



Superior *In vivo* Efficacy of Afucosylated Trastuzumab in the Treatment of HER2-Amplified Breast Cancer

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Abstract

The enhancement of immune effector functions has been proposed as a potential strategy for increasing the efficacy of therapeutic antibodies. Here, we show that removing fucose from trastuzumab (Herceptin) increased its binding to FcγRIIIa, enhanced antibody-dependent cell-mediated cytotoxicity, and more than doubled the median progression-free survival when compared with conventional trastuzumab in treating pre-clinical models of HER2-amplified breast cancer. Our results show that afucosylated trastuzumab has superior efficacy in treating *in vivo* models of HER2-amplified breast cancer and support the development of effector function-enhanced antibodies for solid tumor therapy. *Cancer Res*; 70(11): 4481–9. ©2010 AACR.

Introduction

Herceptin (trastuzumab) is a humanized antibody for treating HER2/ErbB2-overexpressing breast cancer. Trastuzumab was initially approved for the treatment of women with HER2-positive metastatic breast cancer in combination with standard cytotoxic chemotherapy (1) and as a monotherapy (2, 3). Multiple studies have been completed in several large adjuvant or early breast cancer trials for HER2-positive breast cancer (4). A survival benefit was noted after only 2 years of follow-up, which is impressive in breast cancer (4). Multiple mechanisms of action are thought to contribute to the tumor-inhibitory effect of trastuzumab. Binding of trastuzumab to HER2 has a direct inhibitory effect on HER2-amplified tumor cells. Trastuzumab disrupts the ligand-independent HER2-HER3 interaction resulting in rapid inhibition of HER3/PI3K/AKT signaling (5). Ultimately, this leads to an increase in the CDK2 inhibitor, p27, resulting in cell cycle arrest of the cancer cells (5–8). Another primary effect on tumor cells is the ability of trastuzumab to inhibit HER2 ectodomain shedding (9), which may have therapeutic significance. Trastuzumab is also known to have synergistic effects when combined with chemotherapy (10).

In addition to directly inhibiting tumor cell signaling, trastuzumab can also mediate the effector functions of immune cells through the constant region (Fc) of the antibody. As a humanized IgG₁, it binds to Fcγ receptor III (RIII) and is a

potent mediator of antibody-dependent cell-mediated cytotoxicity (ADCC). The ability of trastuzumab to mediate ADCC is strictly related to HER2 density (11). Tumor cells that overexpress HER2 are observed to have 100-fold more HER2 on their cell surface than normal adjacent breast epithelial cells (12). As a result, trastuzumab mediates ADCC very effectively against target cell lines that overexpress HER2 but displays only background activity against cells that express normal levels of HER2. Depleting trastuzumab effector functions by Fc modification or deleting the FcγR functions from the mouse results in significant reduction of trastuzumab efficacy, demonstrating the importance of immune effector functions in trastuzumab response (13). Clinical relevance for effector functions of Herceptin is supported by a report demonstrating that trastuzumab efficacy correlated with the high-affinity FcγRIII genotype (V/V; ref. 14) similar to rituximab-treated patients (15). Furthermore, increased tumor-associated natural killer (NK) cells and lytic capacity of effector cells were detected after trastuzumab treatment (16–18), and trastuzumab response correlated with high *in vitro* ADCC (17, 18) and immune cell infiltration (17).

Together, the aforementioned studies provide a biological and clinical rationale for a strategy in which increasing trastuzumab-FcγRIIIa affinity could enhance therapeutic benefit. Currently, no published reports have shown that enhancing FcγR affinity results in enhanced *in vivo* efficacy for the treatment of established, nonhematologic tumors. Although promising results are described in a few published reports in which treatment commenced at the same time as tumor cell inoculation (13, 19, 20), these studies leave open the question of whether the observed outcomes were due to the effects on tumor implantation. The primary aim of the present study is to determine whether the efficacy of trastuzumab in suppressing the growth of established tumors is augmented by increasing FcγR binding affinity.

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Materials and Methods

Cell lines

Breast cancer cell line BT-474-M1 is an *in vivo*-passaged subclone of BT-474 (American Type Culture Collection; ref. 13). MCF7-neo/HER2 cells were established at Genentech, Inc. KPL-4 breast cancer cells were obtained from J. Kurebayashi (Department of Breast and Thyroid Surgery, Kawasaki Medical School, Kurashiki, Okayama, Japan; ref. 21). SKBR-3 and MCF-10A cells were from American Type Culture Collection. Cell lines were maintained in high-glucose DMEM/Ham's F-12 (50:50) supplemented with 10% fetal bovine serum and 2 mmol/L of L-glutamine.

Analysis of N-linked oligosaccharides

The oligosaccharide profiles of the antibodies were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, as previously described (22).

Fcγ receptor binding ELISA

Soluble human FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA(F158), and FcγRIIIA(V158) (ref. 23), and mouse FcγRI, FcγRII, and FcγRIIIA (Genentech) were expressed in Chinese hamster ovary (CHO) cells (mFcγRI and mFcγRII in 293 cells) as recombinant fusion proteins with Gly-His6-glutathione S-transferase at the COOH terminus of the extracellular domain of the receptor α chains. Soluble mouse FcγRIV was expressed in CHO cells as a recombinant fusion protein with Gly-His8 at the COOH terminus of the extracellular domain of the receptor α chains (Genentech). MaxiSorp 384-well microwell plates (Nunc) were coated with 2 μg/mL of anti-glutathione S-transferase (clone 8E2.1.1; Genentech) or anti-His (penta his antibody, for mouse FcγRIV; Qiagen) in 50 mmol/L of carbonate buffer (pH 9.6), at 4°C overnight followed by a wash (PBS containing 0.05% polysorbate; pH 7.4). Plates were blocked for 1 hour in room temperature with PBS containing 0.5% bovine serum albumin (BSA) and washed. Fcγ receptor [0.25 μg/mL, in PBS containing 0.5% BSA, and 0.05% polysorbate 20 (pH 7.4) in assay buffer] was incubated in plates for 1 hour followed by a wash. To measure binding to the high-affinity FcγRI, IgG antibodies (0.0085–500 ng/mL in 3-fold serial dilution) in assay buffer were added to the plates. To measure binding to the low-affinity FcγRII, FcγRIII, and FcγRIV, IgG antibodies were first incubated with goat F(ab')₂ anti-κ antibody (MP Biomedicals) at a 1:2 (w/w) ratio for 1 hour to form antibody complexes. Complexed IgG antibodies (0.42–25,000 ng/mL in 3-fold serial dilution) in assay buffer were added to the plates, incubated for 2 hours, and washed. Bound IgG was detected after 1 hour of incubation with peroxidase-labeled goat F(ab')₂ anti-human IgG F(ab')₂ (Jackson ImmunoResearch) in assay buffer followed by a wash, incubation with substrate (3',5,5'-tetramethyl benzidine; Kirkegaard & Perry Laboratories), and termination with 1 mol/L of phosphoric acid. The absorbance (450 nm) at the midpoint of the standard curve (mid-OD) was calculated. The corresponding concentrations of standard and samples at this mid-OD

were determined from the titration curves using a four-parameter nonlinear regression curve-fitting program (XLfit).

In vitro ADCC

In vitro ADCC assays were performed as previously described (24). In short, peripheral blood mononuclear cells (PBMC) were separated from the blood of normal volunteers using lymphocyte separation medium (MP Biomedicals). The NK cells were enriched from the PBMCs using anti-CD56 microbeads (Miltenyi Biotec). Target cells (1×10^4) were preincubated with antibodies for 30 minutes in 37°C in serum-free RPMI 1640 supplemented with 0.1% BSA before adding the effector cells in a 25:1 E/T ratio (10:1 when NKs were used as effectors). The cells were incubated for an additional 4 hours before detecting death by measuring the lactate dehydrogenase activity from the medium using Cytotoxicity Detection Kit (LDH; Roche). All measurements were done in quadruplicate. The percentage of cytotoxicity was calculated as follows: % cytotoxicity (experimental lysis – spontaneous effector lysis – spontaneous target lysis) / (maximum target lysis – spontaneous target lysis) × 100.

HER2 binding

Nunc BreakApart Immunomodule plates (Nunc) were coated with 20 ng/mL of HER2-ECD-Ig fusion protein in 50 mmol/L of HEPES (pH 8.2), and 150 mmol/L of NaCl overnight at 4°C. Nonspecific binding was blocked with 2 mg/mL of BSA, 25 mmol/L of Tris (pH 7.5), and 150 mmol/L of NaCl for 2 hours at room temperature. Wells were washed thrice with assay buffer [2 mg/mL BSA and 10 mmol/L HEPES (pH 7.2) in RPMI]. The competitive binding reaction with a dilution series of nonlabeled competitor antibodies and constant ¹²⁵I-trastuzumab was carried out for 2 hours at room temperature. The ¹²⁵I-trastuzumab bound to HER-2 was then detected by a gamma counter and the data were analyzed using the nonlinear regression method of Munson and Rodbard (25). All measurements were done in quadruplicate.

