RESEARCH ARTICLE

Molecular Carcinogenesis WILEY

Fibroblast growth factor-2, derived from cancer-associated fibroblasts, stimulates growth and progression of human breast cancer cells via FGFR1 signaling

Jinyoung Suh[1] | Do-Hee Kim[2] | Yeon-Hwa Lee[1] | Jeong-Hoon Jang[1] | Young-Joon Surh[1,3,4]

[1]Tumor Microenvironment Global Core Research Center and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul, South Korea

[2]Department of Chemistry, College of Convergence and Integrated Science, Kyonggi University, Suwon, Gyeonggi-do, South Korea

[3]Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science and Technology, Seoul National University, Seoul, South Korea

[4]Cancer Research Institute, Seoul National University, Seoul, South Korea

Correspondence
Young-Joon Surh, Tumor Microenvironment Global Core Research Center and Research Institute of Pharmaceutical Sciences, Seoul National University, 08826 Seoul, South Korea.
Email: surh@snu.ac.kr

Abstract

Cancer-associated fibroblasts (CAFs) constitute a major compartment of the tumor microenvironment. In the present study, we investigated the role for CAFs in breast cancer progression and underlying molecular mechanisms. Human breast cancer MDA-MB-231 cells treated with the CAF-conditioned media manifested a more proliferative phenotype, as evidenced by enhanced messenger RNA (mRNA) expression of Cyclin D1, c-Myc, and proliferating cell nuclear antigen. Analysis of data from The Cancer Genome Atlas revealed that fibroblast growth factor-2 (FGF2) expression was well correlated with the presence of CAFs. We noticed that the mRNA level of FGF2 in CAFs was higher than that in normal fibroblasts. FGF2 exerts its biological effects through interaction with FGF receptor 1 (FGFR1). In the breast cancer tissue array, 42% estrogen receptor-negative patients coexpressed FGF2 and FGFR1, whereas only 19% estrogen receptor-positive patients exhibited coexpression. CAF-stimulated MDA-MB-231 cell migration and invasiveness were abolished when FGF2-neutralizing antibody was added to the conditioned media of CAFs. In a xenograft mouse model, coinjection of MDA-MB-231 cells with activated fibroblasts expressing FGF2 dramatically enhanced tumor growth, and this was abrogated by silencing of FGFR1 in cancer cells. In addition, treatment of MDA-MB-231 cells with FGF2 enhanced expression of Cyclin D1, a key molecule involved in cell cycle progression. FGF2-induced cell migration and upregulation of Cyclin D1 were abolished by siRNA-mediated FGFR1 silencing. Taken together, the above findings suggest that CAFs promote growth, migration and invasion of MDA-MB-231 cells via the paracrine FGF2-FGFR1 loop in the breast tumor microenvironment.

KEYWORDS

breast cancer, cancer-associated fibroblasts, fibroblast growth factor 2, fibroblast growth factor receptor 1, tumor microenvironment

1 | INTRODUCTION

The stromal cells represent an important component of the tumor microenvironment and contribute to the progression of cancer.[1,2] Among several types of stromal cells, fibroblasts are prevalent in the tumor microenvironment. Cancer cells activate stromal fibroblasts into cancer-associated fibroblasts (CAFs) through stimulation of paracrine growth factors.[1] CAFs are morphologically and functionally different from normal fibroblasts (NFs) as indicated by their increased rate of proliferation and differential expression of extracellular matrix constituents and growth factors.[1,3] CAFs have significant roles in promotion of growth and invasiveness of cancer cells.[4] It has been reported that tumors with abundant CAFs have poorer prognosis than those with less CAFs.[5] In particular, triple-negative breast cancer (TNBC) patients with stroma-rich tumors have a worse outcome than those with stroma-poor tumors for overall survival.[6] Therefore, molecules secreted by CAFs including CXCL14 and CCL5 are considered potential therapeutic targets for the treatment of TNBC.[7] However, despite emerging evidence for the crucial role of CAFs in tumor microenvironment, the molecular mechanisms by which CAFs regulate proliferation, progression and invasiveness of cancer cells are not fully understood.

CAFs secrete a distinct set of growth factors and cytokines which promote survival, growth, and progression of cancer cells.[8-11] Fibroblast growth factors (FGFs) are a family of multifunctional growth factors involved in a plethora of cellular activities, ranging from proliferation, survival, migration, and differentiation to angiogenesis and tissue repair. Over 20 different FGF isoforms have been identified in mammals, some of which play a role in the pathogenesis of cancer. For instance, stromal expression of FGF10 affects invasive behavior of breast cancer.[12] FGF1 secreted by ovarian CAFs stimulates cell proliferation and migration signaling in a FGF receptor (FGFR) 4-dependent fashion.[13] In addition, FGF2 has been known as a major growth factor derived from CAFs surrounding breast cancer tissue.[3,14] Notably, CAFs isolated from hormone-independent tumors express higher levels of FGF2 compared with hormone-dependent counterparts.[15]

FGF2 is a classical survival factor in multiple cell types.[16] Inappropriate overexpression of FGF2 and its receptors causes aberrant cell proliferation in various malignancies including breast, ovarian, and skin cancer.[17] The biological activity of the FGF2 requires the presence of FGFRs to transduce specific signal transduction.[18] The mammalian FGFR family proteins are encoded by four distinct genes (Fgfr1-4), and their interaction with the corresponding ligand determines the specificity of FGF-induced downstream signaling.[19]

Of the four FGFRs, FGFR1 has been known to exhibit high affinity for FGF2.[20] However, the cross-talk between FGF2 secreted from CAFs and FGFR1 in breast cancer cells has not been elucidated yet. This prompted us to investigate the potential role of the FGF2-FGFR1 axis in breast tumor microenvironment. Here, we report that CAF-derived FGF2 stimulates breast cancer cell proliferation, migration, and progression via FGFR1 signaling.

2 | METHODS AND MATERIALS

2.1 | Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM), Rosewell Park Memorial Institute (RPMI) 1640 medium, and minimum essential medium (MEM) were purchased from Gibco BRL (Grand Island, NY). TRIzol was obtained from Invitrogen (Carlsbad, CA). Primary antibodies for FGFR1 and Cyclin D1 were products of Cell Signaling Technology (Danvers, MA). α-Smooth muscle actin (α-SMA) antibody was obtained by Abcam (Cambridge, UK). Antibodies against FGFR2, and β-actin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The bicinchoninic acid (BCA) protein assay reagent was a product of Pierce Biotechnology (Rockfold, IL).

2.2 | Tissue microarray

Human paraffin-embedded breast cancer tissue array with matched adjacent normal breast tissue (US biomax, Inc; Rockville, MD) was subjected to immunohistochemical and immunofluorescent analysis. Immunohistochemical analysis was performed by Abion CRO (Seoul, South Korea). Four-μm sections of 10% formalin-fixed, paraffin-embedded tissues were placed on glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled twice for 6 minute in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 minute. Slides were then incubated with α-SMA antibody at room temperature for 40 minute in Tris-buffered saline with 0.1% Tween 20 (TBST) and blots were developed using horseradish peroxidase (HRP)-conjugated secondary antibody. The peroxidase binding site were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin.

For immunofluorescence analysis, tissue blocks were deparaffinized with xylene. Following antigen retrieval by heated citrate buffer, sections were permeabilized and blocked according to the standard protocol. After overnight incubation at 4°C with fluorescent conjugated anti-FGF2 or anti-FGFR1 antibody, the tissue section was washed with phosphate-buffered saline (PBS) and then analyzed under a fluorescence microscope.

2.3 | Immunofluorescence staining of cultured fibroblasts

NFs and CAFs were seeded at 5×10^3 cells per well in an eight chamber plate. After overnight incubation, cells were fixed with fixation solution containing 95% methanol and 5% acetic acid (20 minute). The cells were then washed in PBS (twice 5 minute each), permeabilized with 0.2% triton X-100 (5 minute), washed in

PBS (twice 5 minute each), and blocked with 5% bovine serum albumin (BSA) in PBS (30 minute). Polyclonal rabbit anti-α-SMA, diluted at 1:100 with 1% BSA in PBS, was applied overnight at 4℃. This was followed by washing cells in PBS (twice 5 minute each) and then incubation for 1 hour at room temperature with fluorescein isothiocyanate-conjugated anti-rabbit IgG secondary antibody diluted at 1:1000 with 1% BSA in PBS. After washing (twice 5 minute each), cells were treated with propidium iodide. The expression of α-SMA was detected using a confocal microscope (Leica microsystems; Wetzlar, Germany).

2.4 | Gene expression profile analysis

To determine the relationship between FGF2 and α-SMA genes in breast cancers, we selected "Breast Invasive Carcinoma (Provisional)" data set publically available from TCGA data portal (www.cbioportal.org). The clinical significance of *ACTA2* (α-*SMA*) and *FGF2* mRNA expression was evaluated by calculating the Spearman's correlation coefficient.

