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GP369, an FGFR2-IIIb–Specific Antibody, Exhibits Potent Antitumor Activity against Human Cancers Driven by Activated FGFR2 Signaling

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Abstract

Dysregulated fibroblast growth factor (FGF) signaling has been implicated in the pathogenesis of human cancers. Aberrant activation of FGF receptor 2 (FGFR2) signaling, through overexpression of FGFR2 and/or its ligands, mutations, and receptor amplification, has been found in a variety of human tumors. We generated monoclonal antibodies against the extracellular ligand-binding domain of FGFR2 to address the role of FGFR2 in tumorigenesis and to explore the potential of FGFR2 as a novel therapeutic target. We surveyed a broad panel of human cancer cell lines for the dysregulation of FGFR2 signaling and discovered that breast and gastric cancer cell lines harboring *FGFR2* amplification predominantly express the IIIb isoform of the receptor. Therefore, we used an FGFR2-IIIb–specific antibody, GP369, to investigate the importance of FGFR2 signaling *in vitro* and *in vivo*. GP369 specifically and potently suppressed ligand-induced phosphorylation of FGFR2-IIIb and downstream signaling, as well as FGFR2-driven proliferation *in vitro*. The administration of GP369 in mice significantly inhibited the growth of human cancer xenografts harboring activated FGFR2 signaling. Our findings support the hypothesis that dysregulated FGFR2 signaling is one of the critical oncogenic pathways involved in the initiation and/or maintenance of tumors. Cancer patients with aberrantly activated/amplified FGFR2 signaling could potentially benefit from therapeutic intervention with FGFR2-targeting antibodies.
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Introduction

Fibroblast growth factor (FGF) signaling regulates many fundamental biological processes including embryogenesis, tissue and stem cell maintenance, angiogenesis, and wound healing (1–3). The pleiotropic effects of FGFs are mediated through four highly conserved receptor tyrosine kinases (RTK), namely, FGF receptors 1 to 4 (FGFR1–FGFR4; ref. 3). The extracelluar domain of these receptors contains three immunoglobulin-like (Ig) domains (D1, D2, and D3). The second and third Ig domains are critical for ligand binding and specificity, whereas the first Ig domain, absent in certain isoforms, is thought to have an autoinhibitory function. Alternative splicing of the carboxyl half of the third Ig domain yields either the IIIb or the IIIc isoform of FGFR1 to FGFR3 (2, 3). These various isoforms of FGFRs exhibit tissue-specific expression and respond to a different spectrum of the 18 mammalian FGFs (1). For example, FGFR2-IIIb, normally expressed on epithelial cells, can be activated by FGFs of mesenchymal origin, whereas FGFR2-IIIc expressed on mesenchymal tissues responds to ligands secreted by epithelial cells (4, 5).

FGFs initiate intricate and highly regulated signaling events by forming a ternary complex with their cognate receptors and heparan sulfate proteoglycans (1, 2). This results in trans-autophosphorylation of FGFRs at intracellular tyrosine residues and the activation of downstream signaling. One key molecule involved in FGF signaling is FGFR substrate 2α (FRS2α), which, when phosphorylated by activated FGFRs, can recruit other protein complexes to stimulate the Ras/mitogen-activated protein kinase and/or phosphoinositide 3-kinases (PI3K)/Akt signaling networks (1–3). The cellular response to the activated FGF signaling is tissue specific, ranging from proliferation and motility to growth arrest, apoptosis, and differentiation (3).

Dysregulated autocrine and paracrine activation of FGFRs has been reported in a variety of human cancers. A wealth of evidence also supports a critical role of aberrant FGFR2 signaling in cancer, including overexpression of FGFR2 and its ligands, mutations and amplifications of the receptor, and receptor isoform switching. Single-nucleotide polymorphisms (SNP) of *FGFR2* are associated with an increased risk of

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breast cancer development, presumably due to elevated FGFR2 expression (6–8). Potentially activating missense mutations of *FGFR2* have been reported in multiple cancer types, including endometrial, ovarian, breast, lung, and gastric cancers (3, 9–14). Furthermore, the *FGFR2* gene is amplified in a subset of gastric and breast cancers (3, 15–21). The coexpression of FGFR2-IIIb and its ligand, FGF7, in pancreatic and gastric cancers, as well as in lung adenocarcinomas, is associated with poor prognosis (22–24), likely due to aberrant receptor activation through the formation of an autocrine activation loop. Paradoxically, *FGFR2* has also been implicated as a tumor suppressor gene. For example, loss-of-function mutations have been detected in melanomas (25). Decreased expression of FGFR2-IIIb has been reported in several cancer types during tumor progression (3, 26–29), possibly reflecting the physiologic role of FGFR2 in regulating tissue homeostasis (30, 31). These contrasting and context-dependent roles of FGFR2 signaling underscore the complexity of FGFR2 signaling.

To address the role of FGFR2 in tumorigenesis and to explore FGFR2 as a potential therapeutic target, we generated monoclonal antibodies (mAb) against the extracellular ligand-binding domain of FGFR2. Here, we describe the *in vitro* and *in vivo* biological activities of GP369, an FGFR2-IIIb isoform-specific mAb, on human cancer cell lines with FGFR2 dysregulation. GP369 inhibited the ligand-induced phosphorylation of FGFR2 and its downstream signaling, as well as the proliferation driven by FGFR2 overexpression. Administration of GP369 in mice inhibited the *in vivo* growth of human cancer xenografts harboring *FGFR2* amplification. These findings support the hypothesis that dysregulated FGFR2 signaling is one of the many critical tumorigenesis pathways in human cancers. Targeted therapy using FGFR2 mAb could be beneficial for patients with aberrantly activated/amplified FGFR2 signaling.

Materials and Methods

Cell lines and reagents

KATO III, HEC-1-A, AN3 CA, SNU-16, and human lung cancer cell lines were acquired from the American Type Culture Collection (ATCC). FDCP-1, Ba/F3, MFM-223, and endometrial cancer cell lines were obtained from the German Collection of Microorganisms and Cell Cultures. All human cell lines were cultured according to the suppliers' recommendations. FGFs were purchased from R&D Systems. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and heparin were obtained from Sigma-Aldrich.

Screening for functional FGFR2 antibodies

Mice were immunized with a 1:1 mixture of human FGFR2 D2-D3 (IIIb) and D2-D3 (IIIc)-human Fc fusion proteins (see Supplementary Methods for more detailed description). Hybridoma supernatants were screened by MTT assays using FDCP cells expressing FGFR2-IIIb or FGFR2-IIIc in a 96-well plate (70,000 cells per well) with FGF1 (8 ng/mL) and heparin (5 μg/mL).