Analysis of PI3K/AKT pathway activation

Cells were rinsed with PBS and lysed with nondenaturing lysis buffer including 1% Triton (Cell Signaling Technology). Lysates were cleared of insoluble material by centrifugation. AKT phosphorylation was detected by ELISA detecting phosphorylated Ser⁴⁷³ (Cell Signaling Technology).

Cell proliferation

Proliferation/viability of cells was detected using CellTiter-Glo Luminescent Cell Viability Assay (Promega). For the assay, 5×10^3 cells were plated on 96-well plates and incubated overnight for cell attachment before 6 days of treatment with the antibodies. All measurements were done in triplicate.

Mice

Rag2^{-/-} (BALB/c; Taconic) mice were crossbred to FcγRI/III^{-/-} (CD64/CD16 double KO, BALB/c, kindly provided by S. Verbeek; refs. 26, 27), resulting in FcγRI/III/Rag2^{-/-} mice, which were then crossbred to human FcγRIIIa transgenic

Table 1. Oligosaccharide profile of afucosylated trastuzumab

Glycan structure	Total AB (%)	
	Trastuzumab	Afucosylated
	5	
	2	
	8.5	87
	43	
	8	13
	28	
	3	
	2.5	
Total	100	100
Fucosylated	76	0

NOTE: ▲, fucose (Fuc); ◆, galactose (Gal); ●, Mannose (Man); ■, N-acetylglucosamine (GlcNAc).

mice (Bl-6, kindly provided by J. Ravetch; ref. 28) to obtain *FcγRII^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human *FcγRIIIa*) mice. The human transgene is the low-affinity *FcγRIIIa*-F158 allele (Supplementary Fig. S2B). Severe combined immuno-

deficiency (SCID)-beige mice were obtained from Charles River Labs.

Pharmacokinetic studies

Pharmacokinetic studies were performed as previously described (29). In short, a single i.v. 10 mg/kg dose of trastuzumab or afucosylated trastuzumab was injected to *FcγRII^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human *FcγRIIIa*) mice. Serum samples ($n = 4$ /time point) were collected by retro-orbital bleed or cardiac stick after administration of the antibodies (time points: 3 minutes, 1 and 5 hours, and 1, 3, 7, 14, and 28 days). Samples were assayed for trastuzumab by HER2 binding ELISA (29), in which a HER2 extracellular domain coated to a microtiter plate was used to capture the humanized anti-HER2 antibodies in circulation. Time concentration data were analyzed using compartmental pharmacokinetic analysis (Model 8, WinNonlin-Pro v3.2; Pharsight Corporation).

In vivo drug efficacy

To address the requirement of *FcγR* interaction in the trastuzumab response using T-D265A, SCID-beige mice (Charles River Labs) were used. For afucosylated trastuzumab efficacy studies, *FcγRII^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human *FcγRIIIa*) mice were used. Mice xenografted with MCF7-neo/HER2 cells were supplemented with subcutaneous estrogen pellets (0.36 mg, 60-day release; Innovative Research of America) 3 days prior to cell inoculation. Five million MCF7-neo/HER2 cells or 3 million KPL-4 cells were injected into the mammary fat pad in a 1:1 HBSS-matrigel suspension (BD Matrigel, BD Biosystems). When tumor volumes reached 100 to 300 mm³, mice were randomly grouped into treatment cohorts. Dosing is described in the figure legends. Tumor volumes were calculated using the formula: (mm³) = ($L \times W^2$) $\times$ 0.5.

Table 2. Binding of afucosylated trastuzumab to human and mouse *FcγR*

	Relative affinity to mouse <i>FcγR</i>							
	I		II		III		IV	
	Mid-OD*	Fold†	Mid-OD*	Fold†	Mid-OD*	Fold†	Mid-OD*	Fold†
Trastuzumab	197.0	1	4,435	1	6,045	1	138	1
Afucosylated	231.0	0.9	3,690	1.2	6,403	0.9	71	1.9

	Relative affinity to human <i>FcγR</i>									
	I		IIa		IIb		IIIa-F158		IIIa-V158	
	Mid-OD*	Fold†	Mid-OD*	Fold†	Mid-OD*	Fold†	Mid-OD*	Fold†	Mid-OD*	Fold†
Trastuzumab	12.7	1	1,783	1	5,851	1	2,156	1	263	1
Afucosylated	9.2	1.4	1,639	1.1	3,963	1.5	74	29.2	40	6.5

*In ng/mL.

†Fold = mid-OD trastuzumab/mid-OD afucosylated.

Results

Afucosylated trastuzumab has increased affinity to human FcγRIIIa which results in enhanced ADCC

We produced 100% fucose-free (afucosylated) trastuzumab in *FUT8*^{-/-} CHO cells (30). The *FUT8* gene encodes 1,6-fucosyltransferase, which catalyzes the transfer of fucose from GDP-fucose to *N*-acetylglucosamine. The oligosaccharide profile of the antibodies was determined by matrix-assisted laser desorption/ionization time-of-flight analysis, which confirmed the lack of fucose (Table 1). The relative affinity of afucosylated trastuzumab was analyzed by FcγR binding ELISA. Binding to FcγRIIIa-F158 and FcγRIIIa-V158 increased by 29-fold and 6.5-fold, respectively (Table 2). Binding to human FcγRI, FcγRIIa, and FcγRIIb was not markedly altered, indicating that afucosylated trastuzumab has selective enhanced affinity for FcγRIII (Table 2). Similarly, binding to mouse FcγRI, FcγRII, or FcγRIII was not increased and only a minor increase was detected in relative affinity to FcγRIV (Table 2). The increases in binding affinity to mFcγRIV and huFcγRIIIa-V158 were similar (3.3- and 6.2-fold decreased *K_D*, respectively; data not shown) when binding was measured using surface plasmon resonance–based technology (Biacore).

Afucosylated trastuzumab showed increased *in vitro* ADCC. The ability to mediate ADCC improved 1.9- to 7.2-fold (*EC*₅₀; *n* = 5) when F/F effectors were used and 2.1- to 7.7-fold (*n* = 5) when V/V donors were used (Fig. 1A; data not shown). A representative experiment using F/F effector cells is presented in Fig. 1A. ADCC activity increase was statistically significant in 0.01 to 0.1 ng/mL antibody doses (Fig. 1A; *t* test). Despite the effector cell donor–dependent variability, the *EC*₅₀ was consistently lower for afucosylated trastuzumab in each assay. ADCC activity of PBMCs was mediated by NK cells (Fig. 1A). When purified NK cells from F/F donors were used as effector cells, an 11.3-fold enhancement in ADCC was observed for afucosylated trastuzumab (Fig. 1A). Afucosylated trastuzumab did not mediate ADCC of MCF-10A cells which express low/normal levels of HER2 even at high antibody concentrations and using V/V effectors cells (Supplementary Fig. S1A). Together, these results show that afucosylated trastuzumab has increased affinity for human FcγRIIIa, which results in enhanced ADCC.