2.5 | Cell culture

Human breast cancer (MCF-7, MDA-MB-231, and MDA-MB-468) cell lines were maintained in RPMI 1640 and DMEM supplemented with 10% fetal bovine serum (GenDEPOT; Barker, TX) and 100 ng/mL penicillin/streptomycin/fungizone mixture at 37℃ in a humidified atmosphere of 5% CO_2/95% air. CCD-1068sk breast NFs were maintained in MEM supplemented with 10% fetal bovine serum (GenDEPOT) and 100 ng/mL penicillin/streptomycin/fungizone mixture. Primary NFs and CAFs cells (Asterand Bioscience; Detroit, MI) were incubated with 10% fatal bovine serum (Gibco BRL; Grand Island, NY) and 100 ng/mL penicillin/streptomycin/fungizone mixture and other supplements. The cells were plated at an appropriate density according to each experimental scale. All human cell lines have been authenticated within the last 3 years, and experiments were performed with mycoplasma-free cells.

2.6 | Collection of conditioned media

CAFs and NFs were seeded on 100 mm dishes at 1×10^6 cells/mL. Culture medium was removed 24 hour after seeding, and cells were washed once with PBS followed by addition of 8 mL of serum-free medium per well. After 48 hour of incubation, the conditioned media (CM) was collected and passed through 0.2 μm membrane syringe filter to remove any residual cells and debris. For neutralization of FGF2 in the CM of CAFs, CM was preincubated with 25 μg/mL of human FGF2 antibody or its immunoglobulin G (IgG) control (R&D systems; Minneapolis, MN) for 1 hour at room temperature.

2.7 | Enzyme-linked immunosorbent assay

The concentration of FGF2 was determined by the enzyme-linked immunosorbent assay (ELISA) assay (R&D systems). CM of CAFs and NFs was collected and concentrated with Amicon Ultra 50 kDa centrifugal filters (Milipore; Burlington, MA). The concentrated CM was used for measurement of the FGF2 level according to the manufacturer's protocol.

2.8 | Cell migration assay

Cell migration was measured using the culture-inserts ($2 \times 0.22\,cm^2$) supplied from Ibidi GmbH (Regensburg, Germany). To create a wound gap, 100 μL of 1×10^5 cancer cells were seeded on the culture-inserts, which were gently removed using sterile tweezers following an overnight incubation. Cells were then exposed to CMs of fibroblasts (NF-CM and CAF-CM) with or without 5 μM of FGF2-neutralizing antibody (Millipore; Darmstadt, Burlington, MA) for 24 hour. The progression of wound closure was monitored, and distance between gaps was measured under the microscope (Nikon; Tokyo, Japan). All assays were performed in triplicate.

2.9 | Cell invasion assay

The 24-well transwell chamber plate with pores of 8 μm in diameter was used to determine the invasive capability of the cells. Fibroblasts (2×10^4 cells/well) were seeded in the lower compartment. After incubation for 24 hour, cancer cells (1×10^4 cells/well) were seeded in the insert. The lower chamber beneath the insert membrane was incubated in 600 μL of 1% DMEM supplemented with 1% fetal bovine serum in the absence or presence of 5 μM of FGF2-neutralizing antibody. After 36-hour incubation, cells on the upper surface of the membrane were removed by wiping with cotton swabs, and the invaded cells were fixed with 100% methanol and stained with 0.05% crystal violet (Sigma-Aldrich; St Louis, MO). The number of invaded cells was counted in three high power fields under a microscope. The experiment was repeated three times independently.

2.10 | Three-dimensional invasion assay (spheroid invasion assay)

The spheroid invasion assay was performed using a 96-well 3D spheroid BME cell invasion assay kit (Trevigen; Gaithersburg, MD). Briefly, cells were resuspended in the spheroid formation extracellular matrix and MDA-MB-231 cells (1×10^3) and control- or transforming growth factor beta (TGF-β)-activated CCD-1068sk fibroblasts (1×10^3) were plated in the round bottom low attachment 96-well plate. After the cells were left to form spheroids for 3 days, invasion matrix was added to each well and incubated at 37℃ for 3 hour followed by addition of culture medium. Spheroids were visualized under a bright field microscope, 1 day and 6 days after addition of the invasion matrix. Relative invasion was analyzed by

using Image J calculating the threshold the spheroid area and measuring the number of pixels within the area.

2.11 | Western blot analysis

Cells were lysed in radioimmunoprecipitation assay lysis buffer (150 mM NaCl, 0.5% Triton ×100, 50 mM Tri-HCl [pH 7.4], 25 mM NaF, 20 mM EGTA, 1 mM, 1 mM Na_3VO_4, 0.1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail tablets) for 15 minute on ice followed by centrifugation at 13000 g for 20 minute. The protein concentration of the supernatant was measured by using BCA reagent (Thermo Fisher Scientific; Rockford, IL). Protein (30 μg) was separated by 8% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to the polyvinylidene difluoride membrane (Gelman Laboratory; Ann Arbor, MI). The blots were blocked with 5% nonfat dry milk/TBST for 1 hour at room temperature. The membranes were incubated for 4 hour at room temperature with 1:1000 dilution of polyclonal antibody of FGFR1, P-FRS2α, Cyclin D1, lamin B1, and actin. The blots were rinsed three times with TBST for 10 minute each. Washed blots were incubated with 1:5000 dilutions of HRP-conjugated secondary antibody (Thermo Fisher 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).

2.12 | Small interfering RNA transfection

Small interfering RNA (siRNA) oligonucleotides targeting for FGFR1 and FGF2 were purchased from Genolution Pharmaceuticals (Seoul, South Korea). The sense and antisense strands of FGFR1 and FGF2 used are as follows (forward and reverse, respectively): FGFR1; 5′-AUUCAAACCUGACCACAGA-3′ and 5′-UCUGUGGUCAGGUUUGAAU-3′, FGF2; 5′-UAUACUGCCCAGUUCGUUUCAGUGC-3′ and 5′-GCACUGAAACGAACUGGGCAGUAUA-3′. MDA-MB-231 cells (4×10^5/60-mm dish) were transfected with 25 nM of specific or scrambled siRNA oligonucleotides using Lipofectamine RNAiMAX according to manufacturer's instructions (Invitrogen).

2.13 | Reverse transcription-polymerase chain reaction

Total RNA was isolated from each cell by using TRIzol reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol. One μg of total RNA was reverse-transcribed with murine leukemia virus reverse transcriptase (Promega; Madison, WI). The primers employed were (forward and reverse, respectively): α-SMA, 5′-AGCGACCCTAAAGCTTCCCA-3′ and 5′-CATAGAGAGACAGCACCGCC-3′, 791 bp; FAP-α, 5′-AGTTTCAGCGACTACGCCAA-3′ and 5′-GGAAAGCTGTTCCTCGACCA-3′, 379 bp; Cyclin D1, 5′-ACCTGGATGCTGGAGGTCT-3′ and 5′-GCTCCATTTGCAGCAGCTC-3′, 241 bp; c-Myc, 5′-AGCAGCGAC

TCTGAGGAGGAACAAGAA-3′ and 5′-AGGTAGTCCTTCCGAGTGGA-3′, 216 bp; PCNA, 5′- CTTTTCTGTCACCAAATTTGTACC-3′ and 5′-AACTGCATTTAGAGTCAAGACCC-3′, 206 bp; MMP2, 5′-GGCCAAGTGGTCCGTGTG-3′ and 5′-GAGGCCCCATAGAGCTCC-3′, 692 bp; MMP9, 5′-CACTGTCCACCCCTCAGAGC-3′ and 5′-GCCACTTGTCGGCGATAAGG-3′, 263 bp; FGFR1, 5′-AACCTGCCTTATGTCCAGATCT-3′ and 5′-AGGGGCGAGGTCATCACTGC-3′, 209 bp; GAPDH, 5′-AAGGTCGGAGTCAACGGATTT-3′ and 5′-GCAGTGAGGGTCTCTCTCT-3′, 1053 bp. Amplification products were resolved by 1% to 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

2.14 | In vivo xenograft assay

Female Balb/c (nu/nu) mice, 5 weeks of age, were purchased from Orientbio (Seoul, South Korea) and acclimatized for 1 week under standard temperature, humidity, and time lighting conditions at the animal care facility. 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, shmock- or shFGFR1-knockdown-MDA-MB-231 cells and control- or TGF-β-activated CCD-1068sk fibroblasts were coinjected subcutaneously into the flank at a density of 5×10^6 cells in 75 μL PBS and 75 μL Matrigel). Three weeks after injection, tumor volumes were calculated according to the formula: $0.5 \times$ (length) $\times$ (width)^2. Mice were killed after 7 weeks of tumor injection. All the tumors were formalin-fixed, and paraffin-embedded for hematoxylin and eosin (H&E) staining and Masson's trichrome staining.

