Cancer Research

# Interaction between FGFR-2, STAT5, and Progesterone Receptors in Breast Cancer

Juan P. Cerliani[1], Tomás Guillardoy[1], Sebastián Giulianelli[1], José P. Vaque[4], J. Silvio Gutkind[4], Silvia I. Vanzulli[2], Rubén Martins[3], Eduardo Zeitlin[3], Caroline A. Lamb[1], and Claudia Lanari[1]

## Abstract

Fibroblast growth factor (FGF) receptor 2 (FGFR-2) polymorphisms have been associated with an increase in estrogen receptor and progesterone receptor (PR)-positive breast cancer risk; however, a clear mechanistic association between FGFR-2 and steroid hormone receptors remains elusive. In previous works, we have shown a cross talk between FGF2 and progestins in mouse mammary carcinomas. To investigate the mechanisms underlying these interactions and to validate our findings in a human setting, we have used T47D human breast cancer cells and human cancer tissue samples. We showed that medroxyprogesterone acetate (MPA) and FGF2 induced cell proliferation and activation of ERK, AKT, and STAT5 in T47D and in murine C4-HI cells. Nuclear interaction between PR, FGFR-2, and STAT5 after MPA and FGF2 treatment was also showed by confocal microscopy and immunoprecipitation. This effect was associated with increased transcription of PRE and/or GAS reporter genes, and of PR/STAT5-regulated genes and proteins. Two antiprogestins and the FGFR inhibitor PD173074, specifically blocked the effects induced by FGF2 or MPA respectively. The presence of PR/FGFR-2/STAT5 complexes bound to the PRE probe was corroborated by using NoShift transcription and chromatin immunoprecipitation of the *MYC* promoter. Additionally, we showed that T47D cells stably transfected with constitutively active FGFR-2 gave rise to invasive carcinomas when transplanted into NOD/SCID mice. Nuclear colocalization between PR and FGFR-2/STAT5 was also observed in human breast cancer tissues. This study represents the first demonstration of a nuclear interaction between FGFR-2 and STAT5, as PR coactivators at the DNA progesterone responsive elements, suggesting that FGFRs are valid therapeutic targets for human breast cancer treatment. *Cancer Res; 71(10); 3720–31. ©2011 AACR.*

## Introduction

Two-thirds of breast cancers express estrogen (ER) and progesterone receptors (PR) at the time of diagnosis (1); most of these cancers arise in postmenopausal women with low levels of circulating hormones. It has been proposed that paracrine factors participate in the activation of hormone receptors to support hormone-independent tumor growth.

We have recently shown, by using a medroxyprogesterone acetate (MPA)-induced murine breast cancer model (2), that fibroblast growth factor 2 (FGF2) is highly expressed in the stroma of hormone-independent carcinomas and that the FGF receptor (FGFR) inhibitor PD173074 decreased tumor growth (3). Moreover, exogenously administered FGF2 was able to mimic MPA, an effect that was reverted by the antiprogestin RU486 (4). The mechanism by which FGF2, by binding to FGFRs, activates PR is still unknown.

FGFR-2 has been implicated in mammary gland development (5). In a genome-wide association study, an increased risk of developing invasive breast cancer was significantly linked to 4 single nucleotide polymorphisms (SNP) in intron 2 of the FGFR-2 gene (6). Moreover, additional studies in breast cancer cell lines, identified 2 cis-regulatory SNPs that altered the binding affinity for transcription factors OCT-1/RUNX2 and C/EBPb leading to increased FGFR-2 expression (7).

FGFRs trigger the activation of multiple pathways (8) and the activation of SRC, Janus kinase 2 (JAK2), and Signal transducers and activators of transcription (STATs) has recently been reported (9). Moreover, FGFRs have also been found to be localized in the nuclei of several cells (10), however, the role of these nuclear FGFRs remains unclear.

The STAT family of transcription factors mediates cytokine, growth factor, and hormone responses. Following ligand-receptor binding, STATs are activated by tyrosine phosphorylation, resulting in dimerization and translocation to the nucleus (11). Constitutively activated forms of STAT3 and STAT5 have been observed in tumor-derived cell lines and human cancer (12). The overexpression of STAT5 has been

**Authors' Affiliations:** [1]Institute of Experimental Biology and Medicine (IBYME), National Research Council of Argentina (CONICET); [2]National Academy of Medicine; [3]Department of Pathology, Bancario Clinic, Buenos Aires, Argentina; and [4]Oral and Pharyngeal Cancer Branch, NIDCR, NIH, Bethesda, Maryland

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**Corresponding Author:** Claudia Lanari, IBYME-CONICET, Obligado 2490, C1428ADN Buenos Aires, Argentina. Phone: +0540-114-783-2869; Fax: +0540-114-786-2564. E-mail: lanari.claudia@gmail.com

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shown to increase mammary carcinogenesis (13). Also, a clear cross talk between growth factors that input to STAT5 and PR on genes containing GAS sequences has been shown in human breast cancer cells (14, 15). STAT5 (16), PR (17), or FGFR-2 (5) disrupted mammary gland–displayed branching and lobuloalveolar defects, suggesting a link between these proteins in the mammary gland.

The aim of this study was to gain further insight into the mechanisms by which FGF2- and progestin-induced signaling pathways interact, and to corroborate our experimental findings in humans. The results shown herein represent the first demonstration of a relationship between FGFR-2, STAT5, and PR in breast cancer growth that could be therapeutically exploited. The high tumorigenicity of T47D cells transfected with a constitutively active FGFR-2 reinforces a role for FGFR-2 inhibitors in breast cancer.

## Materials and Methods

### Animals

Two-month old virgin female BALB/c mice (IByME Animal Facility) and NOD/SCID mice (Jackson Laboratories) were used. Animal care and manipulation were in agreement with the Guide for the Care and Use of Laboratory Animals.

### Tumors

C4-HI tumors express ER and PR and are maintained by subcutaneous transplantations in untreated BALB/c mice (2). In primary cultures, C4-HI cells are stimulated by MPA (3). Tumor validation was carried out by cytogenetic analysis (2). Primary cultures and cell proliferation assays were carried out as previously described (18).

### Cell lines

T47D cells, purchased from the ATCC, were validated by Genetica DNA Laboratories, Inc. by short tandem repeat profiling. Passages lower than 10 were used. Cells stably transfected with FGFR-2-CA (2 independent bulk transfections) or with the empty vector ($1 \times 10^6$ cells) (19) were inoculated orthotopically along with matrigel, into the mammary gland 4 of NOD/SCID mice ($n = 2$; 4 animals per group).

### Human breast cancer tissue samples

Breast cancer resection specimens from 16 patients submitted for intraoperative biopsies, immediately frozen at −70°C, were provided by the Bancario Hospital. The study was approved by the Institutional Review Board.

### Antibodies and reagents

FGFR-1 (sc-121), FGFR-2 (sc-122), FGFR-3 (sc-123), FGFR-4 (sc-9006), PR (sc-538), BCL-XL (sc-634), STAT5 (sc-835), pSTAT5 (sc-101806), ERK (sc-94), Tissue Factor (TF; sc-30201), ERα (sc-542), MYC (sc-764), and pERK (sc-7383) antibodies were purchased in Santa Cruz Biotech; β-actin (Ab-5), PR (Ab-7), pSer 190 PR (Ab-11), and pSer 294 PR (Ab-12) were obtained from Neomarkers; Cyclin D1 (Clone DCS-6; Thermo Scientific) and AKT (610837) from BD Transduction Lab, and pAKT (N° 4060) from Cell Signaling Technology. Secondary antibodies were from Amersham Pharmacia Biotech or Vector Laboratories. 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodine were purchased from Sigma—Aldrich.

