the redox-sensitive transcription factor, Nrf2 co-localized FGFR1 in the nucleus in MDA-MB-231 breast cancer cells upon FGF2 stimulation which was abolished by NAC treatment (J. Suh and Y.-J. Surh, unpublished observation). Potential interaction between FGFR1 and Nrf2 and its implications for breast cancer growth and progression merit further investigation.

In summary, the results from our present study suggest that paracrine FGF2 stimulation undergoes FGFR1 nuclear localization, which might regulate oncogenic gene transcription presumably through cooperation between CBP and other transcription factors. This might provide a novel molecular mechanism by which CAF-secreted factors affect the oncogenic gene expression of cancer cells in tumor microenvironment. Thus, inhibition of nuclear FGFR1 could be an attractive therapeutic strategy in the management of breast cancer.

## FUNDING

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## CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

## SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.2022.27.1.68.

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