2.15 | Immunohistochemical analysis

The dissected xenograft tumor tissues were prepared for immunohistochemical analysis to detect the expression of Cyclin D1 and proliferating cell nuclear antigen (PCNA). Immunohistochemical analysis was performed by Abion CRO (Seoul, South Korea). Four-μm sections of 10% formalin-fixed, paraffin-embedded tissues were placed on glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated by using microwave and boiled twice for 6 minute in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish nonspecific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 minute. For the detection of respective protein expression, slides were incubated with Cyclin D1 or PCNA antibody at room temperature for 40 minute in TBST, and blots were then developed using HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody, respectively. The peroxidase binding was detected by staining with 3,3′-diaminobenzidine tetrahydrochloride. Finally, counterstaining was performed using Mayer's hematoxylin.

2.16 | Statistical analysis

When necessary, data were represented as means ± SD at least three independent experiments, and statistical analysis between groups was performed using the Student t test. $^*P < .05$; $^{**}P < .01$; $^{***}P < .005$.

3 | RESULTS

3.1 | CAFs are abundant in breast cancer tissues as evidenced by increased expression of the activated fibroblast marker α-SMA

CAFs are characterized by their overexpression of α-SMA, a cytoskeletal protein associated with smooth muscle cells[21] which contributes to morphological transformation from a spindle-like to a stellate shape. As shown in Figure 1A, analysis of the human breast cancer tissue array revealed increased expression of α-SMA compared with normal adjacent tissue. In particular, 95% (37 out of 39) estrogen receptor (ER)-negative breast cancer patients expressed a high level of α-SMA while 55% (33 out of 60) of ER-positive breast cancer patients expressed α-SMA. The higher expression of α-SMA in breast tumors than adjacent normal tissues was verified by immunohistochemical analysis (Figure 1B). We observed α-SMA-positive myoepithelial cells surrounding normal mammary glands which was also described in other studies.[22,23] While NFs showed the typical spindle-shape morphology, CAFs displayed a more heterogeneous appearance with branched cytoplasm surrounding speckle nucleus (Figure 1C). The identity of CAFs and NFs was verified by their differential expression of genes encoding α-SMA and fibroblast activating protein-α (FAP-α) as determned by RT-PCR (Figure 1D). The prevalent overexpression of α-SMA in CAFs was also verified by immunofluorescence analysis (Figure 1E).
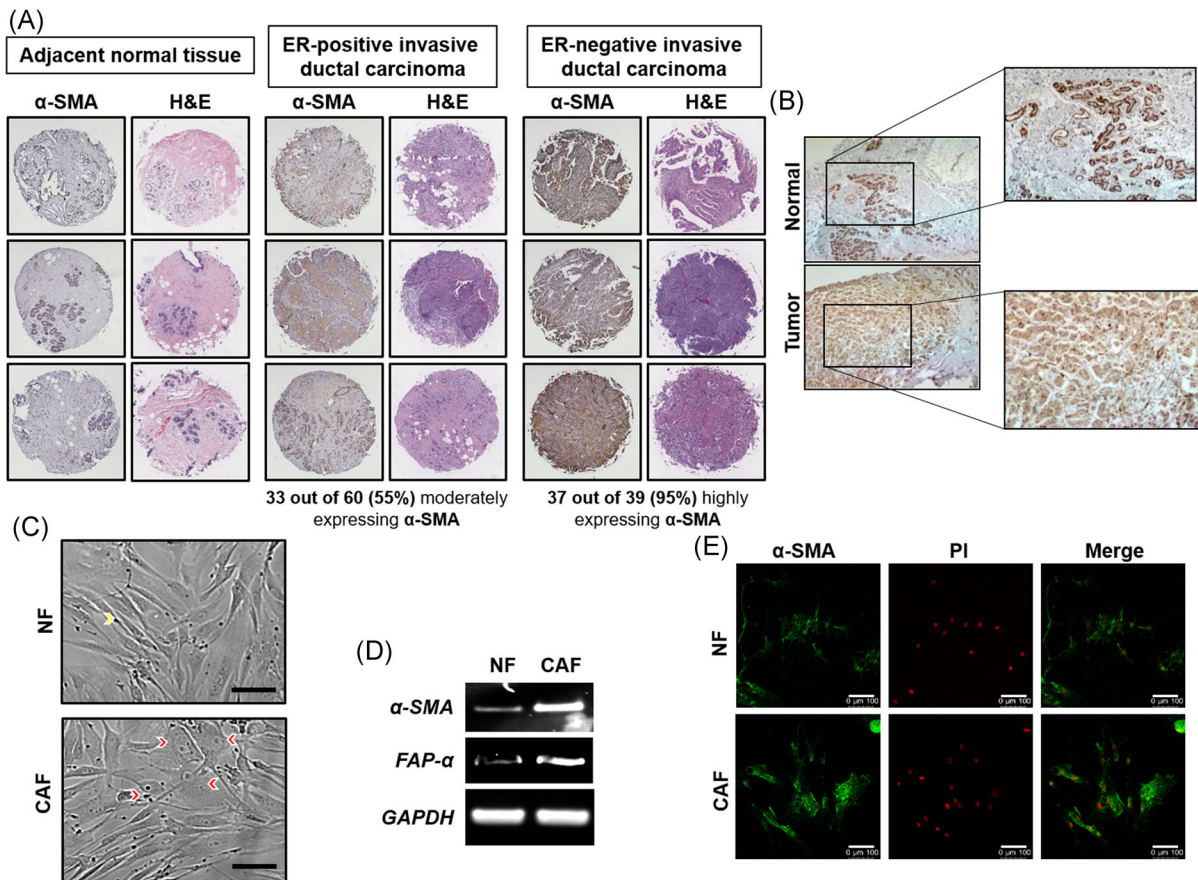

FIGURE 1 Identification of CAFs in human breast cancer tissues. A, Invasive breast carcinomas from a tumor tissue microarray were stained for α-SMA. H&E images were provided by US Biomax Inc. B, The expression levels of α-SMA between infiltrating ductal carcinoma and adjacent normal tissues of breast cancer patients were compared by immunohistochemical analysis. Magnification, ×200. C, Isolated CAFs have distinct morphology characterized by large and plump cells (red arrows) distinguished from NFs which are thin, wavy, and small spindle cells (yellow arrow). Bars, 40 μm. D, The mRNA expression of activated fibroblast markers, such as α-SMA and FAP-α, was measured by RT-PCR in NFs and CAFs. E, Expression of α-SMA in NFs and CAFs was determined by immunofluorescent staining. Bars = 100 μm. CAFs, cancer-associated fibroblasts; H&E, hematoxylin and eosin; mRNA, messenger RNA; NFs, normal fibroblasts; RT-PCR, reverse transcription-polymerase chain reaction; α-SMA, α-smooth muscle actin [Color figure can be viewed at wileyonlinelibrary.com]

3.2 | CAFs stimulate proliferation and invasiveness of MDA-MB-231 breast cancer cells

To determine whether CAFs are involved in cancer cell proliferation, we collected CM from CAFs (CAF-CM) and NFs (NF-CM), and then both media were treated to breast cancer cells. CAF-CM dramatically enhanced the ability of ER-negative MDA-MB-231 cells to migrate compared with NF-CM, whereas CAF-CM barely induced migration of ER-positive MCF-7 cells (Figure 2A). Likewise, MDA-MB-231 cells incubated with CAF-CM were more proliferative than MCF-7 cells (data not shown). Cyclin D1, a well-known cell proliferation marker, is involved in cell migration, and hence ultimately cancer metastasis.[24,25] Incubation of MDA-MB-231 cells in the presence of CAF-CM induced transient upregulation of Cyclin D1 (Figure 2B) and its mRNA transcript (Figure 2C). In addition, expression of two other proliferation marker genes, c-Myc and PCNA was also enhanced upon stimulation with CAF-CM (Figure 2C). Moreover, MDA-MB-231 cells cocultured with CAFs induced expression of these genes and also invasion marker genes (MMP2 and MMP9) and the epithelial to mesenchymal transition marker gene, SNAI1 (Figure 2D).

3.3 | FGF2 production in CAFs is associated with MDA-MB-231 cell migration and invasiveness

To address which secreted factors are involved in breast cancer progression, fibroblast expression of genes encoding representative oncogenic members of FGF family, FGF1, FGF2, and FGF10[26-28] was determined by RT-PCR. As shown in Figure 3A, FGF2 accumulation was most dramatically increased in primary CAFs compared with that in NFs. This result is consistent with other studies demonstrating that CAFs overexpress FGF2 in colon cancer and lung adenocarcinoma.[29,30] To investigate the correlation between FGF2 expression and fibrous stromal characteristics of breast cancer, we analyzed the expression levels of FGF2 and α-SMA in breast cancer patients, and observed a significant correlation (Spearman correlation coefficient; .24, P = 1.43e−13; Figure 3B).