Afucosylated trastuzumab retains FcγR-independent functions of trastuzumab

In addition to the ability to mediate ADCC, trastuzumab has a direct effect on tumor cell signaling which causes cell

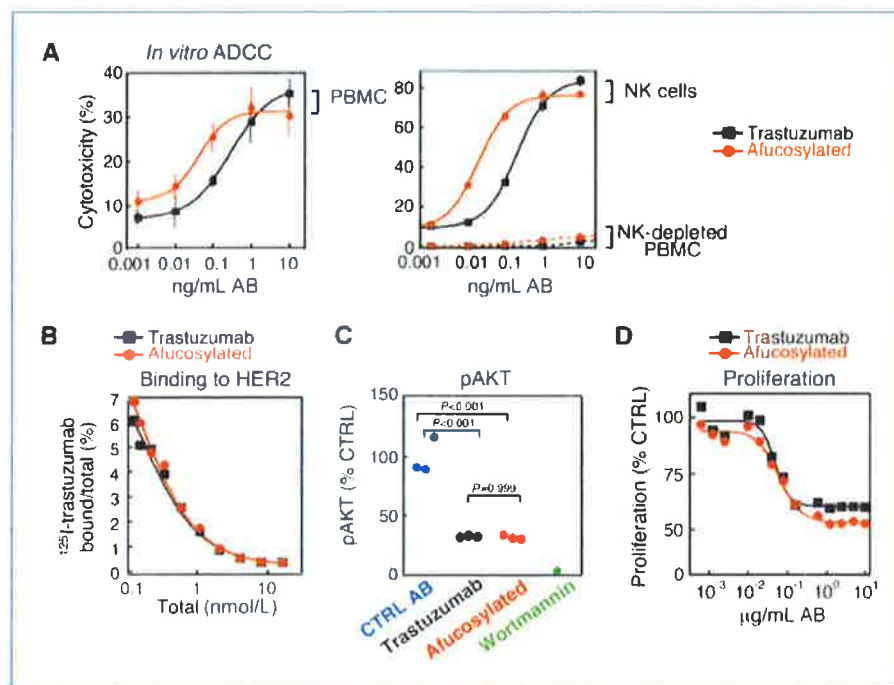


Figure 1. *In vitro* characterization of afucosylated trastuzumab. A, increased affinity to human FcγRIIIa results in enhanced ADCC. The ability of trastuzumab (black symbols) and afucosylated trastuzumab (red symbols) to mediate *in vitro* ADCC by FcγRIIIa-F158 homozygous PBMCs (left), NK cells or NK cell–depleted PBMCs (right) was measured using assay detecting LDH released from lysed cells. HER2-amplified BT474-M1 cells were used as target cells in a 25:1 effector/target ratio (10:1 when NK cells were used as effectors). B to D, removal of fucose does not affect FcγR-independent properties of trastuzumab. B, binding to recombinant extracellular domain of human HER2 was determined by competition binding of ¹²⁵I-trastuzumab with nonlabeled trastuzumab or afucosylated trastuzumab. C, HER2-amplified SKBR-3 cells were treated for 1 h with antibodies (10 μg/mL) or Wortmannin (1 μmol/L). AKT phosphorylation was measured by ELISA detecting phosphorylated Ser⁴⁷³. Statistical significance between groups was determined using Dunnett's test. D, proliferation of SKBR-3 cells was analyzed after 6 days of treatment with trastuzumab or afucosylated trastuzumab using CellTiter-Glo Luminescent Cell Viability Assay.

cycle arrest and inhibition of proliferation. To confirm that carbohydrate modification does not affect HER2 binding, we competed the binding of radioiodinated trastuzumab to HER2 ECD by adding increasing concentrations of noniodinated antibody (trastuzumab or afucosylated trastuzumab) in the reaction (Fig. 1B). The calculated affinities of antibodies were similar for trastuzumab and afucosylated trastuzumab (K_D , 0.13 ± 0.04 and 0.12 ± 0.03 nmol/L, respectively), indicating that lack of fucose does not affect HER2 binding.

To explore the antisenescence properties, SKBR-3 cells were treated with trastuzumab or the afucosylated variant. Treatment with 10 μ g/mL of trastuzumab or afucosylated trastuzumab caused a 69% to 66% reduction in pAKT after 60 minutes of treatment (Fig. 1C). No difference was detected in the effects of afucosylated and conventional trastuzumab treatment ($P = 0.99$; Dunnett's). SKBR-3 proliferation was measured after 6 days of treatment. Both antibodies caused approximately 50% maximal reduction in proliferation (Fig. 1D). The EC_{50} values were 52 ± 8 and 61 ± 9 ng/mL for trastuzumab and afucosylated trastuzumab, respectively. Together, these results confirm that the lack of fucose does not affect the Fc γ R-independent functions of trastuzumab. These functions include HER2 binding, the immediate inhibition of the PI3K pathway, and sustained inhibition of tumor cell proliferation.

Pharmacokinetic properties of afucosylated trastuzumab

Removal of fucose does not affect IgG binding to FcRn (23). However, carbohydrate composition or increased Fc γ R affinity might have an effect on target-independent clearance or drug disposition of the antibody (31). To address this question, we performed pharmacokinetic analysis of afucosylated trastuzumab. For this purpose, the systemic disposition of trastuzumab and afucosylated trastuzumab was evaluated in *Fc γ RI^{-/-}Fc γ RIII^{-/-}RAG2^{-/-}Tg* (human Fc γ RIIIa) mice following a single i.v. dose (10 mg/kg). Serum samples ($n = 4$ /time point) were analyzed for anti-HER2 antibodies 3 minutes to 28 days after administration of the antibodies. Both molecules displayed biphasic systemic disposition, with a rapid initial phase and a prolonged terminal phase (Fig. 2). Afucosylated trastuzumab displayed increased clearance (9.0 versus 6.8 mL/d/kg), decreased terminal half-life (10.1 versus 13.1 days), and decreased AUC_{0-inf} (1,118 versus 1,463 d $\times$ μ g/mL) relative to trastuzumab (Fig. 2). Both groups displayed a central volume of distribution values (V_1) that approximated mouse plasma volume. Taken together, these results show that afucosylated trastuzumab exhibited moderately altered pharmacokinetic dispositions compared with trastuzumab, slightly faster elimination from the circulation, and a modest reduction in half-life.

Fc γ R interaction is required for trastuzumab response in the treatment of established KPL-4 tumor xenografts

The KPL-4 breast cancer cell line harbors HER2 amplification (21) but does not respond to trastuzumab *in vitro* in a proliferation assay (5). A "hotspot" *PIK3CA* mutation (H1047R) generates constitutive PI3K/AKT signals and is

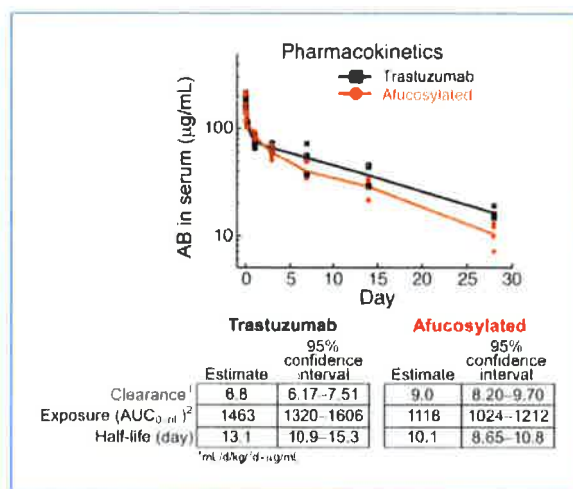


Figure 2. Pharmacokinetic profile of afucosylated trastuzumab. A single i.v. 10 mg/kg dose of trastuzumab (black symbols) or afucosylated trastuzumab (red symbols) was injected to *Fc γ RI^{-/-}Fc γ RIII^{-/-}RAG2^{-/-}Tg* (human Fc γ RIIIa) mice. Serum samples were assayed for trastuzumab by ELISA.

the likely cause for the trastuzumab insensitivity. However, KPL-4 cells are sensitive to the Fc γ R-mediated trastuzumab activity similar to BT474-M1 (Supplementary Fig. S1B). Despite the inability of trastuzumab to inhibit KPL-4 cell proliferation, KPL-4 tumor xenografts respond to trastuzumab when treated with three weekly 15 mg/kg doses (Fig. 3A, left). To confirm that the *in vivo* response is dependent on Fc γ R interaction, we also treated mice with the D265A mutant of trastuzumab (T-D265A). The single alanine substitution impairs binding of trastuzumab in all human and mouse Fc γ R (13). Treatment of mice with T-D265A resulted in almost complete loss of response (Fig. 3A, left), confirming that trastuzumab response in the treatment of KPL-4 tumors were due to the Fc γ R-dependent effects of trastuzumab.

Increased *in vivo* efficacy of afucosylated trastuzumab

To assess the efficacy of afucosylated trastuzumab *in vivo*, we treated established KPL-4 xenografts grown in the mammary fat pads of SCID-beige mice with three weekly 10 mg/kg doses of trastuzumab or afucosylated trastuzumab. Although both trastuzumab and afucosylated trastuzumab inhibited the tumor growth ($P < 0.001$ and $P = 0.003$, respectively; log rank test), no difference was seen between the antibodies ($P = 0.48$; log rank test), indicating that the modest increase in binding affinity to Fc γ RIV was not sufficient to result in increased efficacy (Fig. 3A, right).