MPA, FGF1, and RU486 (RU, Mifepristone), were obtained from Sigma–Aldrich and interleukin 2 (IL-2) in R&D System. FGF2 was a gift from Dr. A. Baldi (IByME), PD173074 (PD) and ZK230211 (ZK, Lonaprisan) were gifts from Pfizer (Groton, CT) or Bayer Schering Pharma (AG, Berlin), respectively.

### *In vitro* experiments

In all assays, cultures were starved for 24 hours by using culture medium (DMEM/F12 without phenol red, Sigma) without serum and were then incubated with 10 nmol/L MPA, 50 ng/mL FGF2, or 20 ng/mL FGF1 with or without the inhibitors.

### Western blots and immunoprecipitations

Extracts and western blots were carried out as previously described (3, 20). ERK was used as a loading control. For coimmunoprecipitation assays, extracts (1 mg) were precleared with A/G protein and centrifuged at 12,000 rpm; the supernatant was incubated overnight with 4 μg of the selected antibody and A/G protein and centrifuged at 3,000 rpm. The pellet was washed and resuspended in cracking buffer.

### Transfections

Proliferating cells were transfected for 24 hours with the vector FGFR-2-CA (5 μg of plasmid DNA/plate) or the empty vector, by using Lipofectamine 2000 (Invitrogen). After 24 hours, cells were washed and incubated with the antibiotic G418 for 3 weeks (400 μg/mL). For propagation, cells were kept under continuous antibiotic selection (200 μg/mL).

### Activation of reporter genes

PRE-Luc or GAS-Luc (2 μg plasmid DNA/well) and 10 ng of a Renilla luciferase expression plasmid, RL-CMV (Promega) were transfected for 6 hours by using Lipofectamine (Invitrogen). The medium was replaced by medium with 10% fetal calf serum (FCS) for 24 hours. Cells were starved for 24 hours and treated with different reagents for 18–24 hours. Luciferase assays were carried out by using a dual-luciferase reporter assay system (Promega).

### Immunofluorescence and colocalization

Frozen sections of breast cancer samples or cells growing on chamber slides were fixed in ice-cold 70% ethanol for 30 minutes, blocked in 10% FCS, and incubated with the FGFR-1–4 or STAT5 antibodies at 1/100 dilutions for 1 hour at room temperature. Slides were washed, incubated overnight at 4°C with the PR/pPR antibodies, then washed, incubated for 1 hour with both secondary antibodies at 1/100 dilutions and mounted with Vectashield (Vector Labs). Negative controls lacked primary antibodies. Cells or tissues were analyzed by using a Nikon Eclipse E800 Microscope by using the Nikon DS-U1 with ACT-2U software. Ten 600× fields were quantified in each sample, and the percentage of cells showing colocalization with respect to the total number of cells was calculated.

### Immunohistochemistry

Antigen retrieval was done by boiling for 4 cycles of 5 minutes in 0.1 M citrate buffer. Primary antibodies were used at 1/100 dilution. Specimens were counterstained with 10% hematoxylin, dehydrated, and mounted.

### RNA preparation and real-time quantitative PCR

Total RNA was isolated from T47D cells by using TRIzol Reagent (Invitrogen) according to manufacture's protocol. RNA was reverse transcribed by using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) according to manufacture's instructions. The resulting cDNA product was used as template for quantitative PCR (qPCR) analysis by using iQSybr Green Supermix and the iCycler PCR thermocycler (Bio-Rad). Specific oligos for human *MYC* (NM_002467.4) and *CCND1* (Cyclin D1; NM_053056.2) were designed by using Primer-Blast (NCBI) and their sequence is detailed in Supplementary Table 1. *GAPDH* (NM_002046.3) expression was used as a control to normalize the data.

### NoShift transcription factor assay

C4-HI cells were arrested for 24 hours and treated with MPA, FGF2, or vehicle for 18 hours. Next, the cells were detached, lysed, and processed according to the manufacturer's instructions (Novagen 71378–3).

### Chromatin immunoprecipitation assays

T47D cells were starved, treated with vehicle or MPA for 45 minutes, washed with PBS, and fixed in 2 mmol/L EGS (Pierce) in 25% DMSO/75% PBS for 20 minites, followed by 1% PFA in PBS for 30 minutes. Chromatin immunoprecipitation (ChIP) assays were carried out by using the HighCell# ChIP kit (Diagenode). Rabbit IgG (sc-2027), PR (sc-7208X), STAT5 (sc-835X), and FGFR-2 (sc-122X) antibodies were obtained from Santa Cruz. Specific oligos for human *MYC* promoter were designed by using Primer-Blast (NCBI; Supplementary Table 1). Data from each IP were normalized to the corresponding inputs of chromatin before immunoprecipitation, normalized to IgG/input data, and expressed as relative to the value of the control experiment.

### Statistical analysis

Differences between means ± SD from more than 2 groups were compared by using one way ANOVA analysis followed by Tukey *t*-tests, and between 2 groups the Student *t*-test. Western blot band intensity and immunofluorescence staining was quantified by using the Image Quant software.

## Results

### The proliferative effects induced by FGF2 and MPA are inhibited by FGFR inhibitors or antiprogestins, respectively

In a previous study, we showed that FGF2 and MPA increased $^{3}$H-thymidine uptake in C4-HI and T47D cells (3). Here we show that the growth of both cell populations was stimulated by MPA, FGF1, and FGF2, and that RU486 and the FGFR inhibitor PD173074 inhibited the stimulation induced by MPA, FGF2, or FGF1 (Fig. 1A and B), confirming a cross talk between FGFR and PR in both human and mouse cells. When cells were counted, 7 days after treatment, similar results were observed (Fig. 1C and D). No differences were found between RU486 or ZK230211, a new antiprogestin with less antiglucocorticoid effects (21; Supplementary Fig. S1). FACS analysis