To assess the functional role of FGF2 in CAF-CM-induced breast cancer cell migration, a wound healing assay was conducted by using FGF2-neutralizing antibody. The concentration of FGF2, determined by ELISA, was much higher in CM from CAFs, compared with that in CM from NFs (Figure 3C). As illustrated in Figure 3D, CAF-CM-promoted MDA-MB-231 cell migration was significantly reduced
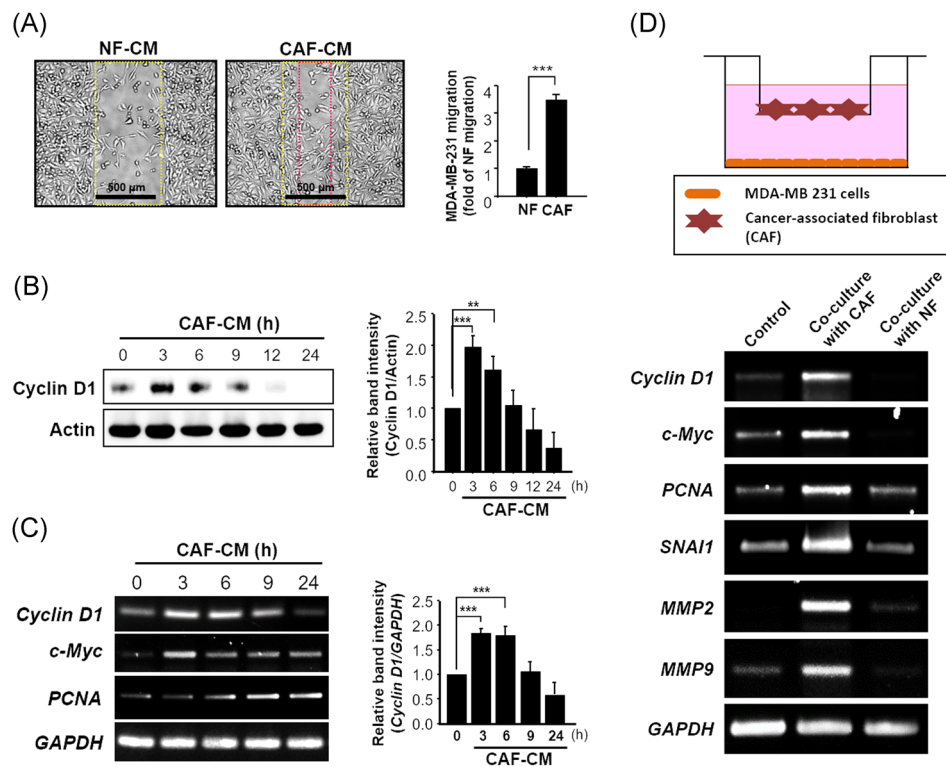

FIGURE 2 Enhancement of breast cancer cell proliferation and migration by CAFs. A, Migrative ability of MDA-MB-21 cells was measured by the wound healing assay. Migration distance of cells stimulated with CAF-CM was increased compared with that of those cells treated with NF-CM. B and C, MDA-MB-231 cells were incubated with CAF-CM for the indicated time periods, and the protein levels of Cyclin D1 was assessed by Western blot analysis (B). The mRNA expression of cell proliferation markers was analyzed by RT-PCR (C). D, MDA-MB-231 cells were cocultured with CAFs and NFs for 24 hour, and the mRNA levels of proliferative and invasive markers were determined by RT-PCR. Data are means ± SD. *P < .05; **P < .01; ***P < .005 . CAFs, cancer-associated fibroblasts; CM, conditioned medium; mRNA, messenger RNA; NFs, normal fibroblasts; RT-PCR, reverse transcription-polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]
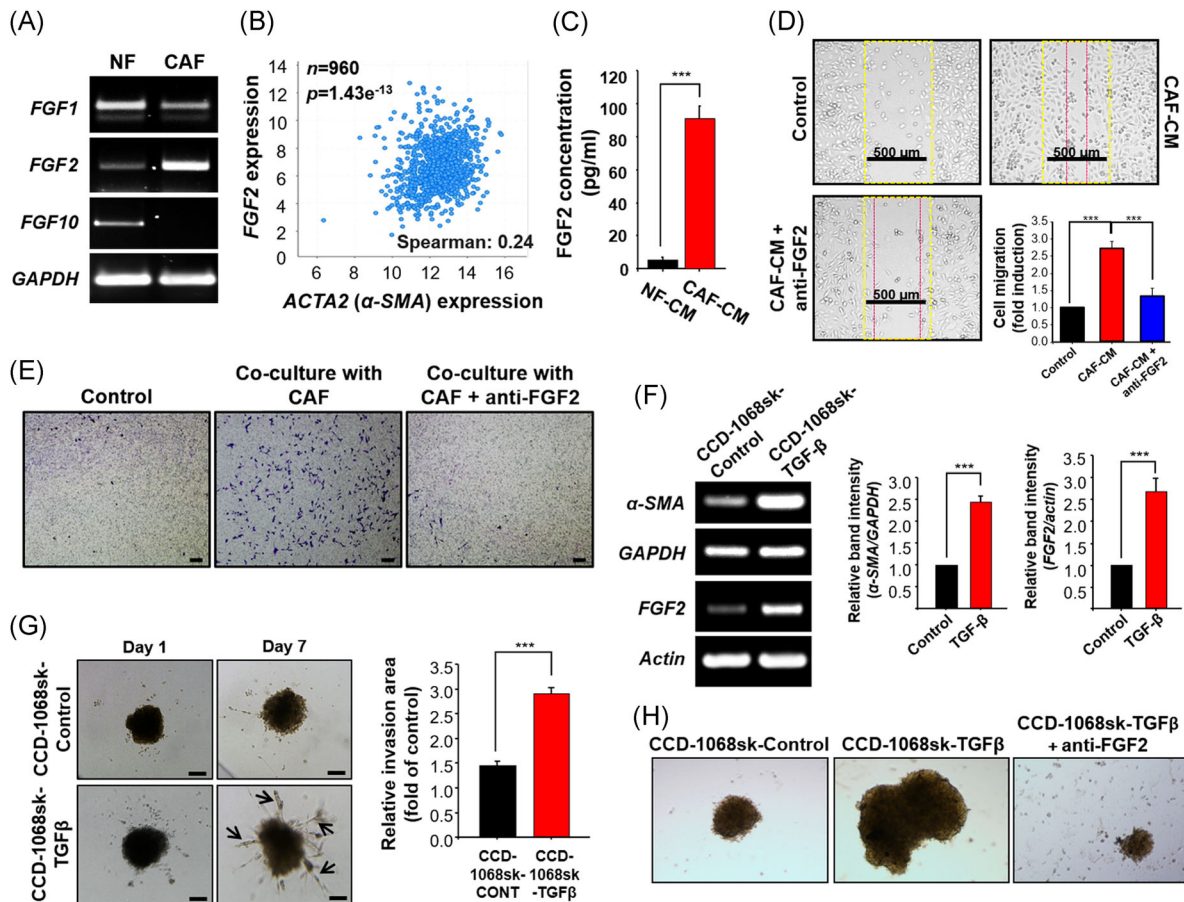
FIGURE 3 Effects of FGF2 produced by CAFs breast cancer cell migration and invasion. A, The mRNA expression of some representative FGFs was determined by RT-PCR in NFs and CAFs. B, Correlation between the mRNA levels of FGF2 and α-SMA in breast cancer patients. To analyze the relationship between *FGF2* and α-*SMA* in breast cancers, we obtained the data from TCGA, Provisional by using www.cbioportal.org. Spearman's correlation coefficient represents positive association between to genes. C, Secretion of FGF2 in NFs and CAFs was measured by the ELISA assay. D, Migrative ability of MDA-MB-231 cells with and without FGF2 antibody treatment was measured by the wound healing assay. The cells were exposed to CAF-CM in the absence or presence of FGF2-neutralizing antibody for 24 hour. Bars = 500 μm. E, The extent of cancer cell invasion was measured by a transwell cell invasion assay in MDA-MB-231 cells. Bars = 100 μm. F, The mRNA expression of activated fibroblast markers was determined by RT-PCR in control and TGF-β-stimulated CCD-1068sk fibroblasts which were preincubated over 4 days with recombinant TGF-β. G, Bright field micrographs of spheroidal aggregates that contain MDA-MB-231 cells cocultured with control or TGF-β-stimulated CCD-1068sk fibroblasts. Protrusions of invasive cancer cells were marked by arrows. Bars = 200 μm. H, Effects of FGF2 antibody neutralization on TGF-β-stimulated formation of spheroidal aggregates. Data are means ± SD. CAFs, cancer-associated fibroblasts; ELISA, enzyme-linked immunosorbent assay; FGF, fibroblast growth factor; mRNA, messenger RNA; NFs, normal fibroblasts; RT-PCR, reverse transcription-polymerase chain reaction; TGF-β, transforming growth factor beta; α-SMA, α-smooth muscle actin. *P < .05; **P < .01; ***P < .005 [Color figure can be viewed at wileyonlinelibrary.com]

when FGF2-neutralizing antibody was added. Likewise, antibody neutralization of FGF2 abolished the invasiveness of these cells cocultured with CAFs (Figure 3E).

