

**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 have been found in a variety of human tumors. We generated monoclonal antibodies (mAbs) 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 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.

## Introduction:

Fibroblast growth factor (FGF) signaling regulates many fundamental biological processes including embryogenesis, tissue homeostasis, metabolism, angiogenesis, and wound healing (1-3). The pleiotropic effects of FGFs are mediated through four highly conserved receptor tyrosine kinases (RTKs), namely FGF receptor 1-4 (FGFR1-4) (3). The extracellular 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 FGFR1-3 (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 cascades by forming a ternary complex with their cognate receptors and heparan sulfate proteoglycans (1, 2). This results in trans-autophosphorylation of FGFRs at specific intracellular tyrosine residues and the activation of downstream signaling. One key molecule involved in FGF signaling is FGFR substrate 2  $\alpha$  (FRS2 $\alpha$ ), which upon phosphorylation by activated FGFRs can recruit other protein complexes to stimulate the Ras/mitogen-activated protein kinase (MAPK) and/or phosphoinositide 3-kinases (PI3K)/Akt signaling networks (1-3). The cellular response to the activated FGF signaling is context dependent and tissue specific, ranging from proliferation and migration to growth arrest, apoptosis, and differentiation (3).

Dysregulated autocrine and paracrine activation of FGFRs have 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 (SNPs) of *FGFR2* are associated with an increased risk of breast cancer development, presumably due to elevated FGFR2 expression (6-8). Missense, potentially activating, mutations of *FGFR2* have been reported in multiple cancer types, including endometrial, ovarian, breast, lung, and gastric cancer (3, 9-14). Furthermore, the *FGFR2* gene is amplified in a subset of gastric and breast cancers (3, 15-21). The co-expression of FGFR2-IIIb and its ligand, FGF7, in pancreatic and gastric cancers, as well as 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 physiological role of FGFR2 in regulating tissue homeostasis (30, 31). These contrasting and context dependent roles of FGFR2 signaling underscore the complexity of the FGFR2 signaling.

To address the role of FGFR2 in tumorigenesis and to explore FGFR2 as a potential therapeutic target, we generated mAbs against the extracellular ligand binding domain of FGFR2. Here we describe the *in vitro* and *in vivo* biological activity 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 proliferation driven by FGFR2 overexpression. Administration of GP369 in mice inhibited the *in*

*in vivo* growth of human cancer xenografts harboring *FGFR2* amplification. These findings support that dysregulated *FGFR2* signaling is one of 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 American Type Culture Collection. FDCP-1, Ba/F3, 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/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 C terminus of the third Ig domain of human *FGFR2*-IIIb were synthesized and biotinylated at their N-termini. *FGFR2* Peptides (1µM) and a negative control peptide, E2 (1µM) were captured on streptavidin Octet biosensors. Sensograms were recorded when peptide loaded biosensors were incubated with GP369 (1µM) in Octet sample diluent.

**Proliferation assay.** For SUM52PE proliferation assay, the cells were seeded in 96-well plates at 5000 cells/well in the growth medium (Ham's F-12, 5% FBS, 5  $\mu$ g/ml insulin, and 1  $\mu$ g/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 mouse IgG 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 10000 cells/well in the complete growth medium (DMEM with 10% FBS) and cultured overnight to allow for adherence. Cells were then incubated in serum free media for 24 hours followed by treatment of 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 SCID mice (Taconic) were inoculated subcutaneously into the flank with  $5 \times 10^6$  cells in 1:1 RPMI 1640 (Invitrogen)/Matrigel (BD Biosciences). Tumor measurements were taken twice weekly using vernier calipers. Tumor volume was calculated using the formula:  $V = 0.5 \times \text{width} \times \text{width} \times \text{length}$ . When tumors approached  $200 \text{ mm}^3$ , mice were randomized into groups of 10 and received GP369 (2-20 mg/kg) or a control murine IgG1 (mIgG, Bio-X-Cell) at 20 mg/kg twice weekly by intra-peritoneal injection. For the MFM-223 *in vivo* studies, 5 week old female NCr nude mice (Taconic) were implanted subcutaneously on the left flank with 0.72 mg 90-day release 17- $\beta$  estradiol pellets (Innovative Research) and inoculated subcutaneously into the right flank with  $10^7$  MFM-223 cells in 1:1 EMEM (ATCC)/Matrigel. When tumors approached  $200 \text{ mm}^3$ , mice were randomized into groups of 10 and treated with 20 mg/kg of mIgG or GP369 by

intra-peritoneal injection twice weekly. All statistical analysis was done using a one-way analysis of variance and Tukey 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's 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 (Figure 1A and Supplementary Table 1). 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 suggests that *FGFR2* amplification in these cells is focal (data not shown). We also surveyed FGFR copy number in a dozen of human endometrial and lung cancer cell lines, some of which also exhibited increased copy number in various FGFR genes (Supplementary Table 1).

The expression levels of FGFRs in the cell lines with *FGFR2* amplification were analyzed by qRT-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 analysis (data not shown) and flow cytometry (Fig 1C).