To further evaluate the activity of the afucosylated trastuzumab variant, we generated *Fc γ RI^{-/-}Fc γ RIII^{-/-}RAG2^{-/-}Tg* (human Fc γ RIIIa) mice that lack murine Fc γ RI (27) and Fc γ RIII (26) and express the human Fc γ RIIIa transgene (28). The transgene expression is controlled by the human Fc γ RIIIa promoter and is expressed in NK cells and macrophages (28). Expression of the transgene in mice was

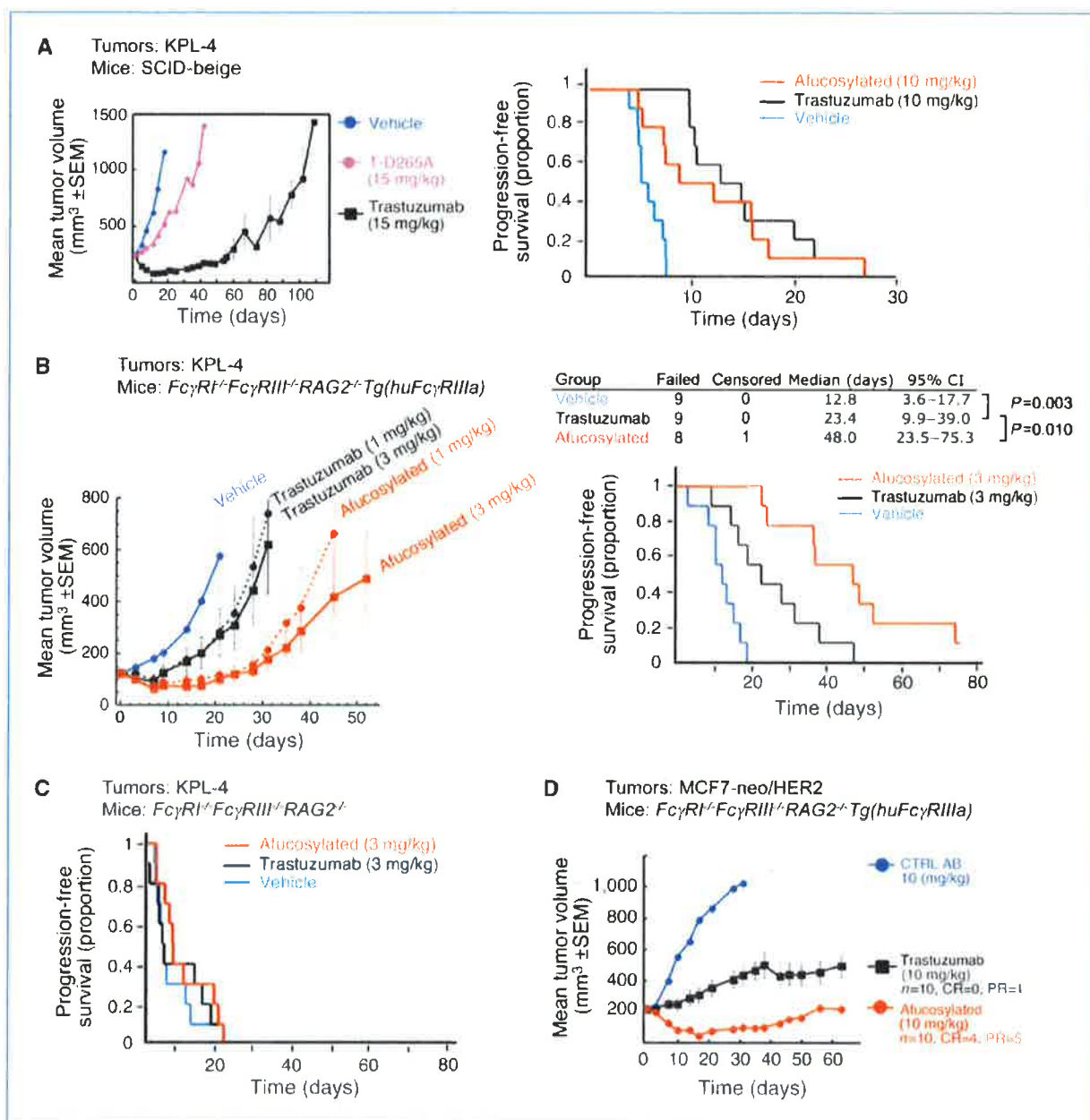


Figure 3. Superior *in vivo* efficacy of afucosylated trastuzumab compared with trastuzumab. **A**, FcγR interaction is required for trastuzumab response in the treatment of KPL-4 tumor xenografts (left). Established KPL-4 tumor xenografts in SCID-beige mice were treated with vehicle (blue), trastuzumab (black), or a trastuzumab variant incapable of FcγR binding (T-D265A; magenta). Intraperitoneal 15 mg/kg, 1×/wk × 3 dosing was started at day 0. A loading dose of 30 mg/kg was administered on the first injection ($n = 8$). Efficacy of afucosylated trastuzumab in SCID-beige mice: pre-established KPL-4 tumors were treated with vehicle (blue), trastuzumab (black), or afucosylated trastuzumab (red). Intraperitoneal 10 mg/kg, 1×/wk × 3 dosing was started at day 0 (right). A loading dose of 20 mg/kg was administered on the first injection ($n = 10$). Time to progression: tumor $V = 2 \times V$ on day 0. **B**, KPL-4 tumor xenograft bearing *FcγRI^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human FcγRIIIa) mice were treated with vehicle (blue), trastuzumab (black), or afucosylated trastuzumab (red). A single 1 mg/kg (dotted lines) or 3 mg/kg (solid lines) dose was administered i.v. at day 0 ($n = 9$; left). Progression-free survival for *FcγRI^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human FcγRIIIa) mice bearing KPL-4 tumors treated with vehicle (blue), trastuzumab (3 mg/kg, black), or afucosylated trastuzumab (3 mg/kg, red; $n = 9$). Dose was administered i.v. at day 0 (right). Time to progression: tumor $V = 2 \times V$ on day 0. Statistical significance was determined using log rank test. **C**, KPL-4 tumor xenograft bearing *FcγRI^{-/-}FcγRIII^{-/-}RAG2^{-/-}* mice were treated with vehicle (blue), trastuzumab (black), or afucosylated trastuzumab (red). Intravenous 3 mg/kg, 1×/wk × 3 dosing was started at day 0 ($n = 10$). Time to progression: tumor $V = 2 \times V$ on day 0. **D**, MCF7-neo/HER2 tumor xenograft bearing *FcγRI^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human FcγRIIIa) mice were treated with control AB (blue), trastuzumab (black), or afucosylated trastuzumab (red). Intravenous 10 mg/kg, 1×/wk × 3 dosing was started at day 0. A loading dose of 20 mg/kg was administered on the first injection. CR, complete response (no detectable tumor); PR, partial response (tumor volume reduced <50% of day 0; $n = 10$).

confirmed by quantitative reverse transcription-PCR of splenocyte mRNA (Supplementary Fig. S2A). Analysis of genomic DNA showed that the transgene is the low-affinity FcγRIIIa-F158 allele (Supplementary Fig. S2B).

For the efficacy studies, KPL-4 xenografts were grown in the mammary fat pads of *FcγRII^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human FcγRIIIa) mice until the tumor size reached an average of 125 mm³. At this point (day 0), mice were randomly grouped for treatment regimens (*n* = 9) and treated with single 1 or 3 mg/kg i.v. doses of trastuzumab, afucosylated trastuzumab, or vehicle. Afucosylated trastuzumab inhibited the growth of KPL-4 tumors more effectively than trastuzumab (Fig. 3B, left). Treatment with 3 mg/kg of trastuzumab caused a significant delay in tumor progression (*P* = 0.003; log rank test; Fig. 3B, right) and the efficacy of afucosylated trastuzumab was significantly improved compared with trastuzumab (*P* = 0.010; Fig. 3B, right). The median progression-free survival more than doubled for mice treated with afucosylated trastuzumab, compared with treatment with trastuzumab (48 versus 23.4 days, respectively; Fig. 3B, right).

We performed similar experiments using *FcγRII^{-/-}FcγRIII^{-/-}RAG2^{-/-}* mice without the human transgene. The KPL-4 tumors were treated with three weekly 3 mg/kg i.v. doses of the antibodies. No significant difference was observed in the response to the antibodies, demonstrating that the increase in the efficacy detected for afucosylated trastuzumab was mediated by the human FcγRIIIa transgene (Fig. 3C).

To ensure that the observed increase in efficacy was not model-dependent, the study was repeated using established MCF7-neo/HER2 xenografts grown in *FcγRII^{-/-}FcγRIII^{-/-}RAG2^{-/-}Tg* (human FcγRIIIa) mice. Similar to KPL-4, these cells overexpress HER2 but do not respond to trastuzumab *in vitro* in a proliferation assay. Again, afucosylated trastuzumab was more potent than trastuzumab in treating MCF7-neo/HER2 tumors, causing four complete responses and five partial responses (Fig. 3D). In contrast, no complete responses and only one partial response was detected in trastuzumab-treated mice. Taken together, these results show that the *in vivo* efficacy of afucosylated trastuzumab is superior to trastuzumab in treating two preclinical models of HER2-amplified breast cancer.

