Redesigning a Monospecific Anti-FGFR3 Antibody to Add Selectivity for FGFR2 and Expand Anti-tumor Activity

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Running Title: Engineering of Dual-Targeting Anti-FGFR2/3 Antibodies
Abstract

Fibroblast growth factor receptors (FGFRs) are attractive candidate targets for cancer therapy because they are dysregulated in several human malignancies. FGFR2 and FGFR3 can be inhibited potentially without disrupting adult-tissue homeostasis. By contrast, blocking the closely related FGFR1 and FGFR4, which regulate specific metabolic functions, carries a greater safety risk. An anti-FGFR3 antibody was redesigned here to create function-blocking antibodies that bind with dual specificity to FGFR3 and FGFR2 but spare FGFR1 and FGFR4. R3Mab, a previously developed monospecific anti-FGFR3 antibody, was modified via structure-guided phage display, and acquired additional binding to FGFR2. The initial variant was trispecific, binding tightly to FGFR3 and FGFR2 and moderately to FGFR4, while sparing FGFR1. The X-ray crystallographic structure indicated that the antibody variant was bound to a similar epitope on FGFR2 as R3Mab on FGFR3. The antibody was further engineered to decrease FGFR4 binding affinity while retaining affinity for FGFR3 and FGFR2. The resulting dual-specific antibodies blocked FGF binding to FGFR3 and FGFR2 and inhibited downstream signaling. Moreover, they displayed efficacy in mice against human tumor xenografts overexpressing FGFR3 or FGFR2. Thus, a monospecific antibody can be exquisitely tailored to confer or remove binding to closely related targets in order to expand and refine therapeutic potential.
Introduction

Fibroblast growth factors (FGFs) and their tyrosine kinase receptors (FGFRs) play key roles in regulating specific pathways during embryonic development, as well as homeostasis of diverse tissues, wound healing processes and certain metabolic functions in the adult animal. In humans there are 4 highly homologous FGFRs (FGFR1-4) and 22 FGFs (FGF1-14 and FGF16-23) (1-4). The FGFRs comprise an extracellular region with 3 immunoglobulin domains (D1, D2 and D3), a single-pass transmembrane region and a split cytoplasmic kinase moiety (1, 5). Alternative splicing gives rise to two major variants of FGFRs 1-3, termed isoforms IIIb and IIIc, which differ in the second half of D3 and consequently in ligand-binding specificity (6).

Dysregulated signaling by FGFRs 1-4 is associated with pathogenesis in several cancer types (2, 3). Genomic FGFR alterations, which include gene amplification, chromosomal translocation and activating mutations, can drive aberrant activation of the FGF pathway and promote neoplastic transformation of normal cells. FGFR2 gene amplification occurs in ~10% of gastric and ~4% of triple-negative breast cancers (7-9), while FGFR3 amplification is associated with specific subsets of bladder cancer (9, 10). Missense FGFR mutations are also found in multiple types of cancer (2, 11). Specifically, amino acid substitutions in the linker region between D2 and D3, e.g. S252W in FGFR2 and S249C in FGFR3, augment FGF-driven signaling and tumor-cell proliferation and represent hot spots for somatic mutation (12, 13). Activating mutations also occur in the tyrosine kinase region of FGFRs (14).

Targeting the FGF-FGFR pathway has been a major area of focus for cancer drug development. This effort has included small-molecule tyrosine kinase inhibitors (TKIs), blocking antibodies, as well as ligand traps (9). Current high-potency FGFR TKIs have limited selectivity for different FGFRs (9), which may impact their therapeutic window. For example, disruption of FGF23 signaling through hetero-complexes of FGFR1 and the co-receptor Klothoβ can lead to hyperphosphatemia and soft tissue calcification in patients...
Engineering of Dual-Targeting Anti-FGFR2/3 Antibodies

(15, 16), whereas blockade of FGF19 signaling through FGFR4 hetero-complexes with Klothoβ can disrupt bile acid metabolism (17). More selective antibodies have been developed to antagonize ligand signaling through individual FGFRs, including FGFR1 (18), FGFR2 (19) and FGFR3 (20). However, antibodies recognizing more than one FGFR have not yet been reported.