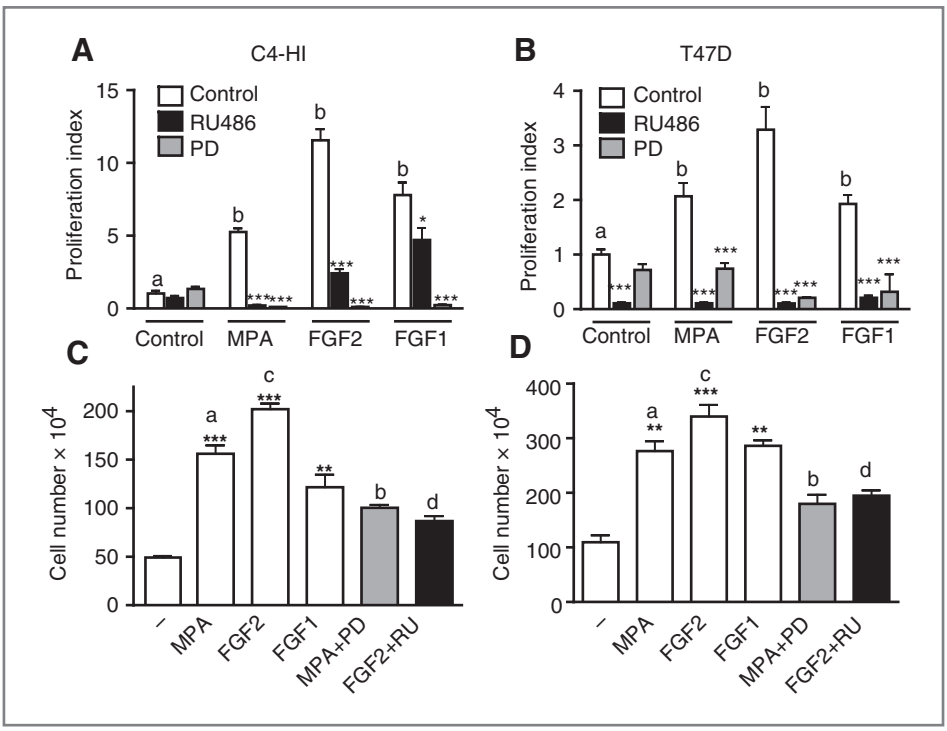


Figure 1. Effects of MPA, FGF2, and their respective inhibitors, on cell proliferation. A, B, $^{3}$H-thymidine uptake assays. Cells were treated for 48 hours with MPA (10nmol/L), FGF2 (50 mg/mL), or FGF1 (20 mg/mL), with or without RU486 (RU; 100 nmol/L) or PD173074 (PD; 0.1 μmol/L). The proliferation index was calculated as experimental cpm/control cpm (mean ± SEM); a versus b: $P < 0.001$ ($n = 3$; octuplicates). C, D, cells treated for 7 days as described above were counted in Neubauer chambers ($n = 2$; triplicates); a versus b: $P < 0.01$; c versus d: $P < 0.001$ (C4-HI) and $P < 0.01$ (T47D). In all cases **: $P < 0.01$ and ***: $P < 0.001$ experimental versus control.

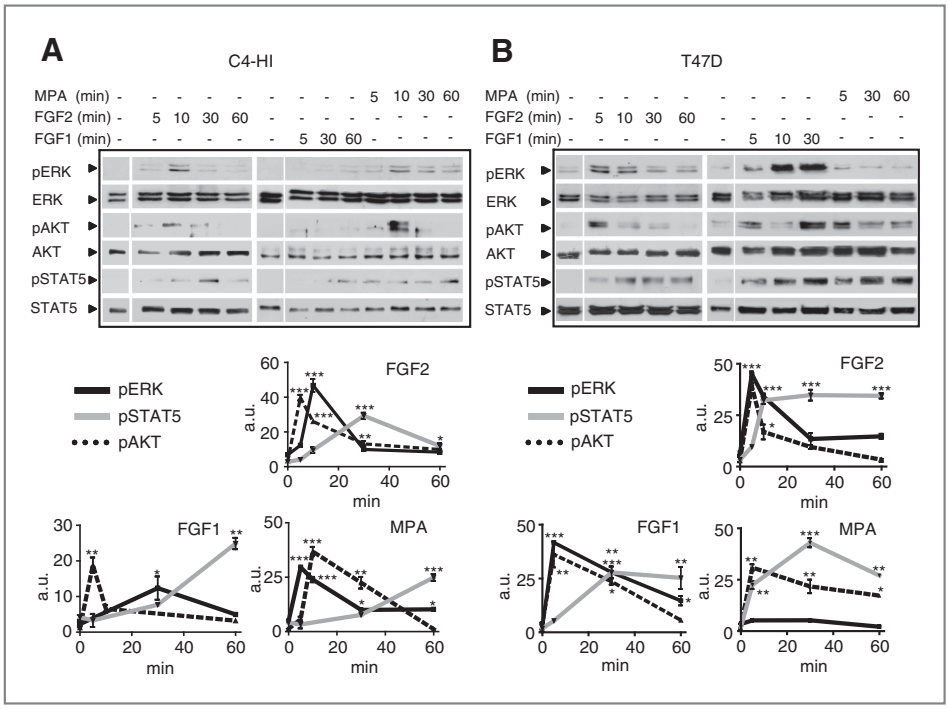


Figure 2. Activation of ERKs, AKT, and STAT5 in C4-HI (A) and T47D (B) cells. Cells were starved for 24 hours and then incubated with MPA (10 nmol/L), FGF2 (50 ng/mL) or FGF1 (20 ng/mL). Top, representative immunoblots are shown. The control lane which was originally beside the 60-minute lane was split and placed in position lane 1 for clarity. Bottom, quantification of the levels of activated proteins in relation to the total amount of protein of the immunoblots. ERKs were used as loading controls. The mean ± SEM of 2 different experiments was plotted. *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ versus control.

confirmed these effects (Supplementary Fig. S2). These results show a cross talk between FGFR and PR in both human and mouse breast cancer cells and suggest that both are key regulators of cell proliferation.

### MPA and FGF2 induce ERK, AKT, and STAT5 phosphorylation

To determine which pathways were involved in the cross talk between FGFR and PR, epithelial C4-HI or T47D cells were serum starved and incubated for short periods of time with FGF2, FGF1, or MPA. In C4-HI cells, FGF2 induced an early increase in pERK and pAKT and a later increase in pSTAT5 (30 minutes; Fig. 2A). A similar pattern was observed in T47D cells, although the activation of STAT5 was earlier and sustained (10 minutes; Fig. 2B). MPA induced in C4-HI a similar activation of ERK and AKT as compared with FGF2, although in this case the activation of ERK was earlier than that of AKT, and the highest activation of STAT5 was observed after 60 minutes of incubation (Fig. 2A). In T47D cells, MPA did not activate ERK but induced an early activation of AKT and a high, early, and sustained increase in pSTAT5 (Fig. 2B), higher than that induced in C4-HI (Fig. 2A). The effect of FGF1 on T47D cells was similar to the one induced by FGF2, although pSTAT5 was increased after 30 minutes of treatment. In C4-HI cells, the increase in pAKT was only observed after 5 minutes of incubation and the activation of STAT5 occurred after 1 hour of treatment (Fig. 2A).

These set of results indicate that FGF2 and MPA turn on common nongenomic pathways in the murine cells and that although in the human breast cancer model there might be a difference regarding the levels of pERK in MPA- or FGF2-treated cells, AKT and STAT5 activation followed the same trend. STAT5 activation prevailed in T47D cells. These cells might be addicted to this pathway as autocrine prolactin activates cell proliferation via activation of STAT5 (22).

### PR interacts with FGFR-2 and STAT5 in cells treated with FGF2 or with MPA

pPR and pSTAT5 act as transcription factors (11, 21) and FGFR-2 may be immunolocalized in the nuclei of ligand-stimulated cells (3, 23, 24). FGF2 binds preferentially to FGFR-2 and it may also bind to other members of the FGFR family (25). Our next aim was to analyze by using confocal microscopy the cellular localization of PR and FGFR-2 in cells treated with MPA, FGF2, or FGF1 and to evaluate the specificity of these results by studying FGFR-1, FGFR-3, and FGFR-4 as compared with FGFR-2.