Epitope mapping of GP369

To determine the epitope of GP369, binding to FGFR2 peptides was measured by biolayer interferometry using an Octet QK instrument (ForteBio). Ten overlapping peptides spanning the COOH terminus of the third Ig domain of human FGFR2-IIIb were synthesized and biotinylated at their NH_2 termini. FGFR2 peptides (1 μmol/L) and a negative control peptide, E2 (1 μmol/L) were captured on streptavidin Octet biosensors. Sensograms were recorded when peptide-loaded biosensors were incubated with GP369 (1 μmol/L) in Octet sample diluent.

Proliferation assay

For SUM52PE proliferation assay, the cells were seeded in 96-well plates at 5,000 cells per well in growth medium [Ham's F-12, 5% fetal bovine serum (FBS), 5 μg/mL insulin, and 1 μg/mL hydrocortisone] and cultured overnight to allow for adherence. Cells were then incubated in reduced-serum medium (Ham's F-12 with 0.5% FBS) and treated with either a control mouse IgG1 (mIgG; Bio X Cell) or GP369 at various concentrations for 5 days. Cell proliferation was assessed by MTT assays. For MCF7 proliferation assay, the cells were seeded in 96-well plates at 10,000 cells per well in complete growth medium (DMEM with 10% FBS) and cultured overnight to allow for adherence. Cells were then incubated in serum-free medium for 24 hours, followed by treatment with various amounts of mouse IgG or GP369 in the absence or presence of FGF7 (25 ng/mL) for 3 days. Cell proliferation was assessed by MTT assays.

Xenograft studies

All mice were treated in accordance with the OLAW Public Health Service Policy on Human Care and Use of Laboratory Animals as well as the ILAR Guide for the Care and Use of Laboratory Animals. In the SNU-16 *in vivo* studies, 10-week-old female C.B-17 severe combined immunodeficient (SCID) mice (Taconic) were inoculated s.c. into the flank with 5×10^6 cells in 1:1 RPMI 1640 (Invitrogen)/Matrigel (BD Biosciences). Tumor measurements were taken twice weekly using vernier calipers. Tumor volume was estimated using the following formula: $V = 0.5 \times \text{width} \times \text{width} \times \text{length}$. When tumors approached ~200 mm^3, mice were randomized into groups of 10 animals each and received GP369 (2–20 mg/kg) or a control mIgG at 20 mg/kg twice weekly by i.p. injection. For the MFM-223 *in vivo* studies, 5-week-old female NCr nude mice (Taconic) were implanted s.c. on the left flank with 0.72 mg of 90-day-release 17β-estradiol pellets (Innovative Research) and inoculated s.c. into the right flank with 10^7 MFM-223 cells in 1:1 EMEM (ATCC)/Matrigel. When tumors approached ~200 mm^3, mice were randomized into groups of 10 animals each and treated with 20 mg/kg of mIgG or GP369 by i.p. injection twice weekly. All statistical analysis was done using a one-way ANOVA and Tukey's multiple comparison test.

Results

Characterization of human cancer cell lines with *FGFR2* amplification

FGFR2 was originally identified as an amplified cDNA from a gastric cancer cell line, KATO III (32). *FGFR2* amplification

was subsequently detected in a number of other cell lines, including gastric cancer cell lines, SNU-16 and Ocum2M (33), and a breast cancer cell line, SUM52PE (15, 34). We queried the Sanger Institute SNP array-based gene copy number database to identify additional *FGFR2*-amplified human cancer cell lines. A few candidate cell lines with potential *FGFR2* amplification were found, including the breast cancer cell line MFM-223 and the endometrial cancer cell line MFE-280. Quantitative PCR on genomic DNA revealed 287 copies of *FGFR2* in MFM-223 (Fig. 1A; Supplementary Table S1). However, there was no discernable alteration in the *FGFR2* copy number in MFE-280. Consistent with previous reports, SUM52PE, KATO III, and SNU-16 exhibited dramatic copy number gains of *FGFR2*. SNP array profiles for MFM-223 and SNU-16 suggest that *FGFR2* amplification in these cells is focal (data not shown). We also surveyed FGFR copy number in a dozen human endometrial and lung cancer cell lines, some of which also exhibited increased copy number in various FGFR genes (Supplementary Table S1).

The expression levels of FGFRs in the cell lines with *FGFR2* amplification were analyzed by quantitative reverse transcription-PCR (RT-PCR). Abundant FGFR2 transcripts, predominantly the IIIb isoform, were detected in all four *FGFR2*-amplified cell lines (Fig. 1B). In addition, these cell lines also express high levels of FGFR2 protein as detected by Western blot analysis (data not shown) and flow cytometry (Fig. 1C).

Identification and characterization of FGFR2 antagonistic antibodies

To investigate the role of dysregulated FGFR2 signaling in tumor cell lines with *FGFR2* amplification, FGFR2-specific monoclonal antibodies were generated. To establish cell-based assays to screen for functional FGFR2 antibodies, we first engineered interleukin-3 (IL-3)–dependent murine Ba/F3 (pro-B) and FDCP-1 (bone marrow) cells to express wild-type (WT) and cancer-associated mutant variants of FGFR2. Previous studies have shown that ectopic expression of FGFRs in these cells confers FGF1-dependent proliferation in the absence of IL-3 (4, 5). As expected, there was no detectable proliferation of FDCP-1 cells stably expressing WT FGFR2 in the absence of IL-3 and FGF1 (data not shown). It is known that FGF1, FGF3, FGF7, FGF10, and FGF22 transduce signals