The previously described monospecific anti-FGFR3 antibody R3Mab effectively blocks binding of FGF1 and FGF9 to both the IIIb and IIIc isoforms of wild-type FGFR3, as well as to certain cancer-associated mutant forms of FGFR3 (20, 21). X-ray structural analysis revealed that R3Mab binds to a specific epitope on FGFR3 that is required for ligand binding. R3Mab displayed potent antitumor activity in mice against human bladder cancer and multiple myeloma tumor xenografts. In this study structure-guided phage display was used iteratively to engineer R3Mab into derivative antibodies that carry dual specificity for FGFR3 and FGFR2 while sparing FGFR1 and FGFR4. The aim of this study was to broaden the potential therapeutic scope beyond that of the parent molecule while avoiding added safety risks. The engineered antibodies displayed inhibition of FGF-stimulated tumor-cell growth in vitro and significant efficacy against human cancer xenografts overexpressing FGFR2 or FGFR3 in vivo.
Materials and Methods

**Generation of FGFR2-binding R3Mab variants by phage library selection**

Random mutations were incorporated into each of the CDR loops H1, H2, H3 or L2 (Table S1) using the method of Kunkel (22). Purified phage suspensions from each library were panned separately against immobilized FGFR2-IIIb proteins for the first round of panning. Eluted phage particles were then pooled together and propagated for subsequent rounds of panning. 96 randomly picked colonies were individually cultured and assayed by phage ELISA to screen for FGFR2 binders. Meanwhile, phagemid DNA was extracted and sequenced from the cultures.

**Library construction and selection for FGFR2 binders that do not bind FGFR4**

The phage display libraries were constructed based on the phagemid displaying the Fab fragment of antibody 2B.1.3. Selected positions in CDR H1, H3 or L2 loops were subject to random mutagenesis (Table S2). For selection of clones that have reduced FGFR4 binding while retaining FGFR2 specificity, in the first round, 1.5 OD of each phage library was mixed with 0.5 nmol/L FGFR4-Fc protein. The mixture was then incubated overnight at 4°C with plate-immobilized FGFR2-IIIb. The second round of panning was similar to the first round except that 1.5 OD of phage preparations were mixed with 10 nmol/L FGFR4-Fc. For the third and fourth rounds, 0.5 OD of phage preparations were mixed with 460 nmol/L FGFR4-Fc protein, and shaken at room temperature (RT) for 20 min before being incubated with coated FGFR2-IIIb. Randomly picked clones were cultured for phage ELISA assays and DNA sequencing as described above.

**Phage ELISA binding assay**
A 384-well MaxiSorp plate was coated E25 (control antibody), FGFR2-IIIb-His, FGFR2-IIIc-His or FGFR4-His in each quadrant. Phage supernatant was added into quadrant after blocking with BSA. Bound phage particles were detected with HRP-conjugated anti-M13 monoclonal antibody (GE Healthcare).

**Surface plasmon resonance assay**

The binding affinities of R3Mab variants for FGFR antigens were determined using a Biacore T100 (GE Healthcare). Anti-human Fc monoclonal antibody was immobilized onto a CM5 biosensor chip. FGFR antigens of various concentrations were injected over captured R3Mab-derived antibodies. Kinetic analyses were performed using the T100 evaluation software to obtain the kinetic and affinity constants.

**Protein expression, purification and structure determination**

The human FGFR2-IIIb ECD (residue 140-369) was expressed as inclusion bodies in *E. coli* BL21(DE3)pLysS cells. The inclusion bodies were washed and dissolved in 6 mol/L Guanidine-HCl, 20 mmol/L Tris pH8, 10 mmol/L TCEP for in vitro folding using the rapid dilution method. The refolding mixture was concentrated and purified through a Heparin affinity column (GE Healthcare), followed by ion exchange chromatography. The 2B.1.3 Fab was expressed and purified as described (20). The FGFR2 and Fab proteins were mixed together at a molar ratio of 1:1 and diluted to 2 mg/mL for crystallization. Crystals were grown at 20 % (w/v) PEG 3350, 0.1 mol/L sodium citrate pH 5.5, and 0.2 mol/L ammonium sulfate using the vapor diffusion method. Diffraction data was collected with a beam wavelength of 1 Å at the ALS and processed to 2.36 Å. Two complexes were found in an asymmetric unit cell. The final model was validated using the program MolProbity.
(23). $R_{\text{work}}$ and $R_{\text{free}}$ values are 19.8% and 24.4%, respectively. No Ramachandran outliers were detected.