***Identification and characterization of FGFR2 antagonistic antibodies.*** In order 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 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 demonstrated 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, 3, 7, 10 and 22 transduce signals through FGFR2-IIIb, whereas FGFR2-IIIc responds to a broader panel of ligands including FGF1, 2, 4, 6, 9, 16, 17, 18 and 20 (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 1).

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, that exhibited marked inhibition of proliferation driven by the IIIb, 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 towards murine, *cynomolgus*, and

human FGFR2-IIIb Fc fusion proteins. No binding to human FGFR2-IIIc, or FGFR1-IIIb or FGFR3-IIIb was detected (data not shown). These results demonstrate 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 binding of GP369 to ten overlapping peptides covering the C-terminal half of the third Ig domain of human FGFR2-IIIb as this is the only region that differs between the IIIb and IIIc form. 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). 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 FGFR-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<sub>50</sub> of approximately 1.4nM) but not by FGFR2-IIIc (Fig. 3A). Breast and gastric cancer cell lines, such as SUM52PE and KATO III, express a C-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).

FGFR2 mutations 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 Ig domains 2 and 3, 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 nM and 4.5 nM, 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. While both cell lines express FGFR2, 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 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 form 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, KATO-III. In the presence of a control mouse IgG, both HCC95 and KATO-III cells formed large colonies in soft agar while the addition of GP369 significantly inhibited the size as well as the number of colonies (supplementary Fig 3).

***GP369 suppresses FGFR2 phosphorylation and downstream signaling.*** We have also investigated the impact of GP369 on the signaling cascades activated by FGFR2. FGF7 induced tyrosine phosphorylation of FGFR2 and led to the subsequent activation of the 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 4B). 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 two hours post incubation with the GP369 antibody, and there was a greater than 60% reduction in the FGFR2 protein level after six hours.

***GP369 inhibits 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, so 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 demonstrated potent tumor growth inhibition when compared to 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. 4A), 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 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 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-based approach and phage display technology have been employed to identify functional blocking antibodies against FGFRs. Utilizing phage display, Sun *et al.* identified antibodies targeting FGFR1, as well as the IIIb and the IIIc isoform of the receptor. Unexpectedly, administration of 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. While 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 domain of the mouse and human FGFR2 shares approximately 98% sequence identity. To our knowledge, FGFR2 or FGFR4 targeting antibodies which exhibit antitumor activity *in vivo* have not been previously reported in 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. Since these cell lines predominantly express the IIIb isoform of FGFR2, we tested the effect of 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 three (43). Previously, a multi-kinase small molecule inhibitor, AZD2171, has exhibited antitumor activity against gastric cancer xenografts overexpressing *FGFR2* (44). However, due to the broad spectrum of targets modulated by AZD2171, *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 to 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 *et al.* reported that *FGFR2* amplification was found exclusively in 4% of estrogen receptor (ER), progesterone receptor (PR), and HER2 triple negative breast cancers (17). They also classified MFM-223 as an *FGFR2*-amplified triple negative cell line. However, low level of ER expression was detected in MFM-223 (45), and more importantly, we found that *in vivo* growth of MFM-223 xenografts was dependent on 17- $\beta$  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 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 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 demonstrated 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 utilize GP369 to determine whether the *in vivo* tumorigenesis of these cancer cells is dependent on these *FGFR2* activating mutations.

In this paper, using an antibody specific for the IIIb isoform of *FGFR2*, we demonstrated that *FGFR2* signaling is one of the key 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.

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### **References:**

1. Beenken A, Mohammadi M. The FGF family: biology, pathophysiology and therapy. *Nat Rev Drug Discov* 2009; 8: 235-53.
2. Eswarakumar VP, Lax I, Schlessinger J. Cellular signaling by fibroblast growth factor receptors. *Cytokine Growth Factor Rev* 2005; 16: 139-49.

3. Turner N, Grose R. Fibroblast growth factor signalling: from development to cancer. *Nat Rev Cancer* 2010; 10: 116-29.
4. Ornitz DM, Xu J, Colvin JS, et al. Receptor specificity of the fibroblast growth factor family. *J Biol Chem* 1996; 271: 15292-7.
5. Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M, Ornitz DM. Receptor specificity of the fibroblast growth factor family. The complete mammalian FGF family. *J Biol Chem* 2006; 281: 15694-700.
6. Easton DF, Pooley KA, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature* 2007; 447: 1087-93.
7. Hunter DJ, Kraft P, Jacobs KB, et al. A genome-wide association study identifies alleles in *FGFR2* associated with risk of sporadic postmenopausal breast cancer. *Nat Genet* 2007; 39: 870-4.
8. Meyer KB, Maia AT, O'Reilly M, et al. Allele-specific up-regulation of *FGFR2* increases susceptibility to breast cancer. *PLoS Biol* 2008; 6: e108.
9. Davies H, Hunter C, Smith R, et al. Somatic mutations of the protein kinase gene family in human lung cancer. *Cancer Res* 2005; 65: 7591-5.
10. Ding L, Getz G, Wheeler DA, et al. Somatic mutations affect key pathways in lung adenocarcinoma. *Nature* 2008; 455: 1069-75.
11. Dutt A, Salvesen HB, Chen TH, et al. Drug-sensitive *FGFR2* mutations in endometrial carcinoma. *Proc Natl Acad Sci U S A* 2008; 105: 8713-7.
12. Greenman C, Stephens P, Smith R, et al. Patterns of somatic mutation in human cancer genomes. *Nature* 2007; 446: 153-8.