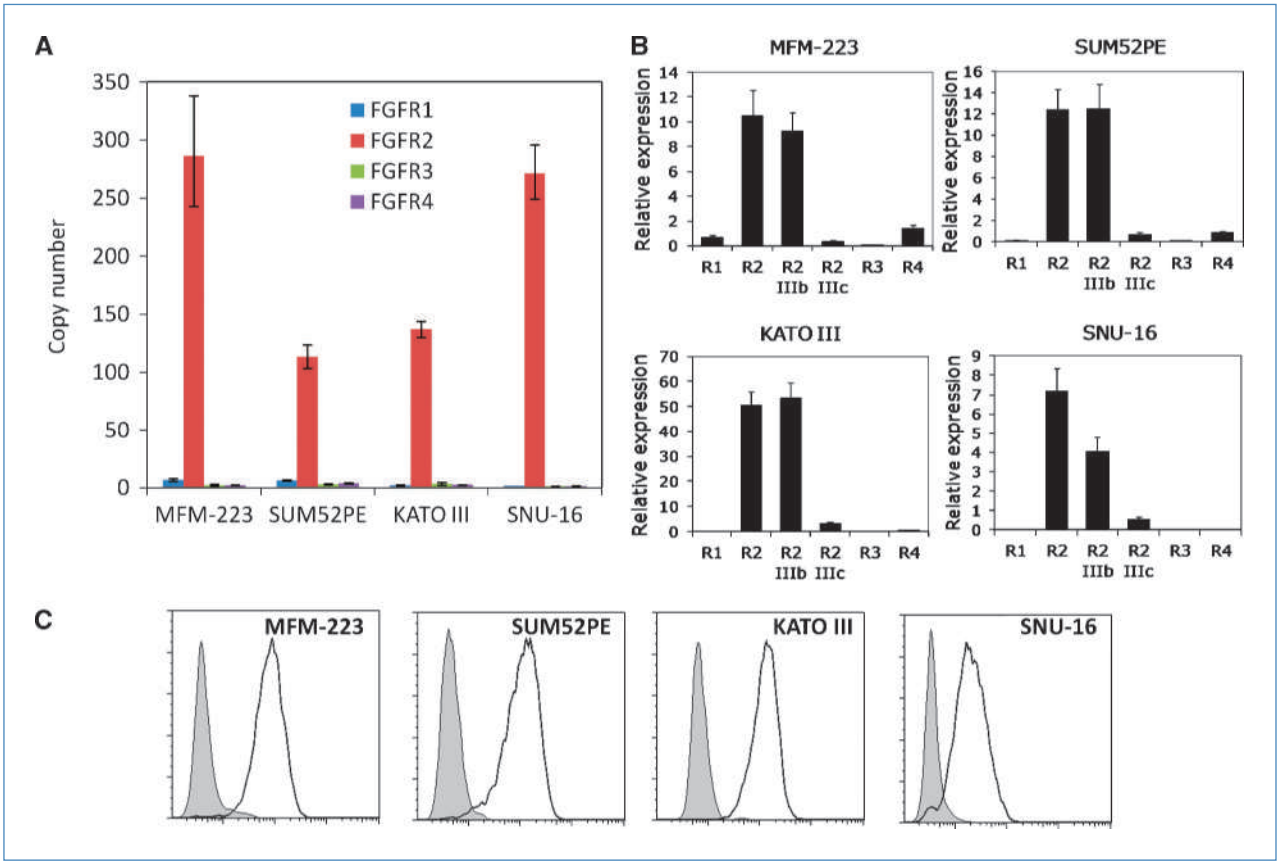

Figure 1. Characterization of human cancer cell lines with *FGFR2* amplification. A, *FGFR* copy number of human cancer cell lines. Quantitative PCR was carried out using primers specific for *FGFRs* or a reference gene, *transketolase* (*TKT*), and copy number was calculated. B, FGFR expression analysis. Quantitative RT-PCR was carried out using primers specific for *FGFRs* and *HPRT*. The expression levels were normalized to *HPRT*. C, FGFR2 cell surface expression by flow cytometry. The cells were incubated with 100 μL of primary FGFR2 antibodies (5 μg/mL) for 1 h, followed by incubation with 1:100 phycoerythrin (PE)-conjugated secondary antibodies (Jackson ImmunoResearch) for 30 min. Analysis of stained cells was performed on a FC500 flow cytometer (Beckman Coulter).