**Cell lines**

SNU16 and MFM-223x2.2 cell lines were obtained from an internal cell bank. The cell line RT112 was obtained from ATCC. The cells were cultured in RPMI medium supplemented with 10% FBS. All cell lines are tested for mycoplasma, cross contamination and genetically fingerprinted when new stocks are generated to ensure quality and confirm ancestry.

**FGF Ligand-Blocking ELISA**

A 96-well MaxiSorp plate was coated anti-human Fc antibody (Jackson ImmunoResearch Lab). FGFR-Fc fusion proteins were incubated after blocking. The plate was washed before being added with the antibody and FGF ligand mixtures. Bound ligand was detected by subsequent incubations of biotinylated anti-FGF antibodies (R&D Biosystems, #BAF273, #BAF251, #BAF969), Streptavidin-HRP (Invitrogen) and the TMB substrate.

**Immunoblotting**

Tumor cells were seeded on tissue culture plates for 24 h, pre-treated with 10 μg/ml anti-FGFR antibodies or control anti-gD antibody, then stimulated with 25 ng/ml FGF-7 (R&D Systems) in the presence of 20 μg/ml heparin (Sigma) for 15 minutes. Total cell lysates were blotted with primary antibodies recognizing various signaling molecules, including: phospho-FGFR (Y653/654), FGFR2, phospho-FRS2 (Y196), FRS2 (Santa Cruz Biotechnology), phospho-ERK1/2 (T202/Y204), ERK1/2, phospho-AKT (S473), AKT,
Engineering of Dual-Targeting Anti-FGFR2/3 Antibodies

phospho-HER3 (Y1289), HER3, phospho-PLCγ1 (Y783), PLCγ1 (Cell Signaling) and β-actin (Sigma).

Xenograft experiments

All procedures were approved by and conformed to the guidelines and principles set by the Institutional Animal Care and Use Committee of Genentech and were carried out in an AAALAC-accredited facility. SNU-16 tumor fragments of about 15-30 mm³ were implanted subcutaneously (s.c.) into the right flanks of 6-8-week-old female Balb/c nude mice (Shanghai Laboratory Animal). 7 million RT-112 bladder carcinoma cells suspended in HBSS with matrigel were inoculated s.c. in the 6-8-week-old female C.B-17 SCID mice (Charles River Lab). When the mean tumor volume reached 100-200 mm³ (day 0), mice were randomized into groups of 6 and treated starting on day 1 with twice weekly intraperitoneal (i.p.) injections of 2B1.3.10, 2B1.3.12 or R3Mab (10, 30 or 50 mg/kg). Control groups were treated with a control human IgG1 antibody diluted in PBS (30 mg/kg). The tumor volumes were measured twice a week.

Statistics

Xenograft data are expressed as mean tumor volumes ± SEM. Unpaired, 2-tailed t test were performed to assess the statistical significance between the experimental and control groups. Time points with P values < 0.005 are considered as significant and marked with asterisks.

Complete details of Materials and Methods are provided in the Supporting Information.
Results

Broadening the binding specificity of an anti-FGFR3 antibody

The objective of this study was to develop an antibody with dual specificity for FGFR3 and FGFR2, but sparing the highly related receptors, FGFR1 and FGFR4, as a potential cancer therapeutic. The starting point for this study was the monospecific antibody R3Mab, which binds to the FGFR3 IIIb and IIIc isoforms with sub-nanomolar affinities (20). R3Mab shows robust inhibition of FGFR3 signaling and tumor growth in vivo (20) and has been studied in phase I clinical trials.