After MPA or FGF2 treatment, there was an increase in nuclear colocalization of PR (green) with FGFR-2 (red), and a discrete colocalization with FGFR-3 (red) in C4-HI cells (Fig. 3A). A similar result was observed in T47D cells (data not shown). Time course experiments with both cell types showed an increased nuclear colocalization, after 5 minutes of MPA-treatment, which was even higher after 20 minutes or 1 hour. After 4 hours, both proteins were downregulated. FGF2 induced a similar colocalization pattern, but this effect was delayed as compared with MPA (Fig. 3B). A colocalization index was calculated with the number of cells showing colocalization in treated groups versus the control group.

Given that (a) STAT5 is localized exclusively in PR+ ER+ mammary gland cells (26), (b) progesterone activates STAT5 (14, 16), (c) FGF2 activates STAT5 in endothelial cells (9), (d) a dominant negative STAT5 inhibits T47D cell proliferation (27), and (e) FGF2 and MPA induce STAT5 phosphorylation in

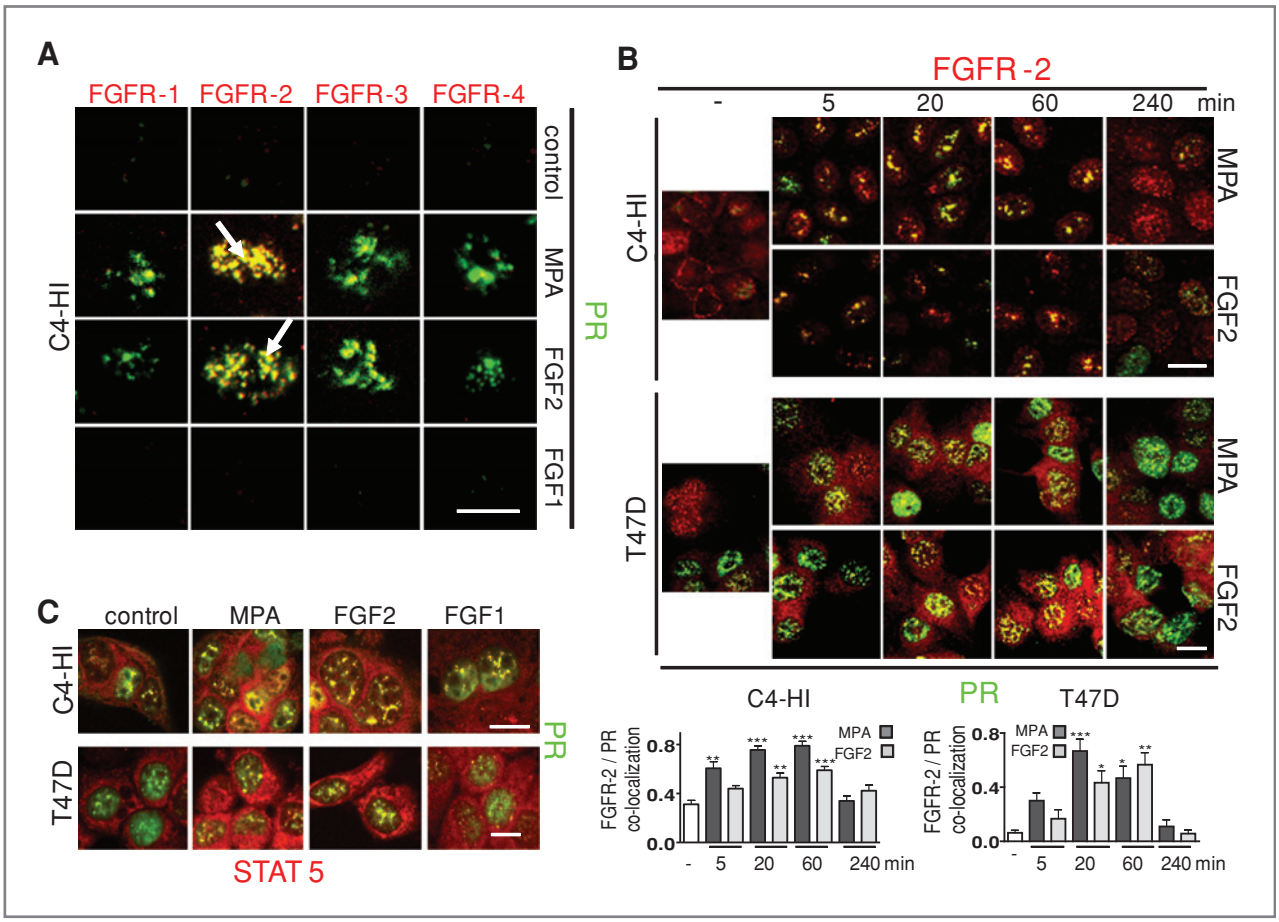


Figure 3. Immunolocalization of PR (green), FGFRs (red), and STAT5 (red) in treated cells. A, confocal images of C4-HI cells after 24-hour treatment. Confocal microscope settings were selected to have unsaturated images in MPA- or FGF2-treated cells. Scale bar: 10 μm; white arrows: nuclear PR and FGFR-2 colocalization. B, time dependent interaction between PR and FGFR-2. Scale bar: 30 μm. Colocalization index: % colocalizing PR + FGFR-2+/total cell number. *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ versus control ($n = 3$; duplicates). C, representative confocal images showing the cellular localization of PR and STAT5 after 24-hour treatment ($n = 3$; triplicates). Scale bar C4-HI: 18 μm, T47D: 15 μm.

C4-HI and T47D cells (Fig. 2), we evaluated the interaction between STAT5 and PR in these 2 models. MPA- or FGF2-treated cells showed an increase in total STAT5 and in nuclear colocalization of STAT5 with PR (Fig. 3C). The increase in nuclear STAT5 was corroborated in Western blots of purified nuclear fractions (not shown). Coimmunoprecipitation experiments by using C4-HI tumors extracts (Fig. 4A) or from FGF2- or MPA-treated T47D cells (Fig. 4B) confirmed the interaction between FGFR-2 (120 kDa), STAT5, and both PR isoforms. Controls were immunoprecipitated with antibody isotypes or β-actin (Supplementary Fig. 3).

These results confirm that the 3 proteins interact with each other in the nuclei of C4-HI and T47D cells.

### MPA and/or FGFs activate PRE or GAS containing reporter genes

To explore the biological activity of these nuclear complexes, we evaluated the ability of MPA and FGFs to activate PRE and GAS genes containing a luciferase (luc) reporter. PRE is the canonical PR DNA binding site and GAS is the STAT DNA binding site. MPA and FGF2 induced an increase in PRE-luc expression in both cells (Fig. 4C). Interestingly, PD173074 prevented most (C4-HI) or part (T47D) of the MPA-induced PR activation, and ZK230211 inhibited the FGF2-induced PRE-luc activation.

IL-2 was used as a positive control for the evaluation of GAS-luc (28). In C4-HI cells, only FGF2 increased GAS-luc expression. However, both MPA and FGF2 increased luciferase expression in T47D cells, and this expression was prevented by the simultaneous incubation with the antiprogestin or with the FGFR inhibitor, respectively.

### MPA and FGF2 regulate the expression of progesterone-regulated genes

To investigate whether the overlapping functions of MPA and FGF2 were observed for endogenous gene products, we evaluated on T47D cells, the mRNA levels of 2 progestin-regulated genes: *CCND1* (29) and *MYC* (30). MPA and FGF2 induced a time course dependent increase in *CCND1* and in *MYC* mRNA expression (Fig. 5A). The increase of the latter was abolished when cells were cotreated with MPA and the FGFR inhibitor or with FGF2 and the antiprogestin (Fig. 5A, bottom).