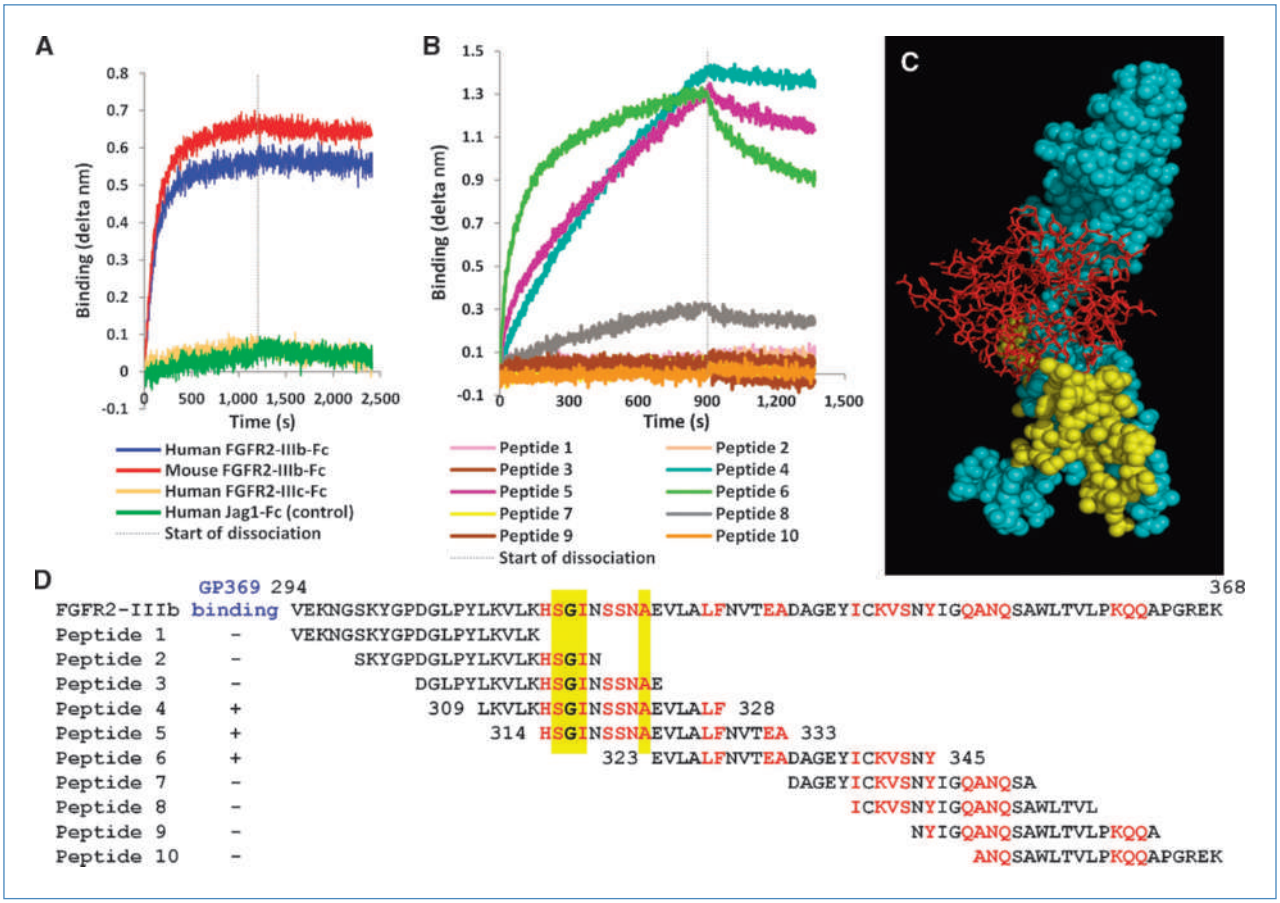

Figure 2. Epitope mapping of GP369. A, specific binding of GP369 to human and mouse FGFR2-IIIb. The extracellular domains of human FGFR2-IIIb, human FGFR2-IIIc, mouse FGFR2-IIIb, and human Jag-1 (control) were expressed as human Fc fusion proteins and captured on anti-hFc Octet biosensors (ForteBio). Sensograms were recorded when the loaded biosensors were incubated with GP369 in Octet sample diluent (ForteBio). B, epitope mapping. Ten overlapping peptides were biotinylated at their NH_2 termini and captured on streptavidin Octet biosensors. Sensograms were recorded when peptide-loaded biosensors were incubated with GP369 in Octet sample diluent. C, GP369 binding epitopes. GP369 binding peptides (from amino acid 309 to 345; yellow) mapped to the structure of FGFR2-IIIb (light blue; D2 on top, D3 at the bottom) in complex with FGF10 (red; PDB structure 1NUN; ref. 35). D, peptide sequences used for epitope mapping. The residues in human FGFR2-IIIb that differ from FGFR2-IIIc are in red. The amino acids involved in binding to FGF10 are highlighted in yellow. The peptides that bind to GP369 are denoted with "+" and those that do not bind are denoted with "–".

through FGFR2-IIIb, whereas FGFR2-IIIc responds to a broader panel of ligands including FGF1, FGF2, FGF4, FGF6, FGF9, FGF16, FGF17, FGF18, and FGF20 (4, 5). Similarly, the proliferation of FDCP-1 cells expressing the IIIb isoform of FGFR2 was stimulated by FGF7 and FGF10 but not by FGF2 and FGF9, whereas the proliferation of those expressing the IIIc isoform was enhanced by FGF2 and FGF9 specifically (Supplementary Fig. S1).

Antibody-secreting hybridomas were generated from mice immunized with the extracellular domain of human FGFR2. Primary screens using the engineered FDCP-1 cell lines identified a highly potent and selective isoform-specific FGFR2 mAb, GP369, which exhibited marked inhibition of proliferation driven by the IIIb isoform, but not the IIIc isoform, of FGFR2.

Octet analysis revealed specific binding of GP369 to human and mouse FGFR2-IIIb, but not to human FGFR2-IIIc or a negative control protein, human Jag1 (Fig. 2A). Surface plasmon resonance confirmed that GP369 exhibited similar affinity toward murine, cynomolgus, and human FGFR2-IIIb Fc fusion proteins. No binding to human FGFR2-IIIc, FGFR1-IIIb, or FGFR3-IIIb was detected (data not shown). These results show that GP369 is an isoform-specific antibody that binds to FGFR2-IIIb from mouse to human.

To understand the molecular interaction between GP369 and FGFR2-IIIb, we tested the binding of GP369 to 10 overlapping peptides covering the COOH-terminal half of the third Ig domain of human FGFR2-IIIb, as this is the only region that differs between the IIIb and IIIc forms. Peptides 4, 5, and 6 showed strong binding to GP369 (Fig. 2B), whereas the seven neighboring peptides did not exhibit any significant binding to GP369 (Fig. 2B). The crystallographic structure of FGFR2-IIIb complexed with FGF10 has revealed critical residues within FGFR2-IIIb involved in direct binding with FGF10 (35). The GP369 binding epitope contains some of these residues important for ligand binding (Fig. 2C). For example, Ser-315, Gly-316, and Ala-322 form hydrogen bonds with

FGF10, whereas Ile-317 contacts FGF10 via hydrophobic interactions. Therefore, binding of GP369 to FGFR2-IIIb is likely to directly block ligand-receptor interaction.

GP369 inhibits WT and cancer-relevant mutant FGFR2-IIIb–driven proliferation

We quantified the potency of GP369 by using FDCP-1 cells expressing either the IIIb or IIIc isoform of FGFR2. GP369 significantly inhibited in a dose-dependent manner the proliferation of FDCP-1 cells driven by FGFR2-IIIb (with an average IC_{50} of ~1.4 nmol/L) but not by FGFR2-IIIc (Fig. 3A). Breast and gastric cancer cell lines, such as SUM52PE and KATO III, express a COOH-terminally truncated variant of FGFR2 with increased transforming activity due to constitutive phosphorylation of FRS2 and activation of downstream signaling (36, 37). GP369 also effectively suppressed the proliferation of FDCP-1 cells driven by this truncated FGFR2 variant (Fig. 3A).

Mutations in *FGFR2* have been detected in many cancers, including about 12% of endometrial tumors (11, 13). Two of the most common mutations in endometrial tumors are S252W, a mutation in the linker region between D2 and D3, which alters ligand specificity and increases the affinity of ligand binding, and N550K, a catalytic domain mutation that enhances kinase activity. GP369 markedly inhibited proliferation driven by both the FGFR2-IIIb S252W and N550K mutant variants with IC_{50} values of 4.4 and 4.5 nmol/L, respectively (Fig. 3A).

To examine the effect of GP369 on human cancer cell lines with FGFR2 dysregulation *in vitro*, we selected two breast cancer cell lines, SUM52PE and MCF7, for further analysis. Both cell lines express FGFR2; however, SUM52PE harbors *FGFR2* amplification whereas MCF7 does not. The *in vitro* proliferation of SUM52PE was not further stimulated by FGF7, an FGFR2-IIIb–specific ligand (data not shown), likely due to the constitutive activation of FGFR2 signaling in these cells. Nevertheless, GP369 significantly blocked the proliferation of SUM52PE in a dose-dependent manner (Fig. 3B). In contrast, the *in vitro* proliferation of MCF7 was stimulated by FGF7, and GP369 effectively blocked the FGF7-induced proliferation of these cells (Fig. 3C).