The antibody redesign strategy here was guided by the previously determined crystallographic structure of the R3Mab Fab fragment in complex with FGFR3-IIIb (PDB 3GRW) (20). This structure indicates that R3Mab interacts with both the D2 and D3 domains of FGFR3-IIIb. Although D2 was subsequently found here to be sufficient for R3Mab binding (see below), initial analyses were based on the contacts on this original structure. Most of the contact surface on the FGFR3-IIIb antigen was contributed by the antibody complementarity-determining regions (CDRs) H3 (46%), H1 (23%) and L2 (22%), with small contributions from CDR H2 and framework region (FR) residues (20) (Fig. S1A). The similarity between FGFR3-IIIb and the intended additional FGFR2-IIIb antigen were compared. Of the total 1421-Å² binding area of R3Mab on FGFR3-IIIb, 399 Å² (28%) accounts for the residue difference between FGFR2-IIIb and FGFR3-IIIb (Fig. S1B). In addition, the D2D3 regions of these two homologs share 68% of protein sequence identity, while their D2 domains share 76% identity (Table S3). Since D3 of the R3Mab-bound FGFR3-IIIb had a different geometry as compared to all other FGFR structures (20), the structures of FGFR2-IIIb and FGFR3-IIIb were superimposed on their D2 regions, which yielded a calculated root mean squared deviation (RMSD) of α-carbon atoms of 0.78 Å. This high degree of structural similarity suggested that it might be feasible to engineer R3Mab to bind and inhibit FGFR2 as well.
To construct a phage display library, mutations were designed that cover most residues in each of the individual heavy-chain CDRs and a selection of the contact residues on all CDRs (Table S1). R3Mab variants displayed as Fab fragments on phage particles were selected for binding to FGFR2-IIIb. Selection on FGFR3 was not performed at this stage to keep the selection stringency low when recruiting binding to FGFR2. After the first round of panning, the phage outputs from the individual libraries were combined and subjected to 3 further rounds of selection. 95 antibody clones, designated as the 2B.1 series, were screened by phage ELISA binding assay. Among these, 81 clones, representing 32 unique sequences, bound to FGFR2-IIIb. All binding clones were apparently derived from the H2 library, because they contained mutations in CDR H2 but not elsewhere (Table 1). The 32 unique antibodies were expressed and purified as corresponding IgG1 molecules. All of them showed substantially improved binding to FGFR2-IIIb relative to R3Mab, with $K_D$ values ranging from 0.3 to 17 nmol/L (Table 1). Remarkably, the mutated H2 sequences contain significant variation, lack clear consensus and differ from R3Mab at 4 or 5 positions (Table 1, Fig. S2). Thus, there appears to be multiple possible solutions to conferring high-affinity binding of FGFR2-IIIb onto R3Mab.

Next six variants were selected for measurements of binding to FGFR3 based on their affinities ($K_D < 3$ nM) for FGFR2 and sequence diversity. All the variants showed improved affinities for FGFR3-IIIb (Table S4). To further assess their ability to inhibit receptor-dependent cell growth, proliferation of MCF7 breast carcinoma cells was assayed either with or without FGF7—a specific ligand for FGFR2-IIIb (1, 19). Variant 2B.1.3 exhibited the greatest antagonist activity, as compared to other variants, which showed less or no inhibition, or even displayed stimulatory effect (Fig. 1). Hence, 2B.1.3 was carried over as a functional antibody for further characterization.

Since all FGFR homologs share nearly 70% sequence identity between each other (Table S3), the engineered variant 2B.1.3 was analyzed for binding to other FGFRs. Mab 2B.1.3 bound FGFR2-IIIc with similar affinity as FGFR2-IIIb (Table 2). Mab 2B.1.3 also...
showed several-fold higher affinity for FGFR3-IIIb and FGFR3–IIIc than did R3Mab, even though the selection strategy used was based on binding to FGFR2-IIIb. Moreover, Mab 2B.1.3 also bound to FGFR4, with a $K_D$ value of 32 nM, yet showed no detectable binding to FGFR1 (Table 2). Therefore, variant 2B.1.3 is trispecific, binding to FGFR2, FGFR3 and FGFR4, but not FGFR1.

**Structural determination of the complex between Mab 2B.1.3 and FGFR2**

To obtain direct insight into how the engineered variant 2B.1.3 acquired specificity for FGFR2, the crystal structure of its complex with FGFR2 was determined (Fig. 2, Table S5). FGFR2-IIIb D2D3 was first generated by expression in *E. coli* and refolding from inclusion bodies and judged to be intact by SDS-PAGE and mass spectrometry. However, in crystals this protein contained only the isoform-independent D2 domain, suggesting proteolysis between D2 and D3 during the crystallization process. The previously determined FGFR3-IIIb:R3Mab complex structure contains both the D2 and D3 domains of FGFR3-IIIb. The whole complex of FGFR2-D2:Mab 2B.1.3 superimposed closely onto the FGFR3-IIIb:R3Mab structure (Fig. S3), with an overall $\alpha$-carbon RMSD of 1.4 Å, indicating that the engineering retained the same binding geometry as the original antibody R3Mab. The FGFR3:R3Mab crystal structure suggests considerable interactions between FGFR3 D3 and the CDR H1 loop. Therefore, to investigate the involvement of D3 in binding, proteins of the D2 domains of FGFR2 and FGFR3 were prepared and their binding affinity to R3Mab and Mab 2B.1.3 measured. Only very minor differences in binding affinity between D2 alone and the D2D3 domains were observed for both receptors (Table S6). Thus, D2 is primarily responsible for binding of R3Mab and its derivatives, whereas D3 plays a minimal role.