13. Pollock PM, Gartside MG, Dejeza LC, et al. Frequent activating FGFR2 mutations in endometrial carcinomas parallel germline mutations associated with craniosynostosis and skeletal dysplasia syndromes. *Oncogene* 2007; 26: 7158-62.
14. Jang JH, Shin KH, Park JG. Mutations in fibroblast growth factor receptor 2 and fibroblast growth factor receptor 3 genes associated with human gastric and colorectal cancers. *Cancer Res* 2001; 61: 3541-3.
15. Heiskanen M, Kononen J, Barlund M, et al. CGH, cDNA and tissue microarray analyses implicate FGFR2 amplification in a small subset of breast tumors. *Anal Cell Pathol* 2001; 22: 229-34.
16. Adnane J, Gaudray P, Dionne CA, et al. BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. *Oncogene* 1991; 6: 659-63.
17. Turner N, Lambros MB, Horlings HM, et al. Integrative molecular profiling of triple negative breast cancers identifies amplicon drivers and potential therapeutic targets. *Oncogene* 2010; 29: 2013-23.
18. Hara T, Ooi A, Kobayashi M, Mai M, Yanagihara K, Nakanishi I. Amplification of c-myc, K-sam, and c-met in gastric cancers: detection by fluorescence in situ hybridization. *Lab Invest* 1998; 78: 1143-53.
19. Mor O, Ranzani GN, Ravia Y, et al. DNA amplification in human gastric carcinomas. *Cancer Genet Cytogenet* 1993; 65: 111-4.
20. Tsujimoto H, Sugihara H, Hagiwara A, Hattori T. Amplification of growth factor receptor genes and DNA ploidy pattern in the progression of gastric cancer. *Virchows Arch* 1997; 431: 383-9.

21. Yoshida T, Sakamoto H, Terada M. Amplified genes in cancer in upper digestive tract. *Semin Cancer Biol* 1993; 4: 33-40.
22. Yamayoshi T, Nagayasu T, Matsumoto K, Abo T, Hishikawa Y, Koji T. Expression of keratinocyte growth factor/fibroblast growth factor-7 and its receptor in human lung cancer: correlation with tumour proliferative activity and patient prognosis. *J Pathol* 2004; 204: 110-8.
23. Cho K, Ishiwata T, Uchida E, et al. Enhanced expression of keratinocyte growth factor and its receptor correlates with venous invasion in pancreatic cancer. *Am J Pathol* 2007; 170: 1964-74.
24. Toyokawa T, Yashiro M, Hirakawa K. Co-expression of keratinocyte growth factor and K-sam is an independent prognostic factor in gastric carcinoma. *Oncol Rep* 2009; 21: 875-80.
25. Gartside MG, Chen H, Ibrahim OA, et al. Loss-of-function fibroblast growth factor receptor-2 mutations in melanoma. *Mol Cancer Res* 2009; 7: 41-54.
26. Diez de Medina SG, Chopin D, El Marjou A, et al. Decreased expression of keratinocyte growth factor receptor in a subset of human transitional cell bladder carcinomas. *Oncogene* 1997; 14: 323-30.
27. Giri D, Ropiquet F, Ittmann M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. *Clin Cancer Res* 1999; 5: 1063-71.
28. Ricol D, Cappellen D, El Marjou A, et al. Tumour suppressive properties of fibroblast growth factor receptor 2-IIIb in human bladder cancer. *Oncogene* 1999; 18: 7234-43.
29. Zhang Y, Wang H, Toratani S, et al. Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis. *Proc Natl Acad Sci U S A* 2001; 98: 11336-40.

30. Grose R, Fantl V, Werner S, et al. The role of fibroblast growth factor receptor 2b in skin homeostasis and cancer development. *EMBO J* 2007; 26: 1268-78.
31. Lin Y, Liu G, Zhang Y, et al. Fibroblast growth factor receptor 2 tyrosine kinase is required for prostatic morphogenesis and the acquisition of strict androgen dependency for adult tissue homeostasis. *Development* 2007; 134: 723-34.
32. Katoh M, Hattori Y, Sasaki H, et al. K-sam gene encodes secreted as well as transmembrane receptor tyrosine kinase. *Proc Natl Acad Sci U S A* 1992; 89: 2960-4.
33. Kunii K, Davis L, Gorenstein J, et al. FGFR2-amplified gastric cancer cell lines require FGFR2 and Erbb3 signaling for growth and survival. *Cancer Res* 2008; 68: 2340-8.
34. Tannheimer SL, Rehemtulla A, Ethier SP. Characterization of fibroblast growth factor receptor 2 overexpression in the human breast cancer cell line SUM-52PE. *Breast Cancer Res* 2000; 2: 311-20.
35. Yeh BK, Igarashi M, Eliseenkova AV, et al. Structural basis by which alternative splicing confers specificity in fibroblast growth factor receptors. *Proc Natl Acad Sci U S A* 2003; 100: 2266-71.
36. Itoh H, Hattori Y, Sakamoto H, et al. Preferential alternative splicing in cancer generates a K-sam messenger RNA with higher transforming activity. *Cancer Res* 1994; 54: 3237-41.
37. Moffa AB, Tannheimer SL, Ethier SP. Transforming potential of alternatively spliced variants of fibroblast growth factor receptor 2 in human mammary epithelial cells. *Mol Cancer Res* 2004; 2: 643-52.
38. Adams GP, Weiner LM. Monoclonal antibody therapy of cancer. *Nat Biotechnol* 2005; 23: 1147-57.