While surveying additional human cancer cell lines with FGFR2 dysregulation, we found that a non–small cell lung cancer cell line, HCC95, expressed high levels of FGFR2 in the absence of *FGFR2* amplification. HCC95 cells formed large colonies in soft agar; therefore, we tested the effect of GP369 on the growth of these cells in soft agar. For comparison, we also tested GP369 on an *FGFR2*-amplified cell line,

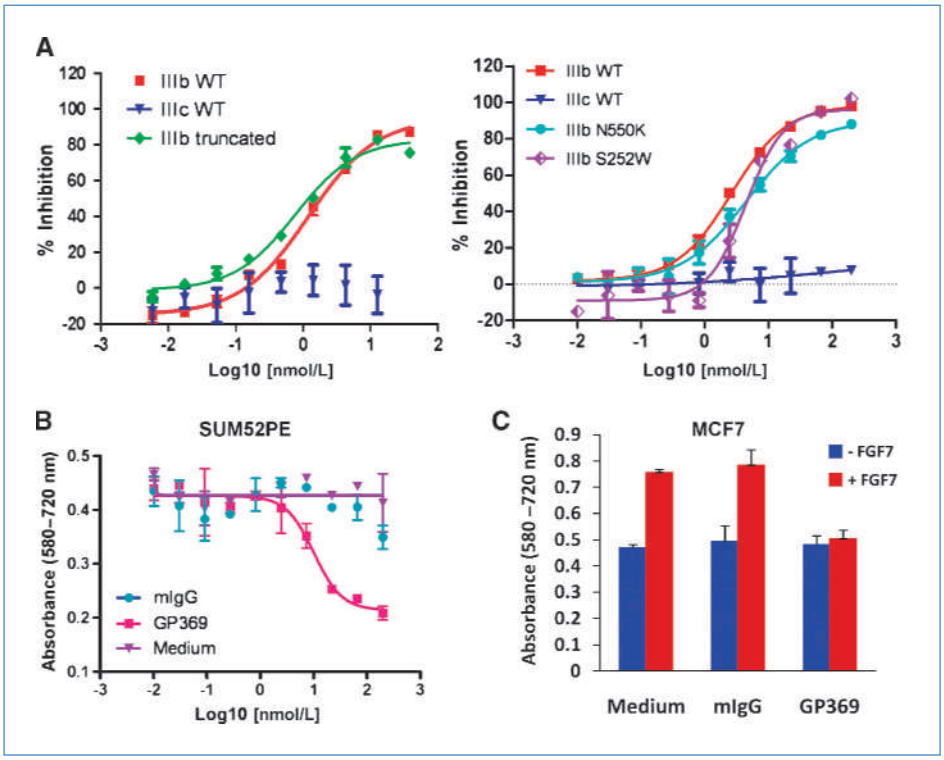

Figure 3. Characterization of FGFR2 antibodies by proliferation assays. A, GP369 suppresses cell proliferation driven by the WT or the COOH-terminally truncated isoform of FGFR2-IIIb or the mutant variants of FGFR2-IIIb. GP369 mAb was added to FDCP-1 cells expressing FGFR2-IIIb, FGFR2-IIIc, a COOH-terminally truncated IIIb isoform, or the S252W and N550K mutant variants in the presence of FGF1 (8 ng/mL) and heparin (5 μg/mL). MTT assays were carried out after 2 d. B, inhibition of the proliferation of SUM52PE cells by GP369. The cells were incubated in reduced serum medium (0.5% FBS) and were either left untreated (medium) or treated with mIgG or GP369 at various concentrations for 5 d. Cell proliferation was assessed by MTT assays. C, inhibition of the FGF7-induced proliferation of MCF7 cells by GP369. The cells were incubated in serum-free medium and were either left untreated (medium) or treated with mIgG or GP369 at 30 μg/mL for 3 d in the absence or presence of FGF7 (25 ng/mL). Cell proliferation was assessed by MTT assays.