The CDR H2 sequence in Mab 2B.1.3, THLGD, is completely different from the parental H2 sequence in R3Mab, IYPTN. As expected, the conformations of the CDR H2 loops in the two Mabs differ substantially (Fig. 2C). Upon aligning the variable domains of
Mab 2B.1.3 onto those of R3Mab (Fig. 2B), the H3 loop also appears twisted by a few degrees, resulting in a distance of 2.6 Å between the Cα atoms of the H3 tip residue Y100b in both structures (Fig. 2C). Accordingly, the position of the FGFR2 D2 domain overall is shifted by ~3 Å from that of the FGFR3 D2 domain. Comparison of the interface between the variants and the FGFR antigens revealed that such reorganizations of the H2 and H3 CDR loops in Mab 2B.1.3 significantly improved packing against the FGFR2 surface. In the parental structure, the shape complementarity score between R3Mab and FGFR3-D2 is 0.731. If the D2 domain of FGFR2 is aligned onto and replaces FGFR3 D2, the shape complementarity score between R3Mab and FGFR2 D2 drops to 0.685. This might explain the lack of R3Mab binding to FGFR2 (Table 2). However, in the new crystal structure, the shape complementarity score between 2B.1.3 and FGFR2-D2 dramatically increased to 0.768, which is consistent with the gain of high-affinity binding to FGFR2 through engineering of R3Mab.

Due to the remarkable similarity among FGFRs, 2B.1.3 cross-reacts with multiple homologs in the family. Although FGFR1 binding was not acquired along with FGFR2 binding, FGFR4 interaction was. Considering that FGFR4 inhibition carries an increased risk of toxicity (17), a second round of engineering was undertaken to eliminate FGFR4 binding.

**Further engineering to remove FGFR4 binding**

To generate a Mab 2B.1.3 derivative that binds FGFR2 and FGFR3 but not FGFR4, it seemed useful to identify antigen residues that likely interact with the antibody but differ between the various FGFRs (Table S7), assuming that Mab 2B.1.3 recognizes all FGFRs in an analogous mode to its interaction with FGFR2. Three phage display libraries were constructed based on the 2B.1.3 Fab template, with random mutagenesis at selected positions on the contacted CDRs H1, H3 and L2 (Table S2). During engineering the focus was on binding to FGFR2 instead of maintaining both FGFR2 and FGFR3, as in the
previous engineering. Therefore, selection was undertaken with immobilized FGFR2-IIIb alone during panning. To counter-select FGFR4 binders, phage particles were incubated with large amounts of soluble FGFR4-Fc protein. The concentrations of FGFR4-Fc were increased up to 0.46 μmol/L for successive rounds of selection (see Materials and Methods). Individual clones from round 4 (n = 96) were assayed by ELISA with FGFR2-IIIb and FGFR4, and ranked by the ratio of FGFR2 to FGFR4 binding-ELISA values. Six clones with the highest FGFR2/FGFR4 binding ratios were sequenced, expressed as IgG and characterized for binding to FGFR2-IIIb and FGFR4 (Table S8). Characterized clones from the H3/L2 libraries 2B.1.3.2, 2B.1.3.4 and 2B.1.3.6 contained mutations only in CDR H3, not CDR L2, whereas characterized clones from the H1/H3 library 2B.1.3.8, 2B.1.3.10 and 2B.1.3.12 contained mutations in both CDR H1 and H3. Although the 4 residues in H3 from L100a to D100d were fully randomized, Y100b remained unchanged, suggesting that the interaction of Y100b with FGFR2 is crucial for binding. In addition, L100a was conservatively mutated to Thr or Ile, and V100c mostly to Asp. The H1/H3 mutants containing an additional H1 mutation of T28P displayed slightly higher affinities for FGFR2. These antibodies bind FGFR2 with $K_D$ values of 1.4 to 6.6 nmol/L, but showed minimal binding to FGFR4 when using concentrations as high as 1 μmol/L for measurements, except that clone 2B.1.3.8 still retained detectable yet weak affinity for FGFR4 (Table S8). The convergence in both sequences and affinities of the 2B.1.3 variants indicated that the last rounds of phage selection had reached the limit of enrichment for binders with desired functions, i.e., diminished FGFR4 binding and retention of tight FGFR2 binding.