Discussion

It has been hypothesized that ADCC is a general mechanism of action for many therapeutic antibodies (32). However, it remains unclear whether ADCC or the engagement of Fcγ receptors significantly contribute to trastuzumab's antitumor effects in patients with breast cancer. After the initial *in vitro* findings, Ravetch and colleagues showed the *in vivo* importance of FcγR-mediated activity of trastuzumab (13). Notably, these studies did not address the role of FcγR interaction in models with established tumors. Instead, treatments were simultaneous with cell inoculation and thus the results might portray the role of effector cell functions on tumor implantation rather than its effect on solid tumor mass. Our findings confirm the results of Clynes and colleagues and extend them to the treatment of pre-established solid tumors.

The ability to increase *in vitro* ADCC by antibody engineering is widely established (33, 34). However, to our knowledge, it has not been previously shown that the increase in antibody-FcγR interaction leads to increased *in vivo* efficacy in the treatment of established nonhematologic tumors. To directly address this question, we generated afucosylated trastuzumab. The approach was selected primarily because the affinity increase is significant and selective for FcγRIII. Moreover, the variant can be readily produced and is completely devoid of fucose. Fucose in the oligosaccharide (at Asn²⁹⁷) of IgG₁ hinders the interaction between carbohydrate of FcγRIIIa (at Asn¹⁶²) and regions of IgG₁ (35). Afucosylation results in increased affinity of the FcγR-IgG₁ interaction. The higher affinity observed by afucosylation is selective for human FcγRIIIa and FcγRIIIb because the other human FcγRs are not glycosylated at the corresponding position (35). Therefore, removing the fucose residue results in increased affinity selectively for FcγRIII but not for other FcγR, FcRn, or C1q (23).

We produced afucosylated trastuzumab in *FUT8^{-/-}* CHO cells (30). One alternative technology to produce afucosylated antibodies in CHO cells is to overexpress GnTIII and ManII (35). Interestingly, the clearance of afucosylated trastuzumab was slightly faster than the clearance of trastuzumab in the transgenic mouse model. Hypothetically, the faster clearance might be a result of differential biodistribution leading to the enrichment of antibodies to immune effector cell-rich organs due to increased FcγRIII affinity. Using a mouse model that expresses a human FcγRIIIa transgene in effector cells, we were able to successfully show that an increase in affinity to FcγRIII results in improved efficacy when treating solid tumors. As with any preclinical finding, confirmation of these results will require validation in human clinical trials.

The superior efficacy of afucosylated trastuzumab is not explained by an increase in antibody exposure. However, a plausible mechanism for more effective tumor growth inhibition may be due to the observation that afucosylated antibodies could escape the inhibitory effect of serum IgG. Reports by Iida and colleagues show that serum IgG markedly inhibits the efficacy of rituximab to deplete B cells (36). This inhibition has been attributed to the ability of IgG to compete for FcγRIII binding. However, due to its higher affinity for FcγRIII, afucosylated IgG might escape the inhibitory effect of serum IgG (36–38).

The differential expression of HER2 in tumors relative to normal tissue is thought to account for Herceptin's favorable safety profile. The activity of trastuzumab in ADCC assays is also specific for only high HER2-expressing target cells (11). To maintain this safety profile, it is critical that any increase in ADCC activity does not result in the cytotoxicity of cells that express normal levels of HER2. Previous reports suggest that low-fucose IgG₁s could mediate ADCC at low antigen densities at which their corresponding high-fucose counterparts do not induce ADCC (39). However, our ADCC analysis using MCF-10A cells (commonly used as a model for nontransformed low HER2-expressing mammary epithelial cells) failed to detect any significant ADCC using afucosylated trastuzumab.

Because trastuzumab does not bind to murine ErbB2 (HER2), the mouse model used in efficacy studies is not suitable for safety studies. However, more detailed safety studies in non-human primates are required. In conclusion, our results support the development of effector function-enhanced antibodies for solid tumor therapy.

Disclosure of Potential Conflicts of Interest

All authors are employees of Genentech, Inc.

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Superior *In vivo* Efficacy of Afucosylated Trastuzumab in the Treatment of HER2-Amplified Breast Cancer

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Keratinocyte growth factor receptor expression in normal colorectal epithelial cells and differentiated type of colorectal cancer

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Abstract. The keratinocyte growth factor receptor, also known as KGFR/FGFR2 IIIb, is mainly localized in epithelial cells and participates in the proliferation of these cells. In the present study, we attempted to clarify the expression and role of KGFR in human colorectal cancer. The KGFR protein was detected in several colorectal cancer cell lines. Immunohistochemically, KGFR was expressed on the luminal surface of epithelial cells in normal colorectal tissues. KGFR was expressed in 35 of 56 (62.5%) colorectal cancer patients and localized at the center of the cancer nests. Cytokeratin 20 (CK 20) colocalized with KGFR. In contrast, Ki-67 localized at the periphery of the cancer nests. Clinicopathologically, a high level of KGFR expression was associated with well-differentiated histological type ($p < 0.0001$) and shallow wall invasion ($p < 0.0174$). These findings indicate that KGFR may play important roles in the differentiation of normal colorectal epithelial cells and establishment of the well-differentiated histological type of colorectal cancer cells.

Introduction

A high percentage of patients with colorectal cancer over-express a number of growth factor receptors, including the hepatocyte growth factor receptor (c-Met), insulin-like growth factor receptor I (IGF-IR), epidermal growth factor receptor (EGFR) and Tie-2 receptor (1-4). The expression of EGFR, which is a receptor of various ligands such as epidermal growth factor (EGF), transforming growth factor (TGF)- α , and heparin-binding epidermal growth factor (HB-EGF,

heregulin and β -cellulin), was reported as an independent predictor of decreased survival of rectal cancer patients (3). A high level of serum soluble Tie-2 in colorectal cancer patients is associated with early metastases (4). The inhibition or down-regulation of these growth factor receptors is considered a novel therapeutic strategy for colorectal cancer. It was reported that the down-regulation of c-Met inhibits colorectal cancer cell growth in liver tissues (1). Cyclooxygenase (COX)-2 inhibitors and/or angiotensin-converting enzyme (ACE) inhibitors suppress colorectal cancer cell growth and invasion via an IGF-IR-dependent pathway (2).

The keratinocyte growth factor receptor (KGFR), also known as fibroblast growth factor receptor (FGFR) 2 IIIb, is a member of the FGFR family. The FGF family includes at least 22 members, which range in molecular mass from 17 to 34 kDa and share a 13-71% amino acid identity (5). Biological activities of the FGFs are mediated by a family of type I transmembrane tyrosine kinase receptors that undergo dimerization and autophosphorylation after ligand binding. Four distinct genes encode closely related FGF receptors, FGFR 1-4. KGFR is the product of an alternative splice variant of the *Fgfr2* gene (6). KGFR is specifically localized in epithelial cells, and cell growth is induced by FGF-1, FGF-3, the keratinocyte growth factor (KGF/FGF-7) or FGF-10 (7). Mice null for the *Fgfr2 IIIb* isoform, but retaining *Fgfr2 IIIc*, show the dysgeneses of the kidneys, salivary glands, adrenal glands, thymus, pancreas, skin, otic vesicles, glandular stomach and hair follicles, and an agenesis of the lungs, anterior pituitary, thyroid, teeth and limbs (8-10). KGFR is expressed in epithelial cells throughout the gastrointestinal tract, and an intraperitoneal injection of KGF enhances normal intestinal epithelial cell growth (11). Regarding cancerous tissues, KGFR expression was reported in pancreatic cancer and colorectal cancer cells (12,13). Pancreatic cancer cell line growth is stimulated by recombinant KGF administration (14). Furthermore, a dose-dependent cell growth stimulated by KGF was observed in well-differentiated colorectal cancer cell lines (15). These lines of evidence suggest that KGFR plays an important role in epithelial cell growth including cancer cells.

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Key words: keratinocyte growth factor receptor, colorectal cancer, Ki-67, cytokeratin 20, immunohistochemistry

Table I. Clinicopathological findings and expression of KGFR.