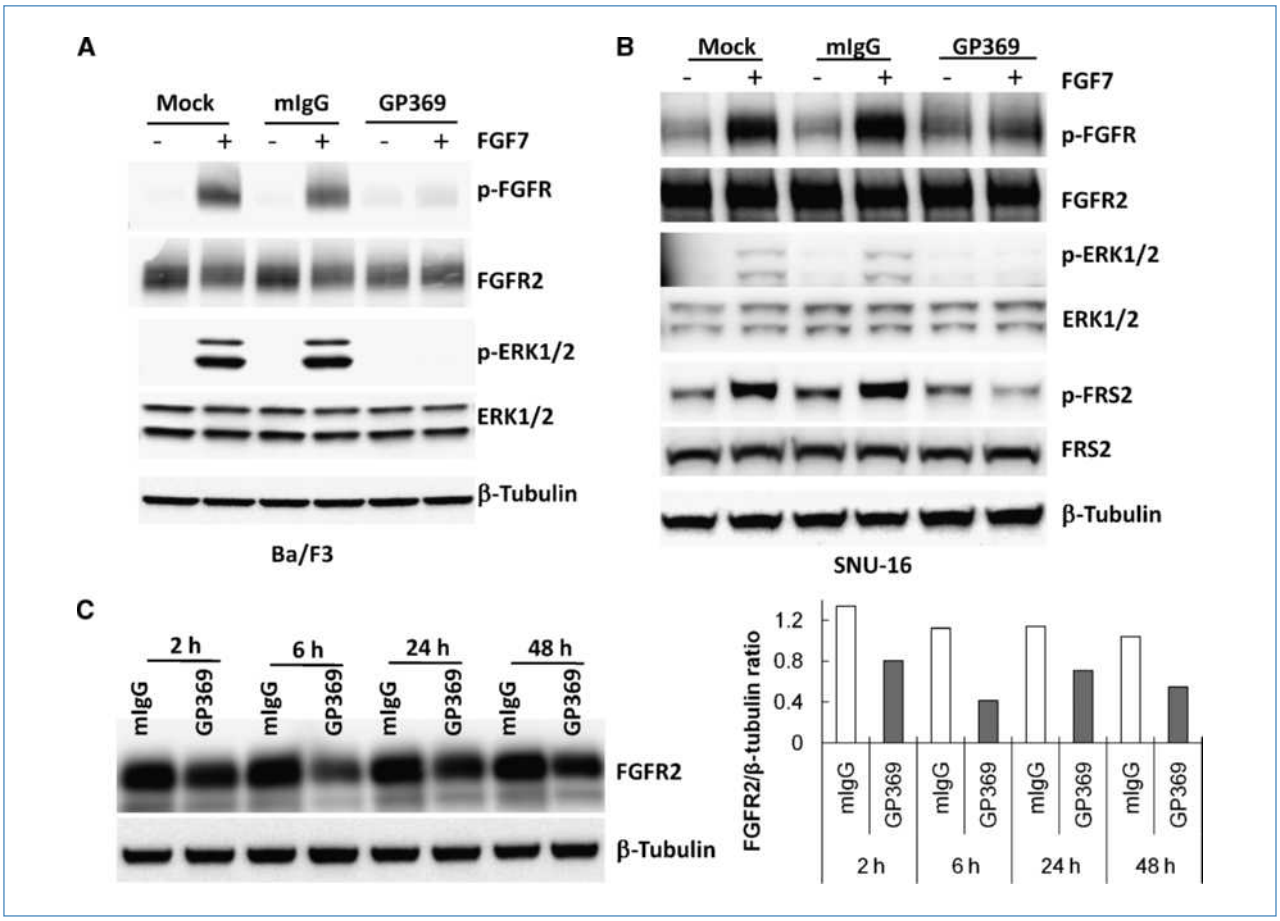

Figure 4. Inhibition of FGFR2 signaling by GP369. A, GP369 suppresses FGF7-induced FGFR2 tyrosine phosphorylation and ERK1/2 phosphorylation in Ba/F3 cells overexpressing FGFR2-IIIb. After 3 h of serum starvation, the cells were treated with PBS (mock) or 5 μg/mL of either mIgG or GP369 for 1 h at 37°C before stimulation by FGF7 (20 ng/mL) and heparin (5 μg/mL) for 15 min. The protein lysates were analyzed by Western blot. B, GP369 blocks FGF7-induced FGFR2 and FRS2 tyrosine phosphorylation and ERK1/2 phosphorylation in SNU-16 cells. The cells were incubated with PBS (mock) or 5 μg/mL of either mIgG or GP369 for 1 h at 37°C before treatment with either heparin (20 μg/mL) or heparin plus FGF7 (30 ng/mL) for 15 min. Western blotting analysis was then carried out. C, downregulation of the FGFR2 protein levels by GP369. SNU-16 cells were treated with 10 μg/mL of either mIgG or GP369. The cells were harvested at various time points. Total levels of FGFR2 and β-tubulin were determined by Western blotting, and densitometric units quantified using Scion Image software.

KATO-III. In the presence of a control mIgG, both HCC95 and KATO-III cells formed large colonies in soft agar, whereas the addition of GP369 significantly inhibited the size as well as the number of colonies (Supplementary Fig. S3).

GP369 suppresses FGFR2 phosphorylation and downstream signaling

We have also investigated the effect of GP369 on the signaling cascades activated by FGFR2. FGF7 induced tyrosine phosphorylation of FGFR2 and led to the subsequent activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2) in both Ba/F3 cells overexpressing FGFR2-IIIb (Fig. 4A) and *FGFR2*-amplified SNU-16 cells (Fig. 4B). GP369 effectively inhibited the ligand-induced tyrosine phosphorylation of FGFR2 and activation of ERK1/2 in these cells (Fig. 4A and B). Tyrosine phosphorylation of FRS2, a key molecule connecting FGFR to downstream signaling pathways, was also attenuated in SNU-16 cells treated with FGF7 (Fig. 4B). In addition, GP369 downregulated the FGFR2 protein level in SNU-16 cells (Fig. 4C). An approximately 40% decrease in the FGFR2 protein level is noticeable 2 hours after incubation with the GP369 antibody, and there was a >60% reduction in the FGFR2 protein level after 6 hours.

GP369 inhibits the *in vivo* growth of cancer xenografts with activated FGFR2 signaling

To assess the functional activity of GP369 *in vivo*, we evaluated the effect of the antibody on the growth of human cancer xenografts harboring *FGFR2* amplification. Of the four *FGFR2*-amplified cell lines examined, only SNU-16 and MFM-223 cells formed tumors in mice; thus, the efficacy of GP369 was tested against these two xenografts.

SNU-16 xenograft tumors were treated with a control mIgG at 20 mg/kg or GP369 at 2, 5, 10, or 20 mg/kg. GP369

treatment showed potent tumor growth inhibition when compared with mIgG-treated controls at all doses evaluated ($P < 0.001$; Fig. 5A). Strikingly, the tyrosine phosphorylation of FGFR2 was barely detectable in GP369-treated tumors (Fig. 5B). Consistent with *in vitro* studies, GP369 reduced the total FGFR2 protein in tumors (Fig. 5B). In contrast to the *in vitro* phospho-RTK profile of SNU-16 cells, which exhibited multiple RTKs with high levels of tyrosine phosphorylation (Supplementary Fig. S4A), FGFR2 was the dominant tyrosine-phosphorylated receptor detected in SNU-16 xenografts treated with a control mIgG (Fig. 5C). A phospho-RTK array confirmed specific inhibition of FGFR2 tyrosine phosphorylation in SNU-16 xenografts by GP369 (Fig. 5C). The predominant high-level tyrosine phosphorylation of FGFR2 in SNU-16 cells *in vivo* might account for the dependence of SNU-16 xenografts on activated FGFR2 signaling as well as their exquisite sensitivity to GP369 treatment.

We next tested the effect of GP369 on the *in vivo* growth of the breast cancer cell line MFM-223. Tumor stasis was achieved in MFM-223 xenografts treated with 20 mg/kg GP369 twice weekly throughout the study ($P = 0.0015$; Fig. 6A). Similar to what we observed in the SNU-16 xenograft study, GP369 drastically decreased the tyrosine phosphorylation of FGFR2, concomitant with a reduction in the total FGFR2 protein in the tumors (Fig. 6B).

The high-affinity binding of GP369 to murine FGFR2-IIIb also allowed us to examine the potential toxicity of targeting FGFR2-IIIb in mice. All treatments were well tolerated without visible signs of toxicity in both xenograft models.

Discussion

The success of mAbs such as trastuzumab and cetuximab as cancer therapeutics has generated great interest in the development of additional antibodies that bind to new RTK targets or novel epitopes of known targets (38). Dysregulated FGFR signaling has been associated with the development of many human malignancies. Therefore, antibodies targeting individual FGFRs could have broad clinical applications.