Considering that greater differential in binding to FGFR2 and FGFR4 as well as fewer mutations are preferable, Mab 2B.1.3.10 and 2B.1.3.12 were selected for further characterization. Both antibodies showed no binding to FGFR1 and retained strong binding to FGFR3 with affinities slightly weaker than 2B.1.3 (Table 2). Therefore, after the second-step engineering, the 2B.1.3 derivatives Mab 2B.1.3.10 and 2B.1.3.12 cross-react with FGFR2 and FGFR3, but show no detectable binding to FGFR4.
Next the abilities of the R3Mab variants to block FGF ligand binding to the specific FGFRs was evaluated. R3Mab blocks FGF ligand binding to both the FGFR3-IIIb and FGFR3-IIIc isoforms. Owing to their different specificities for different FGFRs, the blocking spectrum of each of the new antibodies varied (Fig. 3). All the engineered antibodies showed blocking activities for both FGFR2 and FGFR3, while R3Mab did not inhibit FGF7 binding to FGFR2-IIIb or FGF1 binding to FGFR2-IIIc. Whereas 2B.1.3 strongly inhibited FGF19 binding to FGFR4, 2B.1.3.10 and 2B.1.3.12 did not block the latter interaction, due to substantially diminished FGFR4 affinity.

Redesigned Mab variants inhibit FGFR2- or FGFR3-dependent tumor-cell growth

The newly redesigned variants 2B.1.3.10 and 2B.1.3.12 display dual specificity for FGFR2 and FGFR3. To investigate their biological activities, their effects on receptor-dependent signaling and proliferation in different types of tumor cells were examined. First the new variants were assessed for inhibition of growth of FGFR2-overexpressing tumor cells in vitro. Both the SNU-16 gastric carcinoma and MFM-223x2.2 triple-negative breast carcinoma cell lines have amplification of FGFR2, evident by increased FGFR2 gene-copy numbers and protein over-expression (7). In SNU-16 cells, 2B.1.3.10 and 2B.1.3.12 substantially suppressed FGF7-induced FGFR2 phosphorylation. In addition, the two 2B.1.3 variants markedly reduced phosphorylation of the downstream signaling molecules FRS2α, MAPK, PLCγ1 and AKT (Fig. 4A). Similarly, both variants diminished phosphorylation of FGFR2, FRS2α, MAPK and HER3 in FGF7-treated MFM-223x2.2 cells (Fig. S4A).

Next, the ability of the dual-specific Mab 2B.1.3.10 and 2B.1.3.12 to inhibit in vivo FGFR2-dependent and/or FGFR3-dependent growth of tumor xenografts was investigated. The RT112 cell line expresses FGFR3 but not FGFR2. As anticipated, both Mab 2B.1.3.10 and 2B.1.3.12, which retained the parental specificity for FGFR3 after engineering, as well as the parental antibody R3Mab, suppressed the growth of FGFR3-overexpressing RT112
tumor xenografts (Fig. 4B). The engineered variants 2B.1.3.10 and 2B.1.3.12 in the study, with tumor growth inhibition (TGI) values of 48% and 64%, displayed weaker potency than the parental R3Mab (TGI 82%), which could be possibly due to modified pharmacokinetics. For FGFR2-based efficacy, we turned to the SNU-16 cell line, which expresses readily detectable FGFR2 along with very low FGFR3 levels. Mice bearing SNU-16 xenografts were dosed with non-specific IgG control antibody, the parental R3Mab, or the engineered variants 2B.1.3.10 or 2B.1.3.12. The engineered variants displayed similar TGI values of 63% and 61%, respectively (Fig. 4C). Surprisingly, R3Mab, although not binding to FGFR2, also showed a measurable TGI of 44%. The tumor samples were then collected and analyzed for FGFR2 and FGFR3 expression (Fig. S5). FGFR3 was upregulated in the SNU-16 tumor xenografts in vivo, which may explain the observed inhibitory effect of R3Mab in this model. Regardless, the engineered variants showed significantly stronger activity as compared to R3Mab (p<0.001, day 31). In another experiment, 2B.1.3.10 and 2B.1.3.12 also retarded the growth of MFM-223x2.2 tumor xenografts in mice (Fig. S4B). Collectively, the engineered antibodies can serve as dual agents to effectively inhibit both FGFR2- and FGFR3-dependent cancer cell growth.
Discussion