TGF-β is a representative inducer of fibroblast activation.[31] We established TGF-β-activated fibroblasts (CCD-1068sk-TGF-β) by preincubating CCD-1068sk-immortalized fibroblasts with TGF-β for 4 days.[32] TGF-β-activated fibroblasts acquires altered gene signatures and exhibit CAF-like phenotypic characteristics.[33] Like primary CAFs, these TGF-β-activated fibroblasts overexpressed a CAF marker gene, α-SMA as well as *FGF2* (Figure 3F). Notably, MDA-MB-231 cells attained enhanced invasiveness when cocultured with CAF-like CCD-1068sk-TGF-β fibroblasts in 3D invasion matrix (Figure 3G), and this was nullified by FGF2-neutralizing antibody (Figure 3H).

Based on above findings, we speculate that FGF2 secreted by CAFs binds to a specific receptor present in the MDA-MB-231 cells, thereby regulating their proliferation, and plasticity. To identify the appropriate receptor expressed in MDA-MB-231 cells capable of mediating oncogenic signal transduction triggered by CAF-derived FGF2 in a paracrine manner, the protein expression levels of all four FGFR isoforms (FGFR1, FGFR2, FGFR3, and FGFR4) were measured. As shown in Figure 4A, FGFR1 was found to be predominantly expressed in MDA-MB-231 cells, qualifying them for paracrine interaction with CAFs. Expression of FGFR1 (Figure 4A) and its mRNA transcript (Figure 4B) was also detectable in MDA-MB-468 cells. Unlike MDA-MB-231 cells, however, MDA-MB-468 cells express *FGF2* as well (Figure 4B), a gene

encoding a potential ligand of FGFR1, suggesting an autocrine involvement of latter cells.

Tissue microarray analysis showed that expression of FGF2 and FGFR1 was elevated in breast cancer tissues compared with normal adjacent tissues (Figure 4C). In particular, 42% (15 out of 36) of ER-negative patients coexpressed FGF2 and FGFR1, while the same coexpression was observed in only 19% (12 out of 62) of ER-positive patients (Figure 4D). By analyzing publicly available gene expression profile data (GSE37614), CAFs from TNBC patients were found to have downregulated *FGFR1* and upregulated *FGF2* expression profiles, which further supports the possible paracrine signaling mediated by their protein products in ER-negative breast cancer (Figure S1).

3.4 | FGF2 promotes migration of MDA-MB-231 cells through FGFR1

To determine whether FGF2 could accelerate proliferation and migration of MDA-MB-231 cells through activation of FGFR1 signaling, these cells were incubated with human recombinant FGF2. As shown in Figure 5A, the expression of Cyclin D1 was transiently increased in response to FGF2 stimulation. *FGFR1* gene silencing abrogated the FGF2-induced Cyclin D1 expression at both transcriptional (Figure 5B) and translational (Figure 5C) levels. Furthermore, FGFR1-siRNA-transfected MDA-MB-231 cells exhibited complete suppression of Akt phosphorylation (Figure 5E) and migration (Figure 5E).

3.5 | Activated fibroblasts promote xenograft tumor growth via the FGF2-FGFR1 signaling

To specifically figure out whether FGF2-expressing CAFs could enhance breast tumor growth via FGFR1 signaling, we utilized lentivirus-mediated RNA interference to stably knockdown *FGFR1* in MDA-MB-231 cells (referred to as shFGFR1-MDA-MB-231). Control cancer cells were transduced with scrambled short hairpin RNA (shmock-MDA-MB-231). Athymic nude mice were then coinjected with control- or TGF-β-activated CCD-1068sk fibroblasts and shmock- or shFGFR1-MDA-MB-231 cells. MDA-MB-231 cells coinjected with TGF-β-activated CCD-1068sk fibroblasts formed xenograft tumors (Figure 6A) which were grown much faster (Figure 6B) and larger (Figure 6C) than those derived from the MDA-MB-231 cells coinjected with unactivated fibroblasts. A complex collagen network reflects the presence of fibroblasts which can be depicted by blue staining in Masson's trichrome stain (Figure 6D).
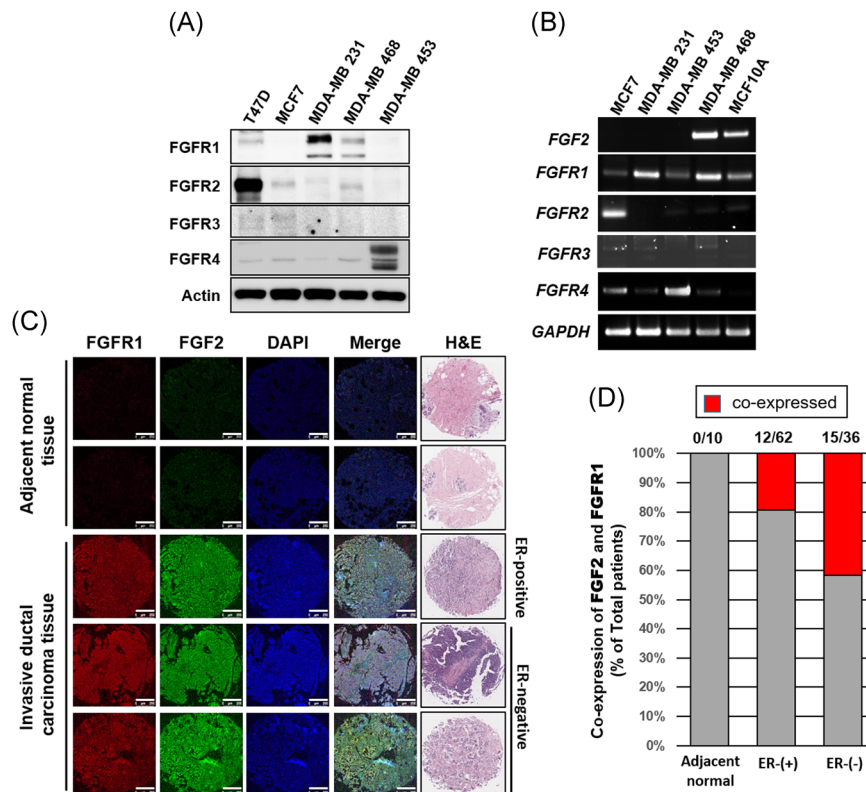