The frequent dysregulation of FGFR signaling in human cancers suggests that targeting and inhibiting individual FGF receptors may have therapeutic benefit. Both immunization

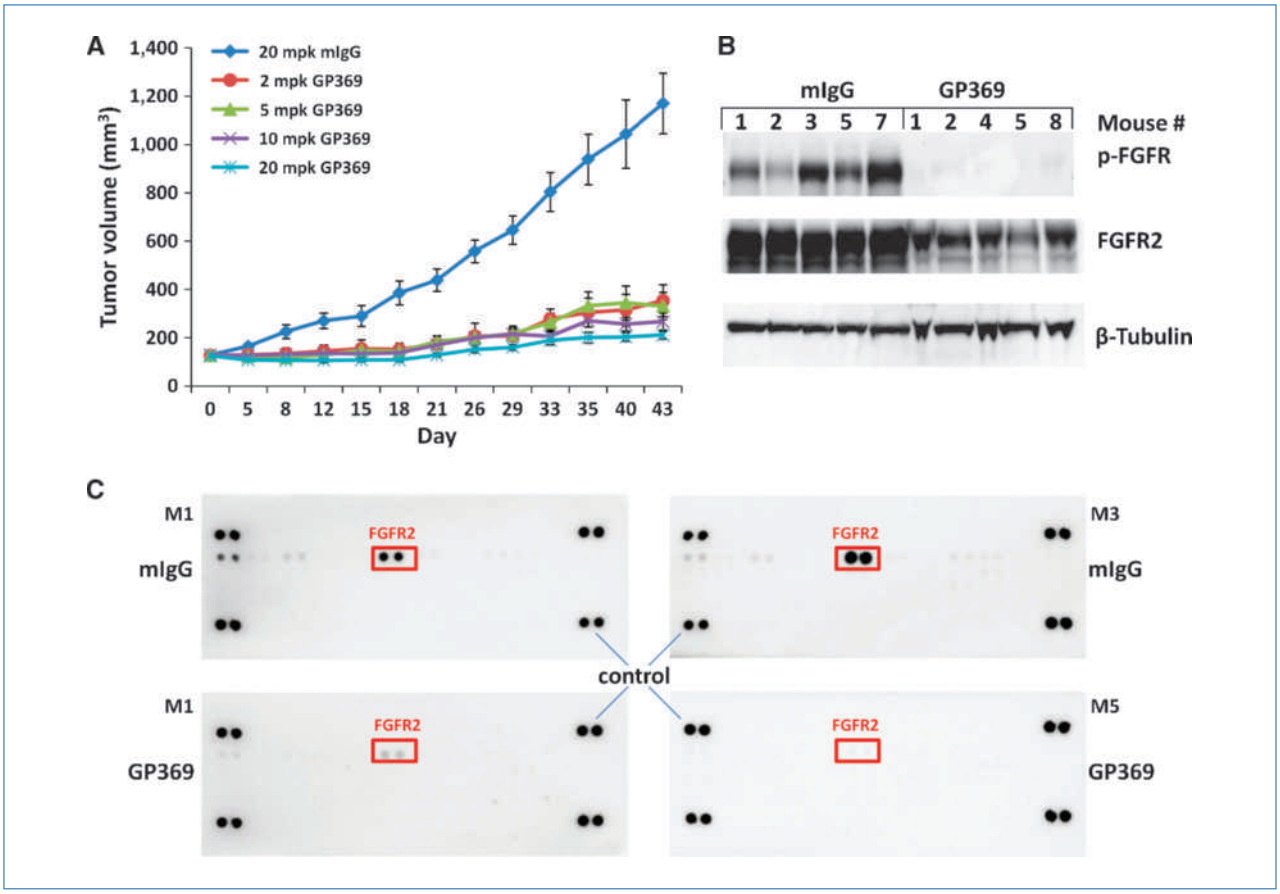

Figure 5. Effect of GP369 on SNU-16 xenografts *in vivo*. A, GP369 inhibits the *in vivo* growth of SNU-16. SCID mice inoculated with SNU-16 cells received mIgG control at 20 mg/kg or GP369 at 2, 5, 10 or 20 mg/kg by i.p. injection twice weekly. B, analysis of total and phospho-FGFR2 in SNU-16 tumors treated with either mIgG or GP369. β-Tubulin was used as a loading control. C, Phospho-RTK status of individual tumors collected at the end of study from mice treated with mIgG (top images from mouse nos. 1 and 3 in the mIgG-treated group) or GP369 (bottom images from mouse nos. 1 and 5 in the GP369-treated group). Each RTK array was probed with 250 μg of protein lysate from an individual tumor.

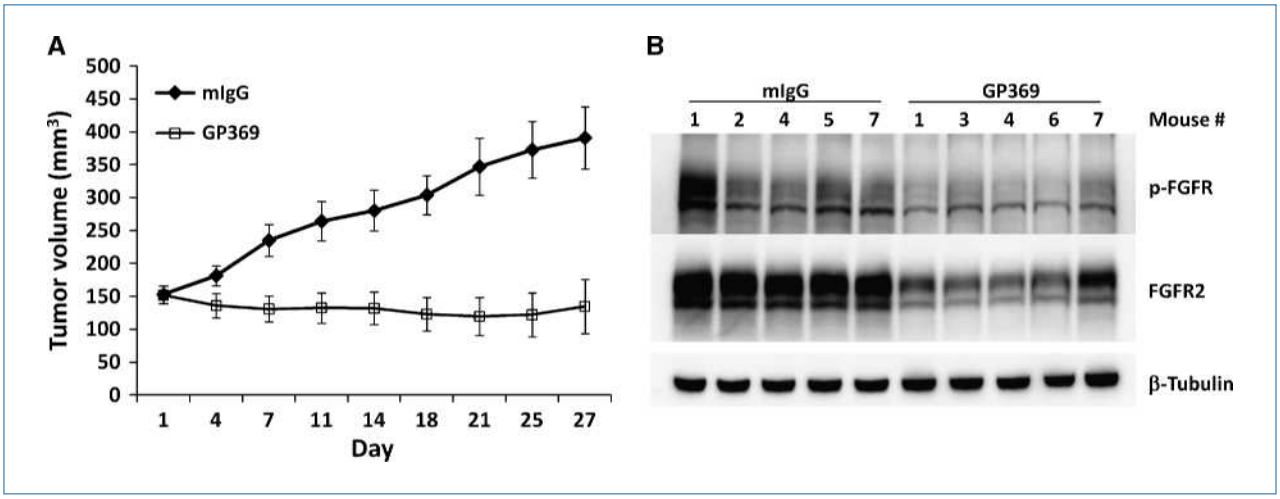

Figure 6. Effect of GP369 on MFM-223 xenografts *in vivo*. A, treatment of MFM-223 xenografts with GP369 results in tumor stasis. Nude mice bearing MFM-223 cells received either mIgG or GP369 at 20 mg/kg by i.p. injection twice weekly. B, analysis of total and phospho-FGFR2 in MFM-223 tumors treated with either the control IgG or GP369. β-Tubulin was used as a loading control.