The FGFR family is associated with versatile normal biologic functions and is additionally implicated in a number of cancer malignancies (2, 3). R3Mab, an antibody that binds monospecifically to FGFR3, was tailored here for binding to other FGFR family members through multiple rounds of engineering, including recruiting desired binding to FGFR2 and removing undesired binding to FGFR4. The first step of engineering was carried out to recruit FGFR2 binding. Each phage library constituted mutagenesis of one contacting CDR, and the range of mutagenesis covered as many residues in that CDR as allowed by library size. Choosing multiple consecutive positions for mutagenesis permitted significant freedom in the CDR backbones. Most of the resulting clones that were able to engage FGFR2 harbored all 5 mutations in CDR H2. The crystal structure demonstrated that the full range of mutagenesis was coupled with complete remodeling of the geometry of the CDR loop. The solutions to spatial reorganizations of a CDR are numerous, as evidenced by the identification of diverse H2 mutants that had gained binding to FGFR2. Such a large variety of solutions are not typically seen as outcomes from standard affinity maturation experiments, whereby the recovered sequences usually contain sparse positions on individual CDRs. Therefore, acquiring additional specificity for homologous antigens may require larger mutagenesis freedom than affinity maturation. As a result, undesired specificity may be acquired through the process, such as the FGFR4 binding in these studies.

The second round of engineering was refinement of specificity to remove unwanted FGFR4 binding. Detailed structural analysis of contact residues between the antibody CDR loops and the antigen surface was used to guide the design of phage display libraries. Selected antibody variants showed reduction in unwanted FGFR4 binding with retention of binding to FGFR2/3. The sequence solutions to this specificity refinement step were much more limited as compared to the first round of engineering. No large backbone conformational changes would be expected at this stage. The refinement step further
demonstrated the ability to exquisitely differentiate binding specificities among closely related antigens antibody engineering.

The dual-specific antibodies generated here bind to two closely related antigens, namely FGFR2 and FGFR3. These 2B.1.3 antibody variants are regular IgG molecules in that they use identical heavy and light chains. They can potentially bind to two FGFR2 isoforms, two FGFR3 isoforms or one FGFR2 and one FGFR3 isoform in a bivalent or monovalent manner respectively. This contrasts to conventional bispecific IgG, which commonly use two different heavy/light-chain pairs to bind to two different antigens in a monovalent manner. The dual-specific antibodies described share some similarities with “two-in-one” antibodies (24). Bostrom et al. randomized all 3 light-chain CDRs of Herceptin and selected for a second specificity as well as the parental specificity. As expected, the second specificity comes from the dominant contributions of light-chain CDRs (24, 25). In one case, although EGFR and HER3 are homologous, the binding epitopes by an anti-EGFR/HER3 “two-in-one” antibody are different (25). The approach described here differs from “two-in-one” antibodies in that it appreciates the sequence and structure similarities between the two homologous antigens, and focuses on a more limited set of mutagenesis so as to retain the parental epitope during engineering.

The antibody engineering presented here started from an existing and extensively characterized antibody, R3Mab, that has potential utility for cancer therapy. Since introduction of the first therapeutic monoclonal antibody in the mid-1980s, there have been many clinically and commercially successful antibody drugs in different disease areas, including trastuzumab, cetuximab, adalimumab, bevacizumab, etc. These antibodies displayed exceptional activities in inhibiting their molecular targets. On the other hand, like the FGFR family, multiple homologous proteins are pursued as molecular targets for their various disease associations. Traditional discovery routes to obtain antibodies targeting a functional epitope, either animal immunization or other display-based library selections, are not guaranteed to be successful. The approach developed here to engineer a known
antibody for acquiring specificity towards homologous targets provides an alternative route for antibody discovery. Moreover, it takes advantage of the favorable properties of previously developed antibodies by maintaining the functional epitopes and presumably the biological functions as well. As the clinical antibody repertoire expands, more antibodies could be re-engineered instead of being discovered ab initio. Potential applications may include protein families that comprise multiple members as disease targets, such as the EGFR (26), TNFR (27), TAM (28, 29), and Eph-Ephrin families (30). As in the traditional discovery processes, engineered antibodies towards homologs should be considered as new molecules, and still need full characterization of their biochemical, biophysical and biologic properties for any potential therapeutic applications.
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Data Deposition