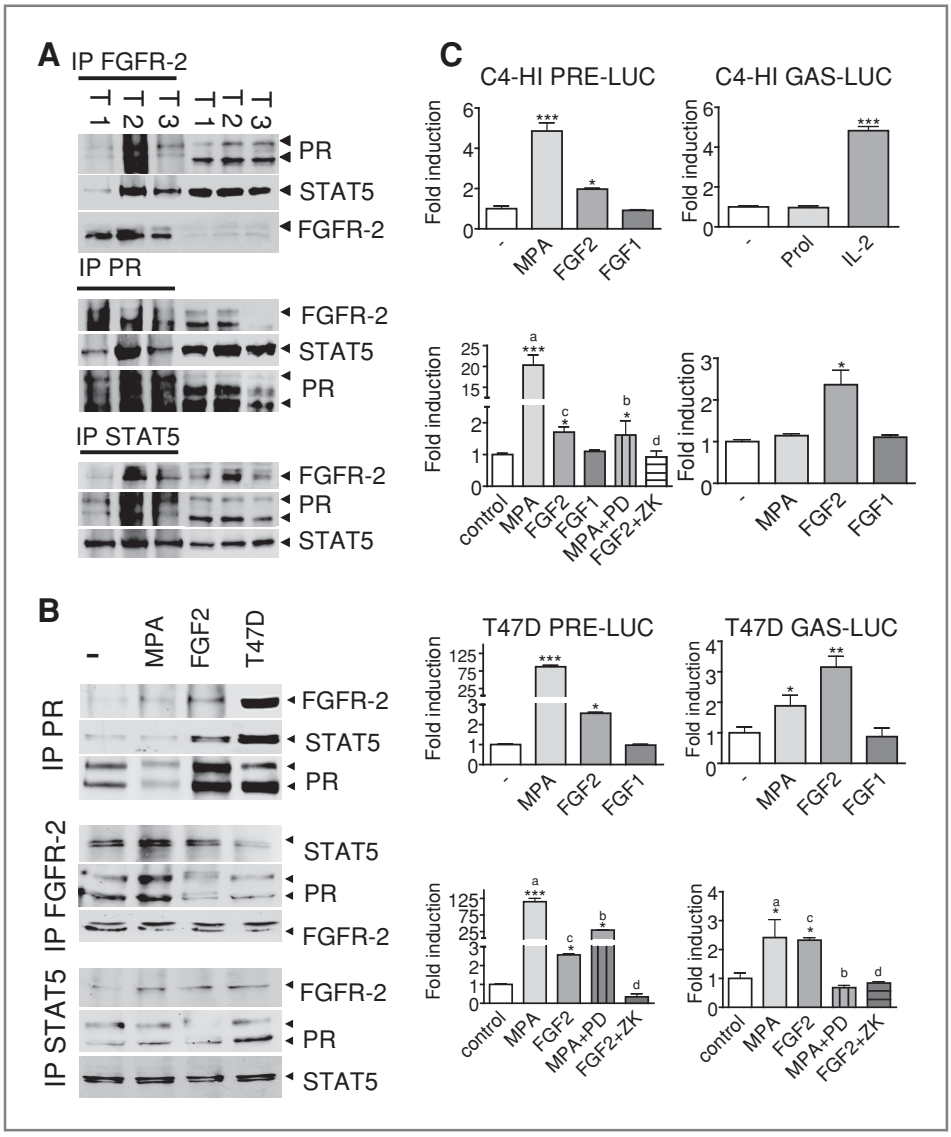


Figure 4. Interaction between PR, FGFR-2, and STAT5. Extracts from 3 C4-HI tumors (A) or T47D cells (B) were immunoprecipitated by using protein G-coupled (PR) or A-coupled (FGFR-2 and STAT5) sepharose beads and immunoblotted with the corresponding antibodies ($n = 2$). Extracts immunoprecipitated with β-actin or with rabbit/mouse anti-IgG were used as controls (Supplementary Fig. 3). C, cells transfected with the empty or the experimental vectors (PRE-luc or GAS-luc) were treated with the different reagents for 24 hours and processed to measure luciferase. IL-2 (1 ng/mL) was used as a positive control ($n = 3$; triplicates). *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ experimental versus control; a versus b: $P < 0.001$ and c versus d: $P < 0.05$. PD: PD173074 (0.1 μmol/L); ZK: ZK230211 (10 nmol/L).

The expression of CCND1, MYC, and several progestin-regulated proteins such as BCL-XL (31), tissue factor (TF; 32), and STAT5 (14) was increased by MPA- or FGF2 and inhibited by PD173074 or RU486, respectively (Fig. 5B). MPA or FGF2 also increased the expression of the progestin-regulated proteins in C4-HI. PD173074 inhibited the stimulation induced by MPA. The effect of RU486 was more selective, because it inhibited the expression of BCL-XL but it did not prevent the stimulation induced by FGF2 in Tissue factor expression (Supplementary Fig. 4A) suggesting a selective role for RU486 under different promoters in C4-HI cells.

### Complexes containing FGFR-2, STAT5, and PR bind to PRE sequences

We have thus far shown (a) a nuclear interaction between PR, FGFR-2, and STAT5 (Figs. 3 and 4A), (b) the activation of a PRE-reporter gene by MPA and FGF2 treatment (Fig. 4C), and (c) the expression of progestin-regulated genes and proteins by both treatments (Fig. 5A and B and Supplementary Fig. 4A). Next, we investigated whether these proteins were forming complexes able to bind PR at the PRE-DNA site by the NoShift transcription factor assay. We showed that PR, FGFR-2, and STAT5 were present in the complexes that bound to the PRE sequence only when the cells were treated with MPA or FGF2 (Supplementary Fig. 4B). When the cells were incubated with MPA, ERα was also part of these complexes. These results suggest that FGFR-2 and STAT5 may be acting as cofactors to regulate genes containing PRE sequences.

To validate these data and to investigate whether this complex occurs on endogenous promoters, we carried out ChIP assays. For this purpose, we focused on the human *MYC* promoter on T47D cells after 45 minutes of MPA treatment. The immunoprecipitated DNA fragments were amplified by qPCR with 3 pairs of primers designed to encompass 2 regions