and phage display–based approaches have been used to identify functional blocking antibodies against FGFRs. Using phage display, Sun and colleagues identified antibodies targeting FGFR1, as well as the IIIb and IIIc isoforms of the receptor. Unexpectedly, administration of pan-FGFR1- or FGFR1-IIIc–specific antibodies in mice resulted in significant weight loss (39). Therefore, the efficacy of these antibodies against human cancer xenografts cannot be adequately assessed in mice. We and others have successfully identified FGFR3 antagonistic antibodies using either immunization and/or phage display methods (40–42). FGFR3 mAbs have been shown to exhibit potent antitumor activities in preclinical models of bladder carcinoma and multiple myeloma with t (4;14) translocation (40–42), highlighting the therapeutic potential of targeting aberrant FGFR3 signaling in cancers. Although antibodies targeting FGFR1 and FGFR3 have been successfully identified, based on our experience, it is challenging to generate functional blocking antibodies against FGFR2 and FGFR4. This is likely due to the high sequence homology between the mouse and human protein and/or the lack of immunogenicity of the ligand-binding domains of these two receptors. In particular, the ligand-binding domains of the mouse and human FGFR2 share approximately 98% sequence identity. To our knowledge, FGFR2- or FGFR4-targeting antibodies that exhibit antitumor activity *in vivo* have not been previously reported in the literature.

To address the role of FGFR2 in tumorigenesis and to explore the therapeutic potential of FGFR2-targeting mAbs, we generated antibodies that specifically inhibit the function of FGFR2 after several attempts of immunizations. We identified *FGFR2*-amplified cell lines that represent gastric and breast cancers, the tumor types known to harbor *FGFR2* amplification. We also identified a number of breast cancer cell lines overexpressing FGFR2 in the absence of receptor amplification. Because these cell lines predominantly express the IIIb isoform of FGFR2, we tested the effect of an FGFR2-IIIb–specific antibody, GP369, on these cells *in vitro* and *in vivo*. GP369 potently blocked FGFR2 signaling and proliferation driven by FGFR2 overexpression. In addition, GP369 consistently inhibited the *in vivo* growth of *FGFR2*-amplified SNU-16 and MFM-223 xenografts.

FGFR2 amplification was previously detected in approximately 3% to 10% of gastric cancers and is associated with a poorly differentiated subtype with poor prognosis (18–21). A recent report showed that up to 25% of gastric cancer patients had *FGFR2* copy number greater than 3 (43). Previously, a multikinase small-molecule inhibitor, AZD2171, was reported to have antitumor activity against gastric cancer xenografts overexpressing FGFR2 (44). However, due to the broad spectrum of targets modulated by AZD2171, the *in vivo* growth inhibitory activity of this molecule could also be attributed to the suppression of other kinases, in addition to FGFR2. The sensitivity of *FGFR2*-amplified SNU-16 xenografts to the FGFR2-IIIb– specific mAb reported here supports an essential role of FGFR2 in tumorigenesis and thus provides a rationale for targeting FGFR2 in gastric cancer patients with *FGFR2* amplification.

Gastric cancer patients with FGFR2-IIIb and FGF7 double-positive tumors had much worse prognosis in comparison with those with single-positive or double-negative tumors (24), suggesting the possible involvement of an FGFR2-IIIb/FGF7 autocrine activation loop in a subset of advanced gastric cancer tumors. The therapeutic potential of targeting the activated FGFR2 signaling in these tumors should be further explored. Gastric cancer is the second most lethal cancer, with 900,000 new cases each year. In a substantial portion of these tumors, the FGFR2 pathway is dysregulated, representing a significant clinical opportunity for FGFR2 inhibitory antibodies.

The *in vivo* activity of GP369 against two *FGFR2*-amplified cancer cell lines with different tissue origins suggests that

FGFR2 amplification may be a common key oncogenic event in a subset of human tumors. Therefore, antibody-based FGFR2 targeting in the subtypes of human cancers harboring *FGFR2* amplification could have clinical benefits. This hypothesis should be tested in additional human cancer xenografts as well as primary tumors with *FGFR2* amplification. Recently, Turner and colleagues reported that *FGFR2* amplification was found exclusively in 4% of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 triple-negative breast cancers (17). They also classified MFM-223 as an *FGFR2*-amplified triple-negative cell line. However, low level of estrogen receptor expression was detected in MFM-223 (45), and more importantly, we found that the *in vivo* growth of MFM-223 xenografts was dependent on 17β-estradiol (data not shown). Therefore, *FGFR2* amplification in breast cancers may not be exclusively present in the triple-negative subtype. It is also worth noting that MFM-223 cells contain a mutation in PIK3CA (Sanger Cancer Genome Project) that leads to activation of the PI3K/Akt pathway, as confirmed by constitutive phosphorylation of Akt (data not shown). Nevertheless, MFM-223 xenografts were still sensitive to GP369, suggesting that constitutive activation of the PI3K/Akt pathway is not sufficient to abrogate the therapeutic response to antibodies targeting FGFR2, which lies upstream of the pathway.

In addition, using a genetically engineered mouse model, we showed that overexpression of FGFR2-IIIb is sufficient to drive tumorigenesis (46), and GP369 can effectively block the growth of these engineered tumors driven by FGFR2 overexpression (data not shown). Therefore, we believe that FGFR2-targeting antibodies have therapeutic potential in cancers with FGFR2 dysregulation in the absence of receptor amplification.

FGFR2 mutations have been found in a variety of tumors. In particular, approximately 12% of endometrial cancers contain mutations in *FGFR2*. Because GP369 can inhibit two of the most common FGFR2 mutations found in these tumors, we will use GP369 to determine whether the *in vivo* tumorigenesis of these cancer cells is dependent on these *FGFR2* activating mutations.

In this article, using an antibody specific for the IIIb isoform of FGFR2, we showed that FGFR2 signaling is one of the important pathways involved in the pathogenesis of human cancers. Our findings provide a rationale for clinically testing the therapeutic potential of FGFR2 antagonistic antibodies in human cancers with activated/amplified FGFR2 signaling.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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