The co-crystal structure of Mab 2B.1.3 and FGFR2-IIIb has been deposited in the Protein Data Bank (PDB) under accession code 4WV1.

REFERENCES

Engineering of Dual-Targeting Anti-FGFR2/3 Antibodies


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<th>Variant ID</th>
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<th>Times found (n)</th>
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Residues that match those in R3Mab are underlined. *ND, not detectable; NA, not available due to protein aggregation.
Table 2. Binding affinities of R3Mab and its variants to all human FGFR homologs

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*ND: not detectable at 500 nmol/L.
Engineering of dual-targeting anti-FGFR2/3 antibodies

Figure Legends

**Figure 1.** Inhibitory effects of engineered 2B.1 antibodies for FGF7-stimulated MCF-7 cell proliferation. Error bars represent SEM.

**Figure 2.** Crystal structure of the complex between FGFR2 D2 domain and the Fab fragment of Mab 2B.1.3. A, FGFR2-D2 (magenta) in complex with Fab 2B.1.3 heavy (green) and light chains (blue). B, overlay of the structures of the complex between FGFR2-D2 and 2B.1.3 (colored as in A) and the complex between FGFR3-D2D3 (yellow) and R3Mab (gray). C, zoom-in representation of the boxed area in B showing the structural differences between the two complexes in the same color scheme.

**Figure 3.** Differential blocking of FGF ligands by R3Mab IgG1 variants. A, blocking of FGF-7 binding to human FGFR2-IIIb. B, blocking of FGF-1 binding to human FGFR2-IIIc. C, blocking of FGF-1 binding to human FGFR3-IIIb. D, blocking of FGF-1 binding to human FGFR3-IIIc. E, blocking of FGF-19 binding to human FGFR4.

**Figure 4.** 2B.1 variants inhibit FGFR2 signaling *in vitro* and suppress xenograft growth *in vivo*. A, blocking of FGF7-stimulated FGFR2 signaling by 2B.1 variants in the gastric cancer cell line, SNU-16. B, effects of R3Mab, 2B.1.3.10 and 2B.1.3.12 on the growth of FGFR3-dependent RT112 bladder cancer xenografts. **** P < 0.0001 for 2B.1.3.10 or 2B.1.3.12 versus control MAb at day 22. n=10 per group; error bars represent SEM. C, effect of 2B.1.3.10 and 2B.1.3.12 on the growth of FGFR2-dependent SNU-16 xenografts compared to control antibody and R3Mab. **** P < 0.0001 for 2B.1.3.10 or 2B.1.3.12 versus control MAb at day 28; *** P < 0.001 for 2B.1.3.10 or 2B.1.3.12 versus R3Mab at day 31. n=10 per group; error bars represent SEM.
Fig. 1

Cell Viability (% of Untreated)

- NO FGF7
- FGF7

Untreated, Ab con, R3Mab, 2B.1.3, 2B.1.95, 2B.1.73, 2B.1.32, 2B.1.88, 2B.1.1
Fig. 4

A

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**RT112 (FGFR3⁺)**

- Control MAb
- R3Mab, 50 mg/kg
- 2B.1.3.10, 50 mg/kg
- 2B.1.3.12, 50 mg/kg

B

Mean tumor volume (mm³)

Time (day)

C

**SNU-16**

- Control MAb, 30 mg/kg
- R3Mab, 30 mg/kg
- 2B.1.3.10, 30 mg/kg
- 2B.1.3.12, 30 mg/kg

Mean tumor volume (mm³)

Time (day)
Redesigning a Monospecific Anti-FGFR3 Antibody to Add Selectivity for FGFR2 and Expand Anti-tumor Activity

Yiyuan Yin, Stevan Djakovic, Scot Marsters, et al.

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