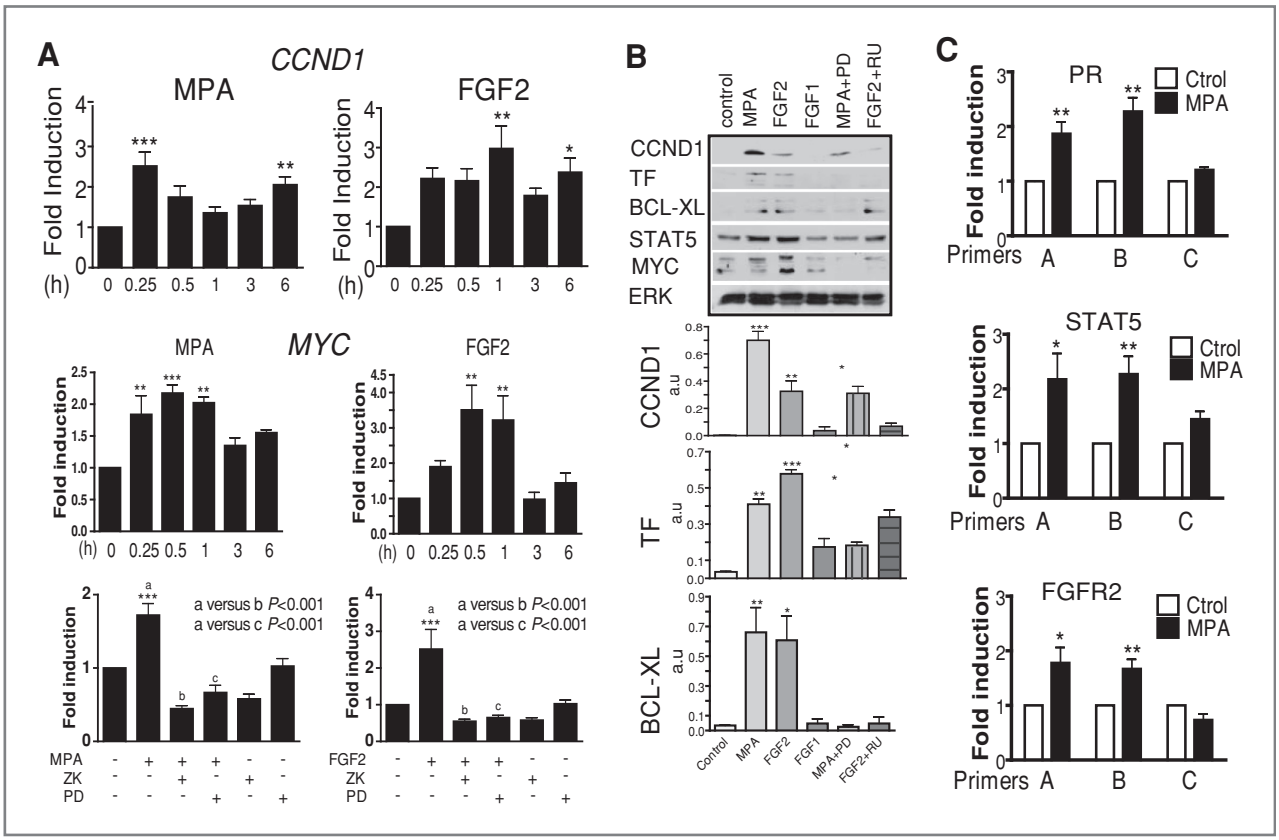


Figure 5. Quantitative PCR, Western blots, and ChIP studies confirm the interaction of PR, FGFR-2, and STAT5 on gene promoters in T47D cells. A, qPCR studies of Cyclin D1 (*CCND1*) and *MYC* of cells under different treatments ($n = 3$). B, cells were treated for 24 hours and extracts processed for Western blotting (top). ERKs were used as a loading control for quantification (bottom). CCND1, a versus b: $P < 0.01$ and c versus d: $P < 0.001$; tissue factor, a versus b: $P < 0.05$ and c versus d: $P < 0.001$; BCL-XL: a versus b and c versus d: $P < 0.05$. C, ChIP of the *MYC* promoter by using primers described in Supplementary Figure 5 designed to include (A, B) or exclude (C) PRE binding sites or hemisites ($n = 4$). In all cases *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ experimental versus control.

on the *MYC* promoter, 1 containing [primers A – PRE-like sequence described by Moore and colleagues (29)], and 1 flanking (primers B – hexanucleotide PRE half-sites) the PRE-like sequences. The recruitment of all 3 proteins to the site at plus 5–6 kb (primers C) was used as a negative control (C; Supplementary Fig. 4). As shown in Figure 5C, a significant increase in PR, FGFR-2, and STAT5 was detected only in regions A and B indicating that the 3 proteins can coexist on the *MYC* promoter after MPA treatment.

### Stable transfections of T47D cells with a constitutively active FGFR-2

All our data suggest that FGFR-2 plays an important role in tumor growth. We have hypothesized that C4-HI cells growing *in vivo* recruit carcinoma-associated fibroblasts, that providing growth factors such as FGF2, activate FGFRs expressed by epithelial cells, which in turn trigger PR signaling (3). Activated FGFRs should induce the growth of HD tumors. T47D cells are nontumorigenic when transplanted into immunocompromised mice in the absence of exogenous hormone supply (32), behaving as HD tumors. Thus, we used stably transfected T47D cells with an activated FGFR-2 (FGFR-2-CA; Fig. 6A) to challenge this hypothesis.

By using these cells, we first evaluated the activation state of PR by using specific pPR antibodies. Ab-12 antibody recognizes pSer 294 PR, a site specifically phosphorylated by ligand-dependent or independent mechanisms, and Ab-11 is specific for pSer 190 PR, a site that is basally phosphorylated although further phosphorylation occurs after activation (33). High levels of pPR expression were observed in untreated FGFR-2-CA cells but not in ZK230211- or PD173074-treated cells. RU486 did not decrease pSer 294 PR phosphorylation (Fig. 6A, bottom). In addition, FGFR-2-CA cells showed similar levels of proliferation as FGF2-treated control T47D cells transfected with the empty vector. Cell proliferation was partially blocked with ZK230211 and RU486, and completely by the FGFR-2 inhibitor (Fig. 6B). These results show that PR is involved in FGFR-2-induced cell proliferation.

FGFR-2-CA cells were transplanted orthotopically into NOD/SCID mice and gave rise to highly invasive poorly differentiated carcinomas (Fig. 6C and D top). The malignant epithelial cells infiltrated the surrounding tissues (Fig. 6D,