39. Sun HD, Malabunga M, Tonra JR, et al. Monoclonal antibody antagonists of hypothalamic FGFR1 cause potent but reversible hypophagia and weight loss in rodents and monkeys. *Am J Physiol Endocrinol Metab* 2007; 292: E964-76.
40. Qing J, Du X, Chen Y, et al. Antibody-based targeting of FGFR3 in bladder carcinoma and t(4;14)-positive multiple myeloma in mice. *J Clin Invest* 2009; 119: 1216-29.
41. Trudel S, Stewart AK, Rom E, et al. The inhibitory anti-FGFR3 antibody, PRO-001, is cytotoxic to t(4;14) multiple myeloma cells. *Blood* 2006; 107: 4039-46.
42. Bai A, Winston WM, Meetze K, et al. Mechanistic Studies of AV370, a Potent FGFR3 Antagonistic Antibody. American Association for Cancer Research 100th Annual Meeting 2009; Abstract #3775.
43. Park JO, Lee J, Jang H, et al. Clinical significance of FGFR2 amplification in gastric cancer American Association for Cancer Research 101st Annual Meeting (Poster #660) 2010.
44. Takeda M, Arao T, Yokote H, et al. AZD2171 shows potent antitumor activity against gastric cancer over-expressing fibroblast growth factor receptor 2/keratinocyte growth factor receptor. *Clin Cancer Res* 2007; 13: 3051-7.
45. Hackenberg R, Lutichens S, Hofmann J, Kunzmann R, Holzel F, Schulz KD. Androgen sensitivity of the new human breast cancer cell line MFM-223. *Cancer Res* 1991; 51: 5722-7.
46. Kabbarah O, Lerner L, Liu Q, et al. Fibroblast Growth Factor Receptor-2 Cancer Models for Biomarker Discovery and Therapeutic Response Prediction. American Association for Cancer Research 100th Annual Meeting 2009; Abstract #1348.

## Figure Legends:

**Figure 1.** Characterization of human cancer cell lines with *FGFR2* amplification. A. *FGFR* copy number of human cancer cell lines. qPCR was carried out using primers specific for *FGFRs* or a reference gene, *transketolase (TKT)*, and copy number was calculated. B. *FGFR* expression analysis. qRT-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  $\mu$ l of primary *FGFR2* antibodies (5  $\mu$ g/ml) for 1 hour followed by incubation with 1:100 phycoerythrin (PE)-conjugated secondary antibodies (Jackson Immune Research) for 30 minutes. 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 and 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 N-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 structure of *FGFR2* IIIb (light blue, D2 on top, D3 at bottom) in complex with FGF10 (red) (PDB structure 1NUN (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 don’t bind are denoted with “-”.

**Figure 3.** Characterization of FGFR2 antibodies by FDCP-1 proliferation assay. A. GP369 suppresses cell proliferation driven by the WT or the C-terminally truncated variant of FGFR2-IIIb, or mutant variants of FGFR2-IIIb. GP369 mAb was added to FDCP-1 cells expressing FGFR2-IIIb, -IIIc, a C-terminally truncated IIIb variant, or the S252W and N550K mutant variants in the presence of FGF1 (8ng/ml) and heparin (5ug/ml). MTT assays were carried out after 2 days. B. Inhibition of proliferation of SUM52PE cells by GP369. The cells were incubated in reduced serum medium (0.5% FBS) and were either left untreated (UT) or treated with mouse IgG (mIgG) or GP369 at various concentrations for 5 days. Cell proliferation was assessed by MTT assays. C. Inhibition of 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 mouse IgG (mIgG) or GP369 at 30 ug/mL for 3 days 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. Following 3 hours of serum starvation, the cells were treated with PBS (mock) or 5 ug/ml of either mouse IgG or GP369 for 1 hour at 37°C prior to stimulation by FGF7 (20 ng/ml) and heparin (5ug/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

ug/ml of either mIgG or GP369 for 1 hour at 37°C prior to treatment with either heparin (20 ug/ml) or heparin plus FGF7 (30 ng/ml) for 15 minutes. Western blotting analysis was then carried out. C. Downregulation of the FGFR2 protein levels by GP369. SNU-16 cells were treated with 10 ug/ml of either mIgG or GP369. The cells were harvested at various time points. Total levels of FGFR2 and  $\beta$ -Tubulin were determined by Western blotting and densitometric units quantified using Scion Image software.