Characteristics	KGFR (+)	KGFR (-)	p-value
Gender			NS ^a
Female	16	5	
Male	19	16	
Age			NS ^b
≤60	13	9	
61-70	14	9	
71-80	6	3	
≥81	2	0	
Histological type			0.0001 ^a
Well	29	2	
Moderately	6	17	
Poorly	0	2	
Macroscopic finding			NS ^a
Type 1	4	1	
Type 2	26	18	
Type 3	5	2	
Depth			0.0174 ^b
MP	4	0	
SS	28	15	
SE	3	6	
Lymphatic invasion			NS ^b
1	16	7	
2	19	10	
3	0	4	
Venous invasion			NS ^b
0	31	15	
1	4	6	
Regional lymph node metastasis			NS ^b
0	16	8	
1	9	6	
2	7	4	
3	0	1	
4	3	2	

NS, not significant. ^aχ² test; ^bMann-Whitney U test.

On the other hand, KGF fails to effectively stimulate colorectal cancer cell line growth established from undifferentiated cancer or from a metastatic nodule (12). FGF-10 does not enhance the growth of several cancer cell lines, including colorectal cancer cell lines (16). In the fetal lungs, dominant negative KGFR blocks airway branching and epithelial cell differentiation, without inhibiting tracheal outgrowth (17). A stable transfectant of the *Kgfr* gene of salivary adenocarcinoma cells induces cancer cell differentiation and apoptosis (18). All these lines of evidence suggest that KGFR may have roles other than cell growth. The roles of KGFR in colorectal

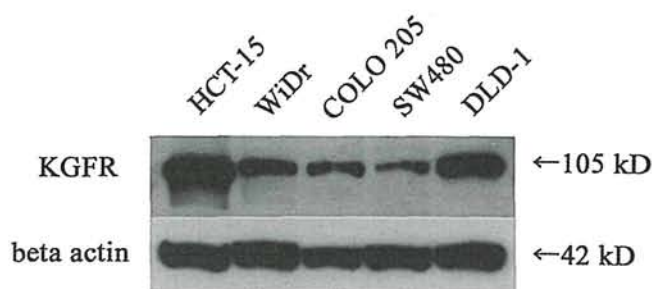


Figure 1. Western blot analysis of KGFR protein in colorectal cancer cell lines. The 105 kDa bands, corresponding to the KGFR protein, were detected in HCT-15, WiDr, COLO 205, SW480 and DLD-1 colorectal cancer cells (upper panel). The β-actin protein with molecular weight of 42 kDa was equally detected (lower panel).

epithelial cells and colorectal cancer cells are not well clarified. In the present study, we attempted to examine the expression and roles of the KGFR protein in normal colorectal tissues and colorectal cancer cells.

Materials and methods

Materials. The following were purchased: anti-rabbit IgG-HRP secondary antibody from Santa Cruz Biotech. (Santa Cruz, CA, USA); Antibiotic-Antimycotic from Invitrogen Corp. (Carlsbad, CA, USA); mouse anti-human cytokeratin 20 (CK 20) monoclonal antibody and mouse anti-human Ki-67 monoclonal antibody from Dako Japan Co., Ltd., (Tokyo, Japan); Histofine Simple Stain Max PO (R) and (M) kits from Nichirei (Tokyo, Japan); an Immobilon PVDF membrane from Millipore (Yonezawa, Japan); M-PER Mammalian Protein Extraction reagent, a BCA protein assay kit and Super Signal West Pico Chemiluminescent substrates from Pierce (Rockford, IL, USA); superfrosted slides with a MAS coat from Matsunami Glass Ind., Ltd., (Osaka, Japan); and a malinol mounting medium from Mutoh Chemical Co. (Tokyo, Japan). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

Human colorectal cancer cell lines. HCT-15, WiDr, COLO 205, SW480 and DLD-1 cell lines were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Cells were grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B at 37°C under a humidified 5% CO₂ atmosphere.

Human colorectal cancer tissues. Colorectal carcinoma tissue samples were obtained from 21 female and 35 male patients during proctocolectomy for colorectal cancer at the Institute of Gastroenterology, Second Hospital of Nippon Medical School (Table I). The mean age of the cancer patients was 63.4 years, with a range of 36 to 87 years. According to Dukes classification, there were 3 patients with Duke's A, 21 with Duke's B and 32 with Duke's C colorectal cancers. Histologically, there were 31 well-differentiated, 23 moderately-differentiated and 2 poorly-differentiated adenocarcinomas. Normal colorectal tissue samples were also

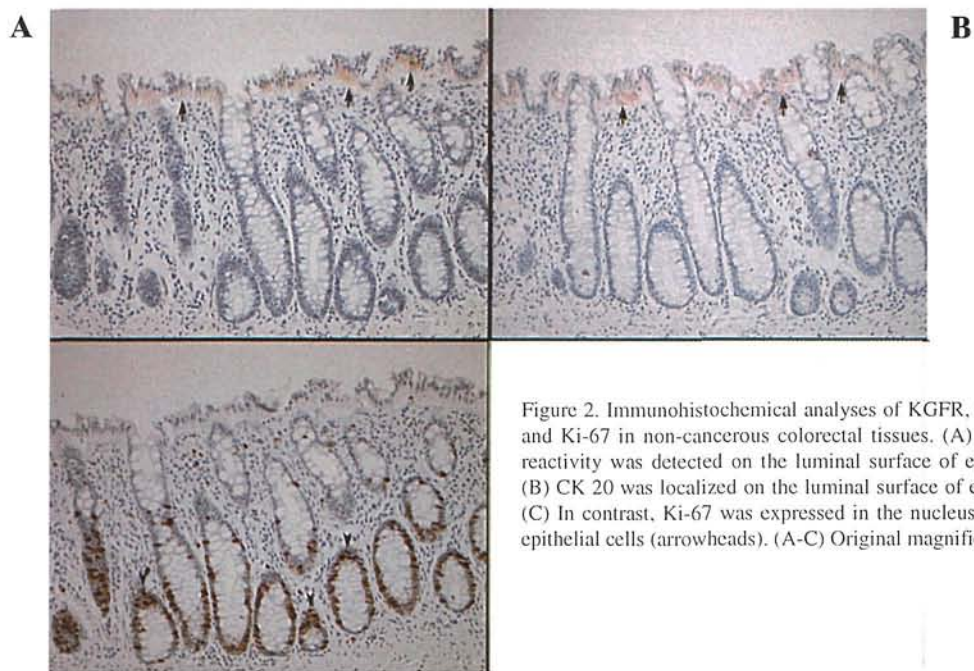


Figure 2. Immunohistochemical analyses of KGFR, cytokeratin 20 (CK 20) and Ki-67 in non-cancerous colorectal tissues. (A) KGFR weak immunoreactivity was detected on the luminal surface of epithelial cells (arrows). (B) CK 20 was localized on the luminal surface of epithelial cells (arrows). (C) In contrast, Ki-67 was expressed in the nucleus of basal and parabasal epithelial cells (arrowheads). (A-C) Original magnification, x200.

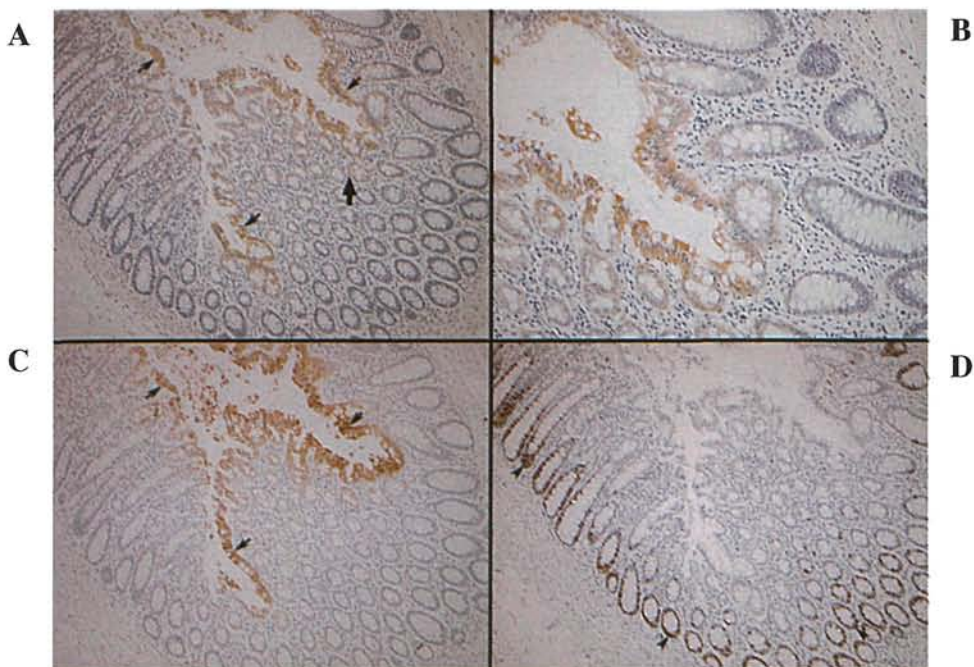


Figure 3. Immunohistochemical analyses of KGFR, CK 20 and Ki-67 in human colorectal tissues forming large crypts close to cancer cells. (A and B) KGFR immunoreactivity was observed on the luminal surface of epithelial cells in large crypts (small arrows). (C) CK 20 was localized on the luminal surface of epithelial cells of large crypts (arrows). (D) Ki-67 was localized in the nucleus of basal and parabasal ductal cells (arrowheads). The large arrow in (A) denotes the area magnified in (B). (A, C and D) Original magnification, x100; and (B) x200.

obtained from the surgical margin at least 10 cm away from each cancerous growth of the same patient. The tissues were fixed in 10% paraformaldehyde solution (PFA) for 18-20 h and then embedded in paraffin. All studies were approved by the Human Ethics Committee of the Second Hospital of Nippon Medical School.