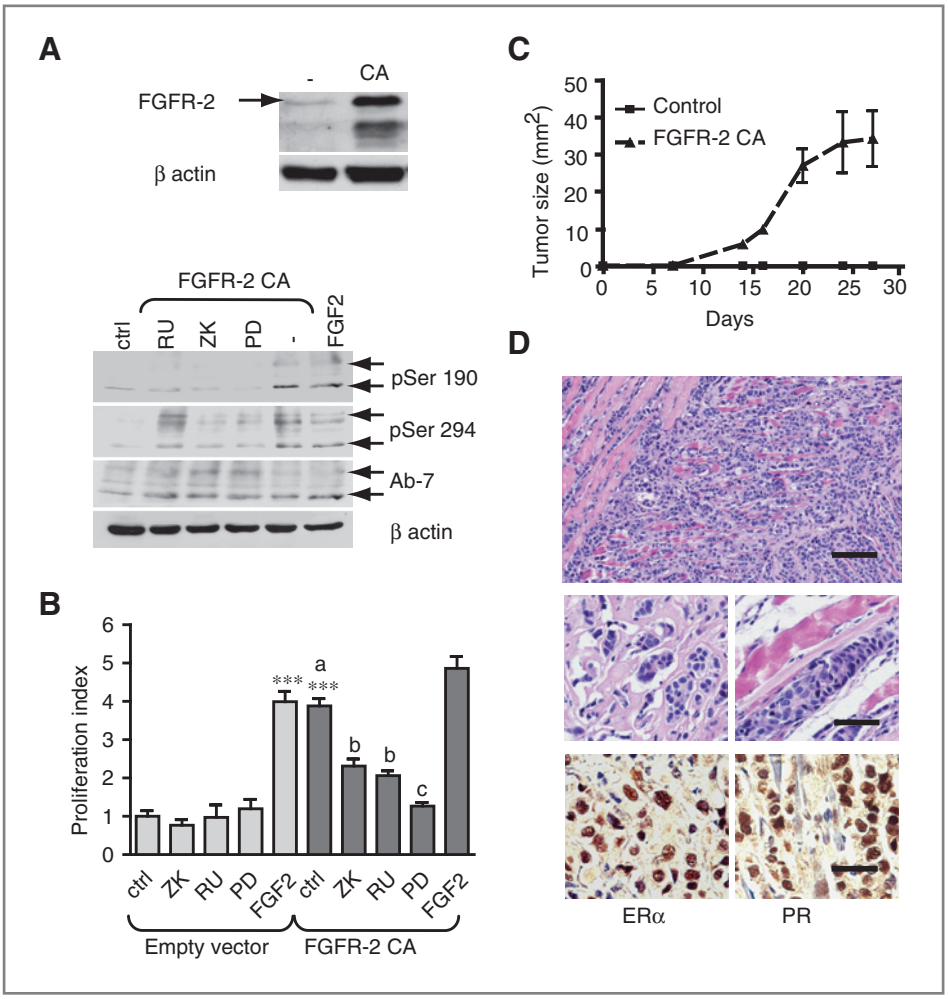


**Figure 6.** *In vitro* and *in vivo* growth of T47D FGFR-2-CA cells A, Western blots. Top, FGFR-2 in gene-transfected cells. Bottom, pPR (pSer 190 and pSer 294) and PR (Ab-7) in extracts from FGFR-2-CA cells treated for 24 hours with ZK (10 nmol/L), RU (10 nmol/L), or PD (0.1 μmol/L) and from mock transfected cells (ctrol) treated with FGF2 or vehicle. B, $^3$H-thymidine uptake index (mean ± SEM) of FGFR-2-CA or control cells treated as described in A for 48 hours ($n = 2$; octuplicates). ***: $P < 0.001$ versus empty vector control; a versus b: $P < 0.05$ and a versus c: $P < 0.001$. C, transfected cells were inoculated orthotopically into SCID/NOD mice. Only FGFR-2-CA cells gave rise to tumors. D, top, low power view of a section of a T47D FGFR-2-CA tumor (scale bar: 150 μm). Middle, right tumor cells invading muscle (scale bar: 70 μm); left, tumor areas of dense extracellular matrix (scale bar: 70 μm). Bottom, immunohistochemistry of ERα (MC-20) and PR (C-19; scale bar: 50 μm).

middle right) and grew surrounded by a dense desmoplastic stroma (Fig. 6D, middle left), a feature shared by many breast cancers. Tumors expressed ERα, PR (Fig. 6D), pSer 190 PR, and also the PR-regulated proteins BCL-XL and Tissue factor (not shown). These results lend support to the protumorigenic role of FGFR-2.

### PR colocalizes with FGFR-2 and STAT5 in the nuclei of breast cancer tissue samples

To evaluate whether the data observed in our mouse model and in the human T47D cells may represent the defining features of a subgroup of breast cancer patients, we studied breast cancer tissue samples from 16 patients (Supplementary Table 2). We found a clear colocalization of FGFR-2, STAT5, and PR/pPR in the nuclei of 4/16 tissue breast cancer samples from postmenopausal patients that had not received hormone replacement therapy. These samples had 70%–90% ERα/PR-positive cells, 2 of them were lobular and 2 ductal invasive carcinomas. In other 2 cases, occasional colocalization between the 3 proteins was also observed. Figure 7A shows a representative ductal carcinoma with a high percentage of ER and PR positive nuclei. Cryostat sections of samples from the same patient show nuclear colocalization between FGFR-2 and PR or pPR (Fig. 7B). PR staining is exclusively nuclear, whereas FGFR was observed in both the nuclear and cytoplasmic compartments. In addition, PR and pPR also colocalized with STAT5 (Fig. 7C). These results indicate that the nuclear interaction of PR and FGFR2/STAT5 is not an artifact of experimental models as it also occurs in a group of breast cancers. We are now extending our studies to a bigger set of patients, to more specifically profile this group.

## Discussion

In this study, we show that progestins and FGF2 exert overlapping functions regarding ERK, AKT, and STAT5 activation, leading to the formation of a nuclear complex between PR and FGFR-2 that involves STAT5. These complexes, which we have detected in experimental mice and human breast cancer, are biologically active and are involved in the modulation of the expression of PR/STAT5-regulated proteins. The protumorigenic role of FGFR-2 was assessed by the stable

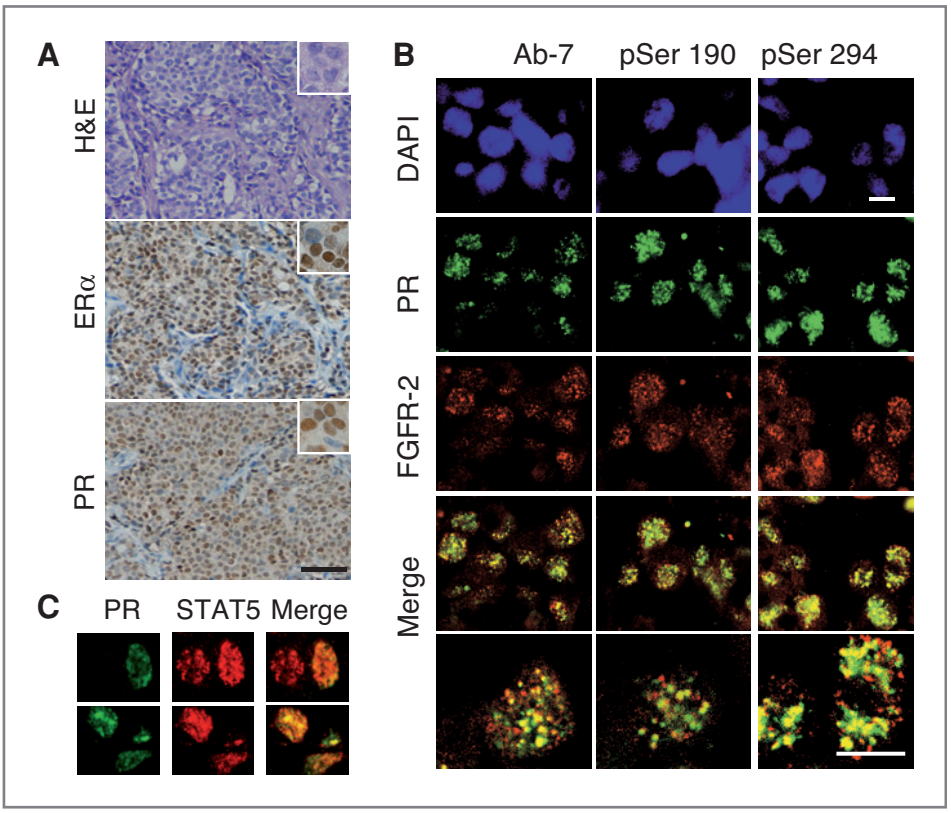


**Figure 7.** Interaction between PR and FGFR-2/STAT5 in breast cancer tissue samples. A, high histological grade ductal carcinoma showing a sheet-like growth pattern (top, H&E) and high levels of ERα (mAb clone 1D5, Invitrogen; middle) and PR (mAb clone PR-2C5, Invitrogen; bottom). Bar, 80 μm. B, immunofluorescence images of frozen sections from the same patient showing nuclear colocalization between PR (Ab-7) or pPR (Ab-11 and Ab-12; in green) and FGFR-2 (in red). Nuclei were counterstained with DAPI. Bottom, high magnification of selected cells; bar, 15 μm. C, images showing colocalization of STAT5 (red) with PR (Ab-7, green; top), and with pSer 190 PR (green; bottom).

transfection of a constitutively active mutated form in nontumorigenic T47D cells. Different types of FGF/FGFR alterations have been already reported in cancer suggesting that FGFRs are potential therapeutic targets in combination with other drugs (34, 35).