FIGURE 4 Differential expression of FGFR isoforms in breast cancer cell lines. A and B, The expression of FGFRs and their mRNA transcripts in different breast cancer cells was analyzed by Western blot (A) and RT-PCR (B) analyses, respectively. C, Immunofluorescent staining of samples from a breast cancer tissue array was performed using anti-FGFR1 and anti-FGF2 antibodies. Images of H&E stained tissue sections were provided by US Biomax Inc. Bars = 200 μm. D, The proportion of tumor tissues vs adjacent normal tissues coexpressing FGF2 and FGFR1 was quantified. FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; H&E, hematoxylin and eosin; mRNA, messenger RNA; RT-PCR, reverse transcription-polymerase chain reaction [Color figure can be viewed at wileyonlinelibrary.com]
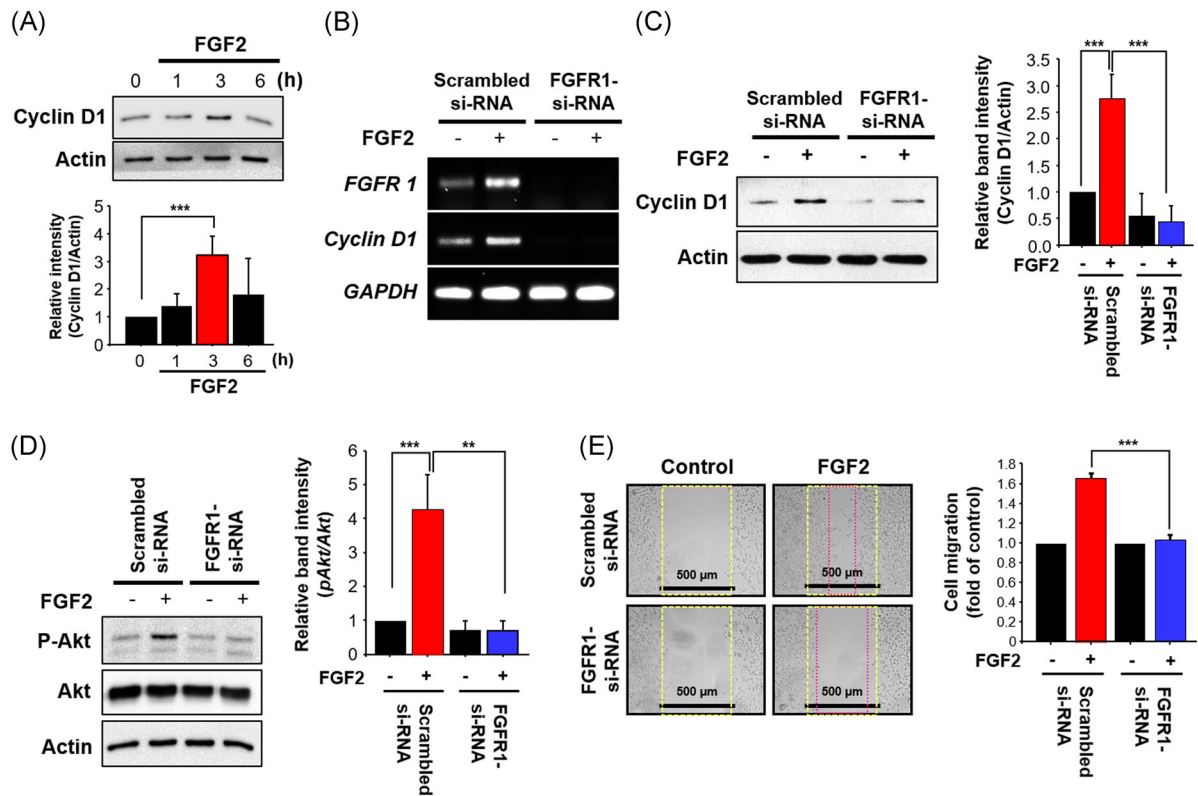
FIGURE 5 FGF2-induced breast cancer cell proliferation and migration mediated through FGFR1 activation. A, MDA-MB-231 cells were treated with FGF2 (20 ng/mL) for indicated time periods. The expression of Cyclin D1 was assessed by Western blot analysis. B and C, MDA-MB-231 cells were transfected with scrambled or FGFR1 si-RNA for 24 hour. Cells were then incubated with 20 ng/mL of FGF2 for 3 hour. The expression of Cyclin D1 was assessed by RT-PCR (B) and Western blot (C) analyses. D, The effects of FGF2 on Akt activation through phosphorylation was assessed by Western blot analysis. E, Scrambled or FGFR1-knockdown MDA-MB-231 cells were exposed to 20 ng/mL of FGF2 for 24 hour. The ability of cancer cell migration was measured by the wound healing assay as described in Materials and Methods. Data are means ± SD. FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA. *$P < .05$; **$P < .01$; ***$P < .005$ [Color figure can be viewed at wileyonlinelibrary.com]

There was increased stromal compartment as indicated by blue-colored background in Masson's trichrome stain in tumors coinjected with activated fibroblasts and shmock-MDA-MB-231 cells (group 3; Figure 6D). These tumors also exhibited significantly elevated expression of the proliferative markers Cyclin D1 and PCNA. In addition, the upregulated expression of FGFR1 as well as Cyclin D1 was evident as assessed by Western blot analysis (Figure 6E). These data indicate that FGF2 produced by activated fibroblasts could facilitate growth of adjacent cancer cells in breast tumor microenvironment via the FGFR1 signaling.

4 | DISCUSSION

Mounting evidence supports the cross-talk between cancer cells and several types of stromal cells including fibroblasts, immune cells and endothelial cells, in the tumor microenvironment.[34] Tumors which contain large proportion of stromal cells have been associated with poor prognosis, independently of other clinicopathological parameters in breast cancer patients.[6] CAFs represent a major component of stroma in the tumor microenvironment, and play an important role in tumor development and progression.[1]

Among many factors secreted in tumor microenvironment, TGF-β is recognized as a master regulator of the trans-differentiation of stromal fibroblasts in the mammary tumor microenvironment.[3,35] Promotion of fibroblasts to activated myofibroblasts by TGF-β confers manifestation of CAF genotypes including elevated α-SMA expression.[36] CAFs increase the frequency of colon tumor-initiating cells, and this effect is dramatically enhanced by TGF-β signaling.[32] Furthermore, poor prognostic colorectal cancer subtypes share a gene program induced by TGF-β in tumor stromal cells.[32] TGF-β1 protein levels are also considered as prognostic marker for breast cancer, especially for TNBC.[37]

It has been reported that CAFs contribute to cell proliferation, angiogenesis and metastasis in breast cancer.[11,38,39] However, a complete understanding of stromal-cancer cell interactions has a long way to go. In tumor microenvironment, CAFs are known to interact with other cells including cancer cells in a paracrine manner.[14] In our present study, CAF-CM rendered TNBC MDA-MB-231 cells more proliferative than non-TNBC MCF-7 cells. This differential effect of CAF-CM on breast cancer cell proliferation has been reported in other studies, demonstrating that MCF-7 cells are less sensitive to CAF-CM in cell proliferation, migration,[40,41] and signaling alterations,[38,42] compared with other breast cancer cells. A variety of
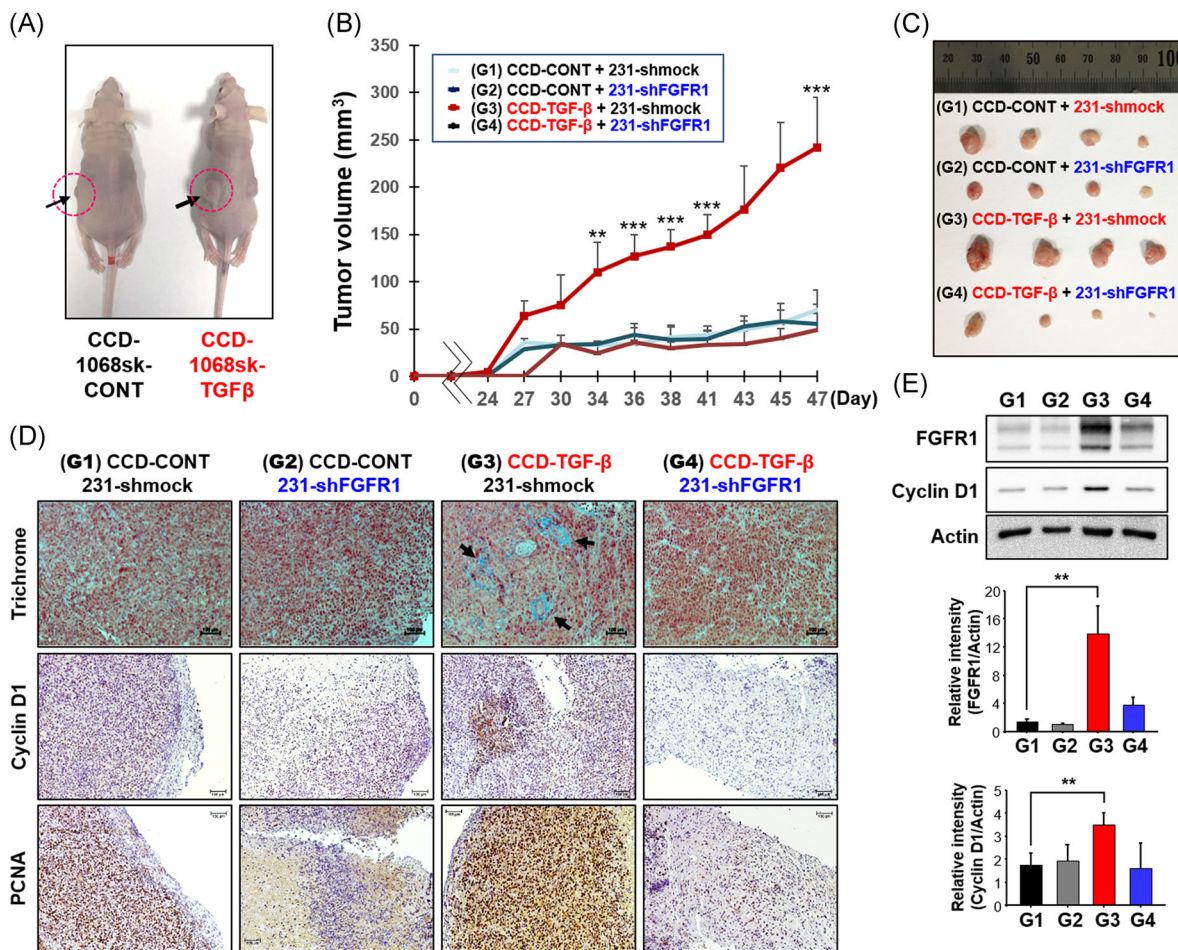
FIGURE 6 Stimulation of tumor formation by activated fibroblasts via FGFR1 signaling. A, The representative photographs of xenograft tumors grown in nude mice. B, Growth curves of xenograft tumors. Mice were coinjected subcutaneously with fibroblasts and MDA-MB-231 breast cancer cells. Group1 (G1): unactivated control-CCD-1068sk fibroblasts and shmock-MDA-MB-231 cells. Group2 (G2): control-CCD-1068sk fibroblasts and shFGFR1-MDA-MB-231 cells. Group3 (G3): TGF-β-activated CCD-1068sk fibroblasts and shmock-MDA-MB-231 cells. Group4 (G4): TGF-β-activated CCD-1068sk fibroblasts and shFGFR1-MDA-MB-231 cells. Tumor volume was measured by using a digital caliper and calculated by the formula; $0.5 \times (\text{length}) \times (\text{width})^2$. C, Appearance of tumors from each treatment group. D, Immunohistochemical analysis in mouse xenograft tumors. A complex collagen network was detected by a blue stain (arrows) in Masson's trichrome staining. The expression of Cyclin D1 and PCNA was analyzed by immunohistochemistry as described in Materials and Methods. Bars = 100 μm. E, The protein expression of FGFR1 and Cyclin D1 was measured by Western blot analysis. Data are means ± SD. FGFR, fibroblast growth factor receptor; PCNA, proliferating cell nuclear antigen; TGF-β, Transforming growth factor beta. *P < .05; **P < .01; ***P < .005 [Color figure can be viewed at wileyonlinelibrary.com]

cytokines including CXCL12 (SDF1),[11] HGF,[43] and IL-6[44] are secreted from CAFs, and contribute to cancer progression.

Although many studies have revealed FGF2 secretion from CAFs,[14,15,30,45] its role in cancer development and progression has not been fully elucidated. FGF2 is a member of FGF family, and an elevated level of its stromal expression is closely correlated with breast cancer progression and recurrence.[46] Microarray analysis of CAFs from TNBC, ER-positive and HER2-positive breast cancer patients showed increased FGF2 expression in CAFs from TNBC whereas FGFR1 was downregulated in the same cells. This indicates that there is a possible paracrine signaling between CAFs and TNBC cells.[47]

FGFR1 is a cognate receptor of FGF2, which is also called basic FGFR or bFGFR,[48] and its amplification drives chemoresistance in breast cancer.[49] FGFR1-targeting small molecules, such as FIIN-2 and

FIIN-4, covalently interact with a conserved cysteine present in the P-loop of FGFR1, thereby reducing the metastatic potential of breast cancer cells.[50] Autocrine FGF2-FGFR1 activation in bone marrow stromal cells increased the secretion of FGF2-laden exosomes, which were subsequently endocytosed by leukemia cells, and thereby protected leukemia cells from tyrosine kinase inhibitors.[51]

We noticed that FGFR1 was expressed primarily in basal-like MDA-MB-231 and MDA-MB-468 breast cancer cells. Although these cells express FGFR1, transmission of FGFR1-mediated signaling, such as FRS2α phosphorylation, cannot be achieved in the absence of external stimulation. Thus, it is likely that these cells require exogenous inducers, such as FGF2 released by CAFs, for their survival in the tumor microenvironment. We observed the expression of FGF2 and FGFR1 in activated fibroblasts and TNBC cells, respectively. FGF2-neutralizing antibody treatment of MDA-MB-231 cells
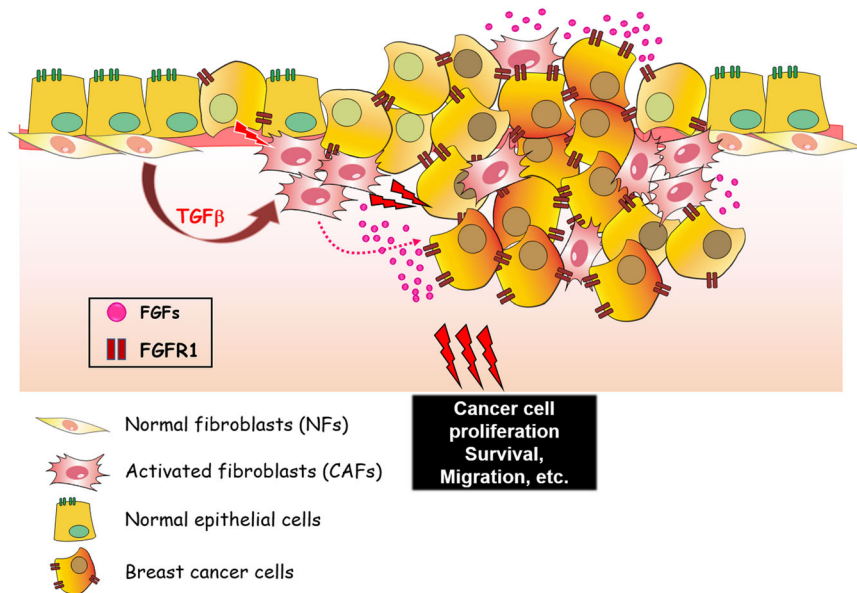
FIGURE 7 A proposed mechanism underlying stromal-to-cancer cell communication via the paracrine FGF2/FGFR1 axis. NFs are activated to produce CAFs, which secrete FGF2. CAF-derived FGF2 binds to FGFR1 in breast cancer cells. This, in turn, transmits the intracellular signal transduction, stimulating proliferation and migration of cancer cells and ultimately tumor growth. CAFs, cancer-associated fibroblasts; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; NFs, normal fibroblasts [Color figure can be viewed at wileyonlinelibrary.com]

attenuated CAF-CM-induced FRS2α phosphorylation responsible for FGFR1 signaling. These findings suggest that FGF2 derived from activated fibroblasts stimulates TNBC cell proliferation and migration through activation of FGFR1 signaling.

CAFs can affect not only cancer cells but also other stromal cells, such as tumor associated macrophages and endothelial cells by secreting FGF2, consequently modulating dynamic tumor microenvironmental conditions, such as promotion of immuno-suppressive status and neovascularization.[29] In an immuno-suppressive tumor condition, antitumor agents have limited activity to kill cancer cells.[52]

The results from our present study suggest that CAFs promote breast cancer cell proliferation, migration, and invasion via the paracrine FGF2-FGFR1 axis. In line with our supposition, Su et al[14] have identified FGF2 and other paracrine secreted factors that regulate breast carcinoma cell mitogenesis. In another study, the activation of FGF2-FGFR1 paracrine signaling triggered the expression of the connective tissue growth factor, leading to the migration and invasion of breast cancer cells.[46] The results demonstrated herein and in the aforementioned studies provide a novel molecular mechanism by which CAF-derived soluble factors affect the growth and progression of breast cancer cells. The FGF2-FGFR1 paracrine loop is important in the cross-talk between cancer cells and CAFs in the breast tumor microenvironment. Therefore, the FGF2-FGFR1 axis could be exploited as a prospective therapeutic target to efficiently disrupt the tumor-promoting interaction between CAFs and breast cancer cells (Figure 7).

ACKNOWLEDGMENTS

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be available from the corresponding author upon reasonable request.

ORCID

Young-Joon Surh http://orcid.org/0000-0001-8310-1795

REFERENCES

1. Kalluri R, Zeisberg M. Fibroblasts in cancer. *Nat Rev Cancer*. 2006;6(5):392-401.
2. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-674.
3. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. *Nature*. 2004;432(7015):332-337.
4. Kawase A, Ishii G, Nagai K, et al. Podoplanin expression by cancer associated fibroblasts predicts poor prognosis of lung adenocarcinoma. *Int J Cancer*. 2008;123(5):1053-1059.
5. Aboussekhra A. Role of cancer-associated fibroblasts in breast cancer development and prognosis. *Int J Dev Biol*. 2011;55(7-9):841-849.
6. de Kruijf EM, van Nes JG, van de Velde CJ, et al. Tumor-stroma ratio in the primary tumor is a prognostic factor in early breast cancer patients, especially in triple-negative carcinoma patients. *Breast Cancer Res Treat*. 2011;125(3):687-696.
7. McCuaig R, Wu F, Dunn J, Rao S, Dahlstrom JE. The biological and clinical significance of stromal-epithelial interactions in breast cancer. *Pathology*. 2017;49(2):133-140.
8. Erez N, Truitt M, Olson P, Arron ST, Hanahan D. Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-κB-dependent manner. *Cancer Cell*. 2010;17(2):135-147.
9. Giannoni E, Bianchini F, Masieri L, et al. Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. *Cancer Res*. 2010;70(17):6945-6956.
10. Martinez-Outschoorn UE, Trimmer C, Lin Z, et al. Autophagy in cancer associated fibroblasts promotes tumor cell survival: role of hypoxia, HIF1 induction and NF-κB activation in the tumor stromal microenvironment. *Cell Cycle*. 2010;9(17):3515-3533.

11. Orimo A, Gupta PB, Sgroi DC, et al. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell.* 2005;121(3):335-348.

12. Chioni AM, Grose R. FGFR1 cleavage and nuclear translocation regulates breast cancer cell behavior. *J Cell Biol.* 2012;197(6): 801-817.

13. Sun Y, Fan X, Zhang Q, Shi X, Xu G, Zou C. Cancer-associated fibroblasts secrete FGF-1 to promote ovarian proliferation, migration, and invasion through the activation of FGF-1/FGFR4 signaling. *Tumour Biol.* 2017;39(7):1010428317712592.

14. Su G, Sung KE, Beebe DJ, Friedl A. Functional screen of paracrine signals in breast carcinoma fibroblasts. *PLoS One.* 2012;7(10): e46685.

15. Giulianelli S, Cerliani JP, Lamb CA, et al. Carcinoma-associated fibroblasts activate progesterone receptors and induce hormone independent mammary tumor growth: a role for the FGF-2/FGFR-2 axis. *Int J Cancer.* 2008;123(11):2518-2531.

16. Ornitz DM, Itoh N. Fibroblast growth factors. *Genome Biol.* 2001;2(3): REVIEWS3005.

17. Chandler LA, Sosnowski BA, Greenlees L, Aukerman SL, Baird A, Pierce GF. Prevalent expression of fibroblast growth factor (FGF) receptors and FGF2 in human tumor cell lines. *Int J Cancer.* 1999; 81(3):451-458.

18. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM. Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 1991;64(4): 841-848.

19. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev.* 2005;16(2): 139-149.

20. Ornitz DM, Yayon A, Flanagan JG, Svahn CM, Levi E, Leder P. Heparin is required for cell-free binding of basic fibroblast growth factor to a soluble receptor and for mitogenesis in whole cells. *Mol Cell Biol.* 1992;12(1):240-247.

21. Micallef L, Vedrenne N, Billet F, Coulomb B, Darby IA, Desmouliere A. The myofibroblast, multiple origins for major roles in normal and pathological tissue repair. *Fibrogenesis Tissue Repair.* 2012;5(Suppl 1):S5.

22. Russell TD, Jindal S, Agunbiade S, et al. Myoepithelial cell differentiation markers in ductal carcinoma in situ progression. *Am J Pathol.* 2015;185(11):3076-3089.

23. Mezawa Y, Daigo Y, Takano A, et al. CD26 expression is attenuated by TGF-β and SDF-1 autocrine signaling on stromal myofibroblasts in human breast cancers. *Cancer Med.* 2019;8(8):3936-3948.

24. Fusté NP, Fernández-Hernández R, Cemeli T, et al. Cytoplasmic cyclin D1 regulates cell invasion and metastasis through the phosphorylation of paxillin. *Nat Commun.* 2016;7:11581.

25. Li Z, Wang C, Prendergast GC, Pestell RG. Cyclin D1 functions in cell migration. *Cell Cycle.* 2006;5(21):2440-2442.

26. Lungu G, Covaleda L, Mendes O, Martini-Stoica H, Stoica G. FGF-1-induced matrix metalloproteinase-9 expression in breast cancer cells is mediated by increased activities of NF-κB and activating protein-1. *Mol Carcinog.* 2008;47(6):424-435.

27. Theodorou V, Boer M, Weigelt B, Jonkers J, van der Valk M, Hilkens J. Fgf10 is an oncogene activated by MMTV insertional mutagenesis in mouse mammary tumors and overexpressed in a subset of human breast carcinomas. *Oncogene.* 2004;23(36):6047-6055.

28. Yiangou C, Gomm JJ, Coope RC, et al. Fibroblast growth factor 2 in breast cancer: occurrence and prognostic significance. *Br J Cancer.* 1997;75(1):28-33.

29. Hegab AE, Ozaki M, Kameyama N, et al. Effect of FGF/FGFR pathway blocking on lung adenocarcinoma and its cancer-associated fibroblasts. *J Pathol.* 2019;249(2):193-205.

30. Knuchel S, Anderle P, Werfelli P, Diamantis E, Ruegg C. Fibroblast surface-associated FGF-2 promotes contact-dependent colorectal cancer

31. Löhr M, Schmidt C, Ringel J, et al. Transforming growth factor-β1 induces desmoplasia in an experimental model of human pancreatic carcinoma. *Cancer Res.* 2001;61(2):550-555.

32. Calon A, Lonardo E, Berenguer-Llergo A, et al. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet.* 2015;47(4):320-329.

33. Calon A, Espinet E, Palomo-Ponce S, et al. Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation. *Cancer Cell.* 2012;22(5):571-584.

34. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell.* 2012;21(3): 309-322.

35. Taylor MA, Lee YH, Schiemann WP. Role of TGF-β and the tumor microenvironment during mammary tumorigenesis. *Gene Expr.* 2011; 15(3):117-132.

36. Micke P, Ostman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer.* 2004; 45(Suppl 2):S163-S175.

37. Ding MJ, Su KE, Cui GZ, et al. Association between transforming growth factor-β1 expression and the clinical features of triple negative breast cancer. *Oncol Lett.* 2016;11(6): 4040-4044.

38. Tsuyada A, Chow A, Wu J, et al. CCL2 mediates cross-talk between cancer cells and stromal fibroblasts that regulates breast cancer stem cells. *Cancer Res.* 2012;72(11):2768-2779.

39. Tyan SW, Kuo WH, Huang CK, et al. Breast cancer cells induce cancer-associated fibroblasts to secrete hepatocyte growth factor to enhance breast tumorigenesis. *PLoS One.* 2011; 6(1):e15313.

40. Camp JT, Elloumi F, Roman-Perez E, et al. Interactions with fibroblasts are distinct in Basal-like and luminal breast cancers. *Mol Cancer Res.* 2011;9(1):3-13.

41. Yu Y, Xiao CH, Tan LD, Wang QS, Li XQ, Feng YM. Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling. *Br J Cancer.* 2014;110(3): 724-732.

42. Gao MQ, Kim BG, Kang S, et al. Stromal fibroblasts from the interface zone of human breast carcinomas induce an epithelial-mesenchymal transition-like state in breast cancer cells in vitro. *J Cell Sci.* 2010; 123(Pt 20):3507-3514.

43. Deying W, Feng G, Shumei L, Hui Z, Ming L, Hongqing W. CAF-derived HGF promotes cell proliferation and drug resistance by up-regulating the c-Met/PI3K/Akt and GRP78 signalling in ovarian cancer cells. *Biosci Rep.* 2017;37(2):BSR20160470.

44. Valencia T, Kim JY, Abu-Baker S, et al. Metabolic reprogramming of stromal fibroblasts through p62-mTORC1 signaling promotes inflammation and tumorigenesis. *Cancer Cell.* 2014; 26(1):121-135.

45. Santolla MF, Vivacqua A, Lappano R, et al. GPER mediates a feedforward FGF2/FGFR1 paracrine activation coupling CAFs to cancer cells toward breast tumor progression. *Cells.* 2019;8(3): 223.

46. Visscher DW, DeMattia F, Ottosen S, Sarkar FH, Crissman JD. Biologic and clinical significance of basic fibroblast growth factor immunostaining in breast carcinoma. *Mod Pathol.* 1995;8(6): 665-670.

47. Tchou J, Kossenkov AV, Chang L, et al. Human breast cancer associated fibroblasts exhibit subtype specific gene expression profiles. *BMC Med Genomics.* 2012;5:39.

48. Blanckaert VD, Hebbar M, Louchez MM, Vilain MO, Schelling ME, Peyrat JP. Basic fibroblast growth factor receptors and their prognostic value in human breast cancer. *Clin Cancer Res.* 1998;4(12): 2939-2947.

49. Turner N, Pearson A, Sharpe R, et al. FGFR1 amplification drives endocrine therapy resistance and is a therapeutic target in breast cancer. *Cancer Res.* 2010;70(5):2085-2094.

50. Brown WS, Tan L, Smith A, Gray NS, Wendt MK. Covalent targeting of fibroblast growth factor receptor inhibits metastatic breast cancer. *Mol Cancer Ther.* 2016;15(9):2096-2106.

51. Javidi-Sharifi N, Martinez J, English I, et al. FGF2-FGFR1 signaling regulates release of leukemia-protective exosomes from bone marrow stromal cells. *eLife.* 2019:8.

52. Galon J, Bruni D. Approaches to treat immune hot, altered and cold tumours with combination immunotherapies. *Nat Rev Drug Discov.* 2019;18(3):197-218.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Suh J, Kim D-H, Lee Y-H, Jang J-H, Surh Y-J. Fibroblast growth factor-2, derived from cancer-associated fibroblasts, stimulates growth and progression of human breast cancer cells via FGFR1 signaling. *Molecular Carcinogenesis*. 2020; 59:1028–1040. https://doi.org/10.1002/mc.23233