Western blot analysis. The anti-KGFR antibody used was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids of the human KGFR protein as previously reported (Accession no. Q96KL9) (18). The proteins from colorectal cancer cell lines were subjected

to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to Immobilon PVDF transfer membranes, which were then incubated for 16 h with the anti-KGFR antibody. The membranes were sequentially washed and incubated with secondary HRP-conjugated anti-rabbit IgG antibody for 60 min. After washing, the blots were visualized by enhanced chemiluminescence. To confirm specificity of the positive band, the anti-KGFR antibody was preincubated with a blocking peptide of KGFR prior to Western blot analysis, and then subjected to Western blot analysis following the same protocol, as described above.

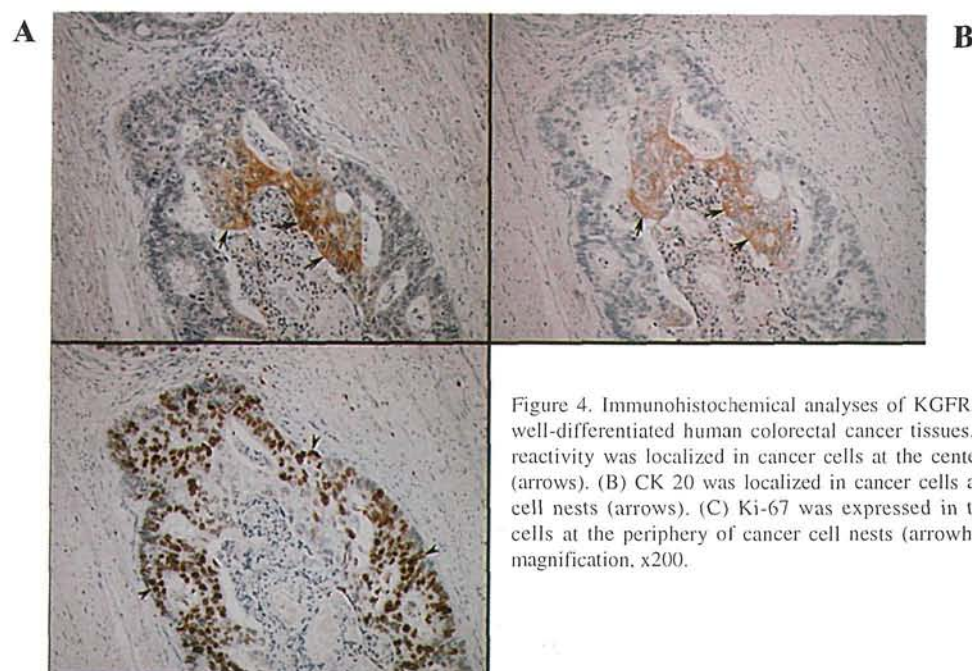


Figure 4. Immunohistochemical analyses of KGFR, CK 20 and Ki-67 in well-differentiated human colorectal cancer tissues. (A) KGFR immunoreactivity was localized in cancer cells at the center of cancer cell nests (arrows). (B) CK 20 was localized in cancer cells at the center of cancer cell nests (arrows). (C) Ki-67 was expressed in the nucleus of cancer cells at the periphery of cancer cell nests (arrowheads). (A-C) Original magnification, x200.

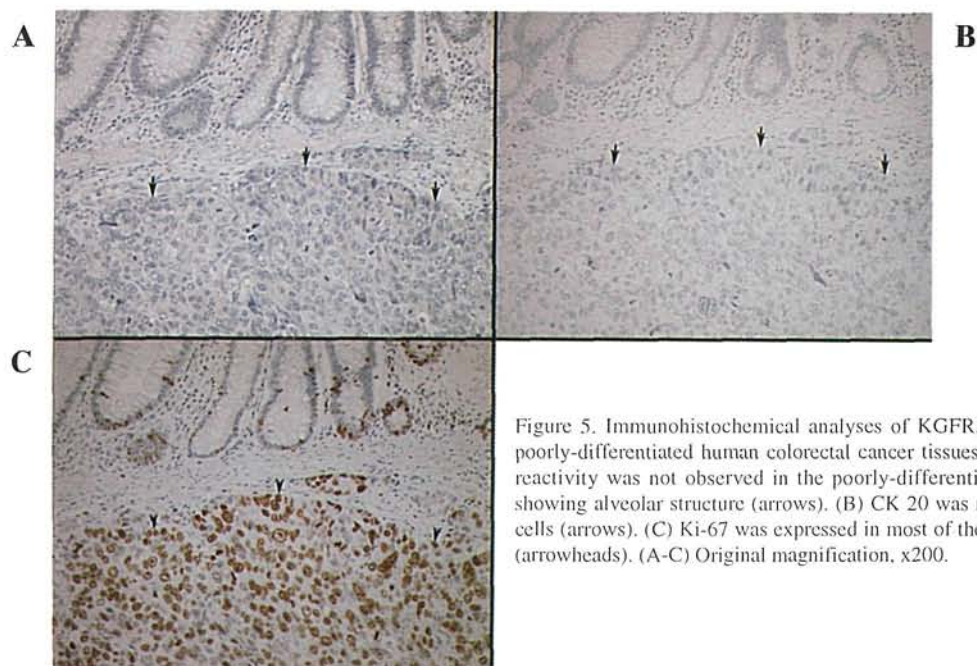


Figure 5. Immunohistochemical analyses of KGFR, CK 20 and Ki-67 in poorly-differentiated human colorectal cancer tissues. (A) KGFR immunoreactivity was not observed in the poorly-differentiated adenocarcinoma, showing alveolar structure (arrows). (B) CK 20 was not localized in cancer cells (arrows). (C) Ki-67 was expressed in most of the nuclei of cancer cells (arrowheads). (A-C) Original magnification, x200.

Immunohistochemistry. The same anti-KGFR antibody used for Western blot analysis, and anti-Ki-67 and anti-CK 20 antibodies were utilized for immunohistochemistry. Paraffin-embedded sections (3 μ m) were subjected to immunostaining using a Histofine Simple Stain Max PO (R) kit for KGFR, and a MAX PO (M) kit for Ki-67 and CK 20. After deparaffinization, the tissue sections for Ki-67 and CK 20 were preheated in 10 mmol/l citrate buffer (pH 6.0) for 15 min at 121°C in an autoclave oven. Then, the endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min. The tissue sections were incubated with the anti-KGFR (1:2000 in dilution), -Ki-67 (1:100 in dilution) or -CK 20 (1:100 in dilution) antibody in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 16 h at 4°C. The bound

antibodies were detected with the Simple Stain Max PO (R) or (M) reagent using diaminobenzidine tetrahydrochloride as the substrate. The sections were counterstained with Mayer's hematoxylin.

Statistical analysis. To analyze the correlation between KGFR expression and clinicopathological features, the χ^2 test or Mann-Whitney U test was used. A value of $p < 0.05$ was considered statistically significant.

Results

Western blot analysis. To characterize KGFR protein expression in colorectal cancer cell lines, total protein was extracted from HCT-15, WiDr, COLO 205, SW480 and

DLD-1 cells. Western blot analysis of these cell lines using the anti-KGFR antibody showed a single 105-kDa band corresponding to the KGFR protein (Fig. 1, upper panel). A 42-kDa band corresponding to the β -actin protein was also detected in all five cancer cell lines (Fig. 1, lower panel). Western blot analysis using the anti-KGFR antibody with a blocking peptide of KGFR did not yield the 105-kDa band (data not shown).