We investigated the ERK and AKT pathways because they are classic nongenomic pathways activated by both FGFs [refs. 36, 37; (reviewed in refs. 34, 38)] and by MPA (15, 39–41), focusing on STAT5 which plays a role in mammary tumorigenesis (11, 13) and in progesterone-mediated effects on the normal mammary gland (16, 26). We report that FGF2 and MPA induced ERK, AKT, and STAT5 activation, in agreement with a recent report showing that FGF2 activates STAT5 in endothelial cells by a SRC- and JAK2-dependent mechanism (6). MPA activation of JAK1–2 and STAT3 phosphorylation has previously been described in our breast cancer model (42). However, to our knowledge, this is the first report to show FGF2-mediated STAT5 activation in breast cancer. STAT5 encodes 2 highly related proteins: isoforms STAT5a and STAT5b (11). Both isoforms play different roles: STAT5a is involved in normal mammary gland differentiation and STAT5b is involved in growth hormone-mediated effects (22). In the current study, we used antibodies that cross-react with both isoforms.

The presence of splice variants of FGFR-3 and other RTKs in the nuclei has already been reported (10). Several reports have also suggested that peptide hormones such as prolactin, which usually activates STAT5, may in parallel with the cell-surface activation of STAT, induce ligand-activated membrane receptor endocytosis and retrotranslocation to the nucleus. Although less information is available regarding nuclear FGFR-2, PKC-, and MAPK-dependent nuclear translocation was shown for FGF2-FGFR-2 in osteoblasts (23) and, accordingly, FGFR-2 was also observed in the nuclei in lung cancers (24). Here we report that FGF2 and MPA induce nuclear colocalization of PR with FGFR-2/STAT5. Although we have used the Ab-7 PR antibody which recognizes PR-A in immunofluorescence studies (43), we have confirmed by immunoprecipitation the involvement of both PR isoforms.

We then evaluated the functionality of these complexes by using PRE- and GAS-luc reporter assays. It has already been reported that GR (44) and PR (14) may act as coactivators of STAT5. Interestingly, we found that MPA only activated PRE-luc in C4-HI cells, indicating that even if STAT5 is activated, it has a higher affinity or it is tethered to other complexes, making it unavailable for GAS binding. FGF2 activated both PRE- and GAS-luc. When we moved to a more physiologic setting, *CCND1* and *MYC* mRNA were also increased by both ligands as well as all the MPA/STAT5-regulated proteins studied (30, 45). The FGFR inhibitor and 2 different antiprogestins not only inhibited cell proliferation induced by MPA or FGF2, respectively, but also inhibited the induction of luciferase expression, mRNA, and protein expression. The specificity of this FGFR inhibitor has been confirmed (46) and we have previously shown in our model, that PD and siRNA FGFR-2 exert the same inhibitory effects (3). The specific effect of

antiprogestins has been confirmed by using antisense oligonucleotides to PR to block the stimulatory effects induced by MPA and FGF2 (2).

In a previous report, we showed that MPA or FGF2 induced an electrophoretic mobility gel shift by using a PRE probe (3). We now confirmed that STAT5 and FGFR-2 can act as coactivators of PR, because they can bind to PR when it is bound to PRE sites as judged by reporter experiments and NoShift transcriptional assays, as well as in the context of chromatin complexed DNA (ChIP assays). Our studies suggest that PR is functionally linked to FGFR-2 and STAT5, and that FGFR-2 interacts with STAT5. However, whether these proteins form a ternary complex, or associate in multimolecular complexes remains to be elucidated. Although the regulation of *MYC* expression is very complex (47), we have focused our studies on the *MYC* promoter as it exhibits a PRE-like site that is involved in the PR regulation of this gene (29). In this regard, the regions studied lack STAT5 binding sites, hence the observed association of STAT5 to the region nearby the PRE hemisites on MPA stimulation raises the possibility of the existence of a functional cross talk between PR and STAT5 at this site within the *MYC* promoter.

The data reported here suggest that the nongenomic effects induced by FGF2 and MPA, such as ERK and AKT activation, need to be supported by genomic effects, which will eventually determine cell fate. FGF2 is a better stimulator of cell proliferation than MPA, probably because it activates the PR pathway and GAS responsive elements. Another possibility is that FGF2 may activate other FGFRs in these breast cancer cells that participate enhancing nongenomic effects. We provide evidence that in hormone-dependent tumors, hormones are the main drivers of cell proliferation, but in hormone-independent neoplasias, carcinoma-associated fibroblasts provide growth factors that activate FGFR-2. Activated FGFR-2 activates PR and STAT5, which in turn activate progestin responsive genes. In the presence of antagonists, tumors may become refractory to endocrine therapy by switching to STAT5-dependent mechanisms. Thus, we propose that FGFR-2 inhibitors might be used together with endocrine therapy to delay the onset of hormone resistance.

The prooncogenic role of FGFR-2 was further documented in the experiment in which highly invasive malignant phenotype was induced in T47D cells when stably transfected with the constitutively active FGFR-2. This construct has a point mutation that turns cysteine 278 into phenylalanine, inducing dimerization and phosphorylation of the receptor. This is one of the cranyosinostosis mutations in the FGFR2 extracellular domain observed in the Crouzon and Pfeiffer syndromes and it has been used to transform 3T3 fibroblasts (48). The T47D FGFR-2-CA cells overexpress constitutively active FGFR-2 still expressing ERα, PR, and pPR, and at least *in vitro* they are inhibited by antiprogestins. Further studies are necessary to investigate whether this overexpression of activated FGFR-2 overrides with time the need of PR to promote tumor growth.

Finally, the fact that FGFR-2 colocalizes with PR and STAT5 in human breast cancer tissue samples suggests that our studies, which were conducted by using a reliable murine breast cancer model and one of the few human breast cancer models expressing PR, may represent what is occurring in a subset of breast cancer patients. The increased expression of FGFR-2 observed in breast cancer patients has been associated with increased RUNX-2 which is also regulated by FGF2 and STAT5 (49). The presence of pSTAT5 in the nuclei of breast cancer tissue samples has been reported (50). The link between all these data will increase our understanding of human breast cancer. The results reported here underscore the use of FGFR-2 inhibitors in combination with endocrine therapy in hormone-related breast cancers.

## Disclosure of Potential Conflicts of Interest

J.P. Cerliani, T. Guillardoy, and S. Giulianelli: Fellows, CONICET C.A.; Lamb and C. Lanari: Members of the Research Career, CONICET.

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