**Figure 5.** Effect of GP369 on SNU-16 xenografts *in vivo*. A. GP369 inhibits *in vivo* growth of SNU16. 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 intra-peritoneal injection twice weekly. B. Analysis of total and phospho-FGFR2 in SNU-16 tumors treated with either the control IgG or GP369.  $\beta$ -tubulin was used as a loading control. C. Phospho-RTK status of individual tumors collected at the end of study from mice administered with mIgG (the top two panels from mouse No. 1 and 3 in the mIgG-treated group) or GP369 (the bottom two panels from mouse No. 1 and 5 in the GP369-treated group). Each RTK array was probed with 250 ug 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 intra-peritoneal injection twice weekly. B. Analysis of total and phospho-FGFR2 in MFM-223 tumors treated with either the control IgG or GP369.  $\beta$ -tubulin was used as a loading control.

**Figure 1**

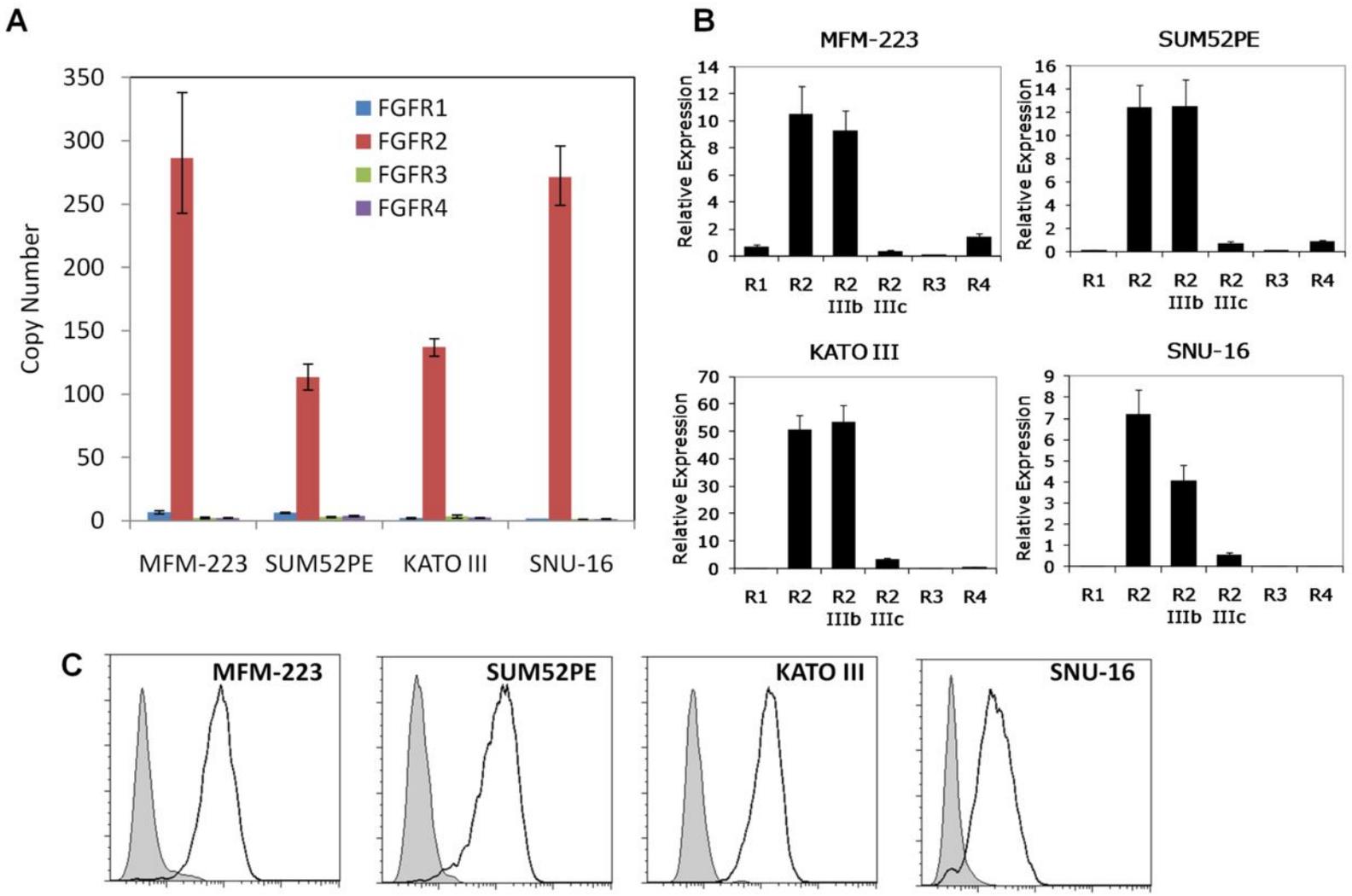
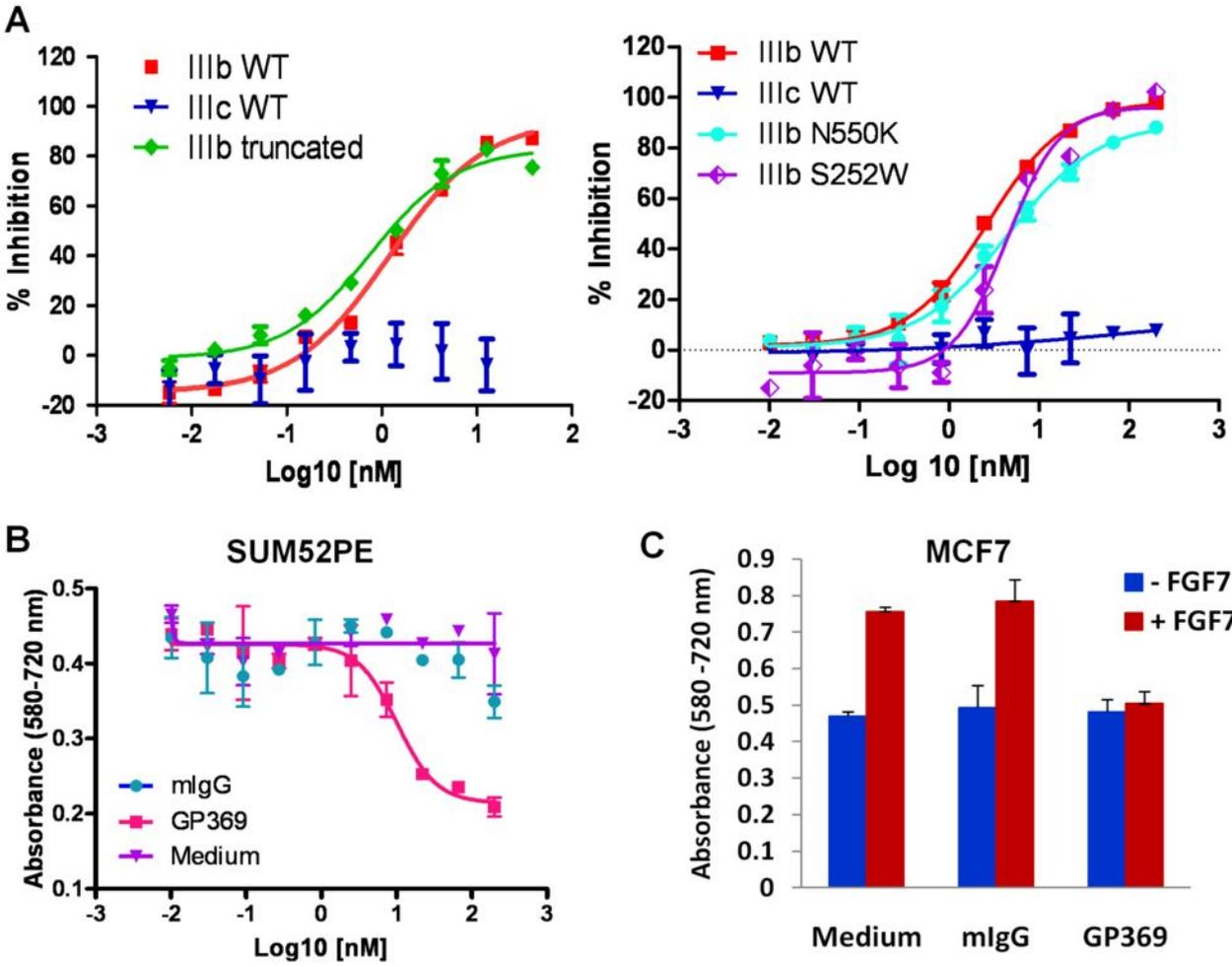
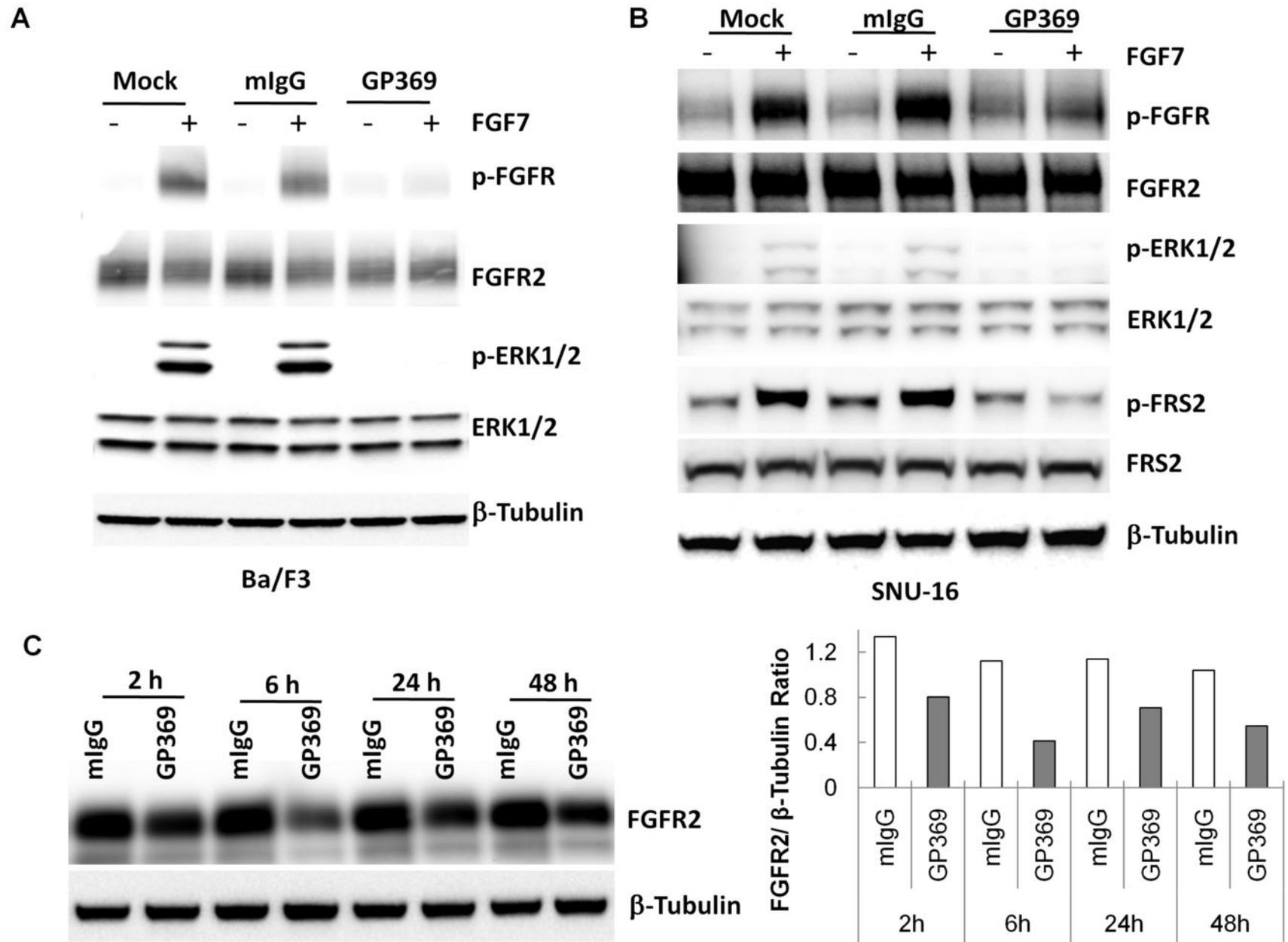




Figure 3



**Figure 4**



**Figure 5**

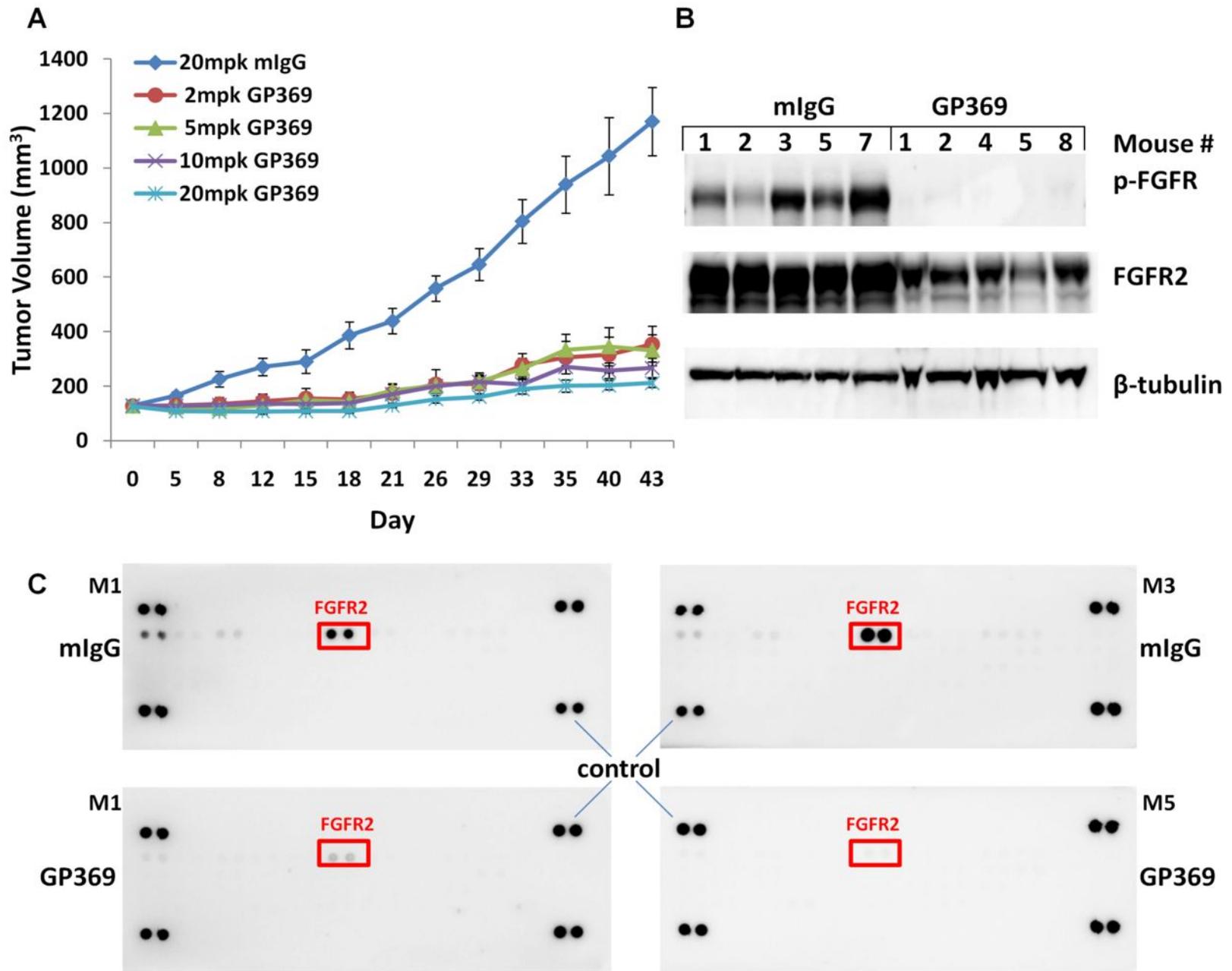
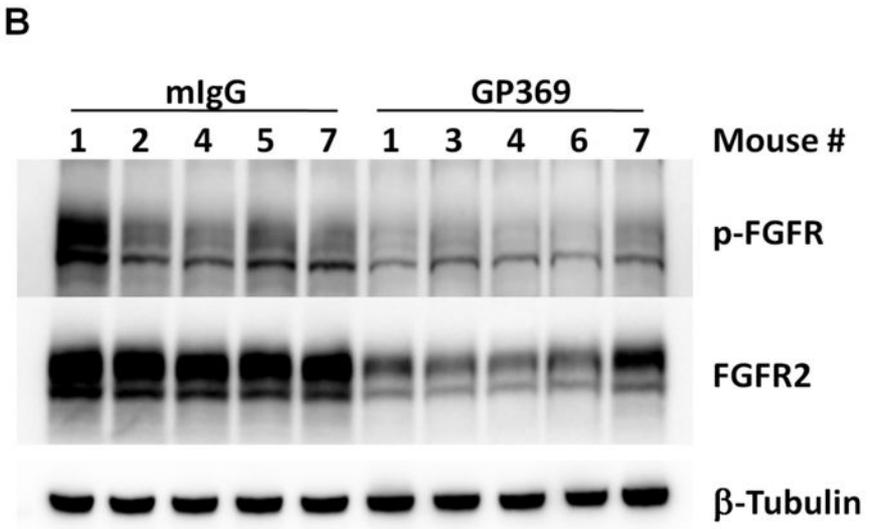
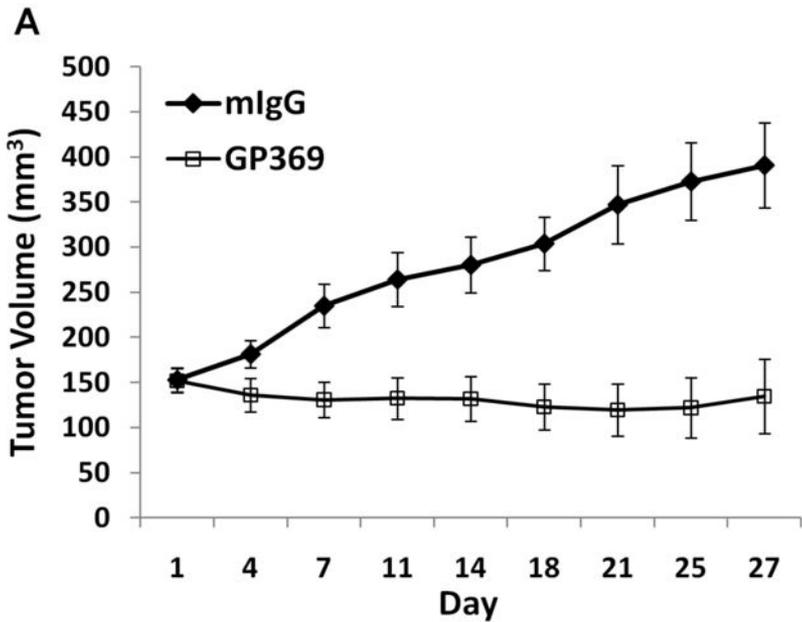


Figure 6



# Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

## GP369, an FGFR2-IIIb specific antibody, exhibits potent antitumor activity against human cancers driven by activated FGFR2 signaling

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