Immunohistochemistry. To confirm the localization of the KGFR protein in human colorectal cancer tissues, immunohistochemical staining was performed. In non-cancerous colorectal tissues, a weak KGFR immunoreactivity was detected in the cell membrane and cytoplasm of the luminal surface of epithelial cells (Fig. 2A, arrows). CK 20 localized on the luminal surface of epithelial cells similar to KGFR in serial sections (Fig. 2B, arrows). In the serial sections, Ki-67 immunoreactivity was detected in the nuclei of ductal cells in the basal and parabasal regions (Fig. 2C, arrowheads). In proliferating large crypts of colorectal tissues adjacent to cancer cells, KGFR was highly expressed on the luminal surface of epithelial cells (Fig. 3A, arrows; and 3B). CK 20 was localized on the luminal surface of epithelial cells of large crypts similar to KGFR (Fig. 3C, arrows). On the contrary, Ki-67 was localized in the basal and parabasal epithelial cells (Fig. 3D, arrowheads). In well-differentiated colorectal cancer tissues, KGFR strongly localized at the center of the cancer cell nests (Fig. 4A, arrows). CK 20 colocalized with KGFR in cancer cells at the center of the cancer cell nests (Fig. 4B, arrows). In contrast, Ki-67 was localized in the nucleus of cancer cells at the periphery of the cancer cell nests (Fig. 4C, arrowheads). In poorly-differentiated colorectal cancer tissues, Ki-67 was localized in the nucleus of most of cancer cells (Fig. 5C, arrowheads). In contrast, KGFR and CK 20 immunoreactivities were not observed in cancer cells (Fig. 5A and B, respectively, arrows). The detailed clinicopathological features of 56 patients are presented in Table I. KGFR expression was statistically associated with well differentiated histological type ($p < 0.0001$) and shallow wall invasion ($p < 0.0174$). No other significant association was detected in this study.

Discussion

The KGFR protein, almost identical in molecular weight to that in human colorectal tissues, was highly expressed in all five colorectal cancer cell lines (12). The KGFR protein expression level was higher in DLD-1 and HCT-15 cells than in other cell lines. These two cell lines are strongly suggested to genetically originate from the same patient (19,20). DLD-1 and HCT-15 synthesize carcinoembryonic antigen (CEA) and the cells are suggested to originate from well-differentiated adenocarcinomas. KGF induces the growth of well-differentiated colorectal cells, but does not stimulate undifferentiated cancer cell growth (12,15). An increased expression level of KGFR in some well-differentiated adenocarcinomas may be associated with a stimulatory growth effect of ligands on colorectal cancer cells. To our knowledge, this is the first report of KGFR protein expression in colorectal cancer cell lines.

The Ki-67 antigen, a cell proliferation marker, is expressed in the nuclei of cells during all the active phases of the cell cycle (G1, S, G2 and M phases), and its expression was examined in colorectal tissues. Interestingly, KGFR was not localized in proliferating cells in normal colorectal tissues, but on the luminal surface of post-proliferation epithelial cells. Many types of growth factor synthesized by colorectal cancer cells are considered to stimulate the growth of crypts close to cancer cells. However, KGFR expression was not detected in proliferating cells of large proliferating crypts close to cancer cells. KGFR was highly expressed on the luminal surface of post-proliferation epithelial cells of crypts. Colorectal epithelial cells are considered to proliferate in the proliferation zone at the basal region of glands, and then migrate to the surface of glands. After proliferation, the cells differentiate to mature ductal cells, which have villi on the luminal surface and synthesize mucin. KGFR-transfected salivary adenocarcinoma cells, which overexpress the KGFR protein, showed a decreased tyrosine-phosphorylation activity of FGF receptor substrate 2, and thus differentiated. KGFR-transfected adenocarcinoma cells exhibited duct-like and acinar-like structures, and newly-synthesized α -amylase and lactoferrin. The growth rate of transfected cancer cells decrease and many cells undergo apoptosis (18). On the other hand, recombinant KGF induces the motility of breast cancer cells, whereas KGFR inhibitors effectively inhibit cancer cell motility (21,22). The results of our study may provide new findings that support the notion that KGFR plays roles in post-proliferation events, such as cell differentiation, cell motility or apoptosis on the luminal surface of normal colorectal cells.

KGFR expression in colorectal cancer tissues was detected in 62.5% of cancer patients, and KGFR localization in cancer tissues showed a characteristic pattern. Proliferating cells localized at the periphery of the cancer cell nests, however KGFR was localized at the center of the nests. Immunohistochemically, c-Met was reported to be localized in the invasive front of colon cancer tissues and its localization suggests that c-Met may play a role in the growth of cancer cells (23). KGFR was not detected in proliferating cancer cells, and its localization pattern may indicate that KGFR is expressed in post-proliferation and differentiated cancer cells. KGFR-negative cases showed deeper invasion of colon than KGFR-positive cases. Furthermore, the KGFR expression level was significantly high in patients with well-differentiated adenocarcinoma, suggesting that KGFR is associated with the formation or maintenance of the duct-like structure.

KGFR-positive cells in normal and cancerous colorectal tissues colocalized with CK 20. CK 20 is an intermediate filament-forming cytoskeletal protein, and was reported to be localized on the luminal surface of epithelial cells in the stomach and intestines (24,25). CK 20 is expressed in most colorectal cancer cells, and its expression is one useful diagnostic marker of colorectal cancer (25). CK 20 colocalized with KGFR in non-proliferating cells in colorectal cancer cell nests. The *Kgfr* and *Ck 20* genes in the chromosomes are located in 10q26 and 17q21.2, respectively (26,27). The promoter regions of the mouse *Kgfr* gene are the SpI-binding sites CCGCCC and GGGCGG, which are present in the EGFR promoter and platelet-derived growth factor (PDGF) α . An

additional motif, GGAGG, is also present in the promoter of human epidermal growth factor receptor (HER) 2/neu (28). However, it is unclear whether the promoter region of the *Ck 20* gene contains consensus sequences similar to that of the *Kgfr* gene. The correlation between KGFR and CK 20 protein expressions remains unclarified. Growth factors or cytokines may contribute to the coexpression of KGFR and CK 20. Further examination using KGFR or CK 20 inhibition techniques, such as the use of morpholino antisense oligonucleotides or siRNA in colorectal cancer cells, may be useful in clarifying the relationship between KGFR and CK 20 expressions.

In summary, KGFR was expressed in colorectal cancer cell lines, and was detected in differentiated cancer cells at the center of cancer cell nests. In normal colorectal tissues, KGFR was expressed on the luminal surface of epithelial cells. KGFR colocalized with CK 20, but not with Ki-67 in normal and colorectal cancer tissues. KGFR may play important roles in the differentiation of normal colorectal epithelial cells and the establishment of the well-differentiated histological type of colorectal cancer cells.

Acknowledgements

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REIVINDICACIONES

1. Una composición que comprende una pluralidad de anticuerpos anti-FGFR2IIIb, donde cada anticuerpo anti-FGFR2IIIb comprende regiones variables de cadena pesada y cadena ligera, donde la región variable de cadena pesada comprende:
 - (i) HVR-H1 que comprende la secuencia de aminoácidos de la SEQ ID NO: 6;
 - (ii) HVR-H2 que comprende la secuencia de aminoácidos de la SEQ ID NO: 7; y
 - (iii) HVR-H3 que comprende la secuencia de aminoácidos de la SEQ ID NO: 8;y la región variable de cadena ligera comprende:
 - (iv) HVR-L1 que comprende la secuencia de aminoácidos de la SEQ ID NO: 9;
 - (v) HVR-L2 que comprende la secuencia de aminoácidos de la SEQ ID NO: 10; y
 - (vi) HVR-L3 que comprende la secuencia de aminoácidos de la SEQ ID NO: 11;donde al menos 95% de los anticuerpos anti-FGFR2IIIb en la composición están afucosilados.
2. La composición de la reivindicación 1, donde el dominio variable de cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 4.
3. La composición de la reivindicación 1 o la reivindicación 2, donde el dominio variable de cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 5.
4. La composición de la reivindicación 1, donde el dominio variable de cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 4 y el dominio variable de cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 5.
5. La composición de cualquiera de las reivindicaciones 1 a 4, donde la cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 2.
6. La composición de cualquiera de las reivindicaciones 1 a 5, donde la cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 3.
7. La composición de la reivindicación 1, donde la cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 2 y la cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 3.
8. La composición de cualquiera de las reivindicaciones 1 a 7, donde los anticuerpos son anticuerpos monoclonales.
9. La composición de cualquiera de las reivindicaciones 1 a 8, donde los anticuerpos son anticuerpos quiméricos.

10. La composición de cualquiera de las reivindicaciones 1 a 9, donde los anticuerpos son anticuerpos humanizados.
11. La composición de cualquiera de las reivindicaciones 1 a 10, donde los anticuerpos carecen de fucosa en Asn297.
12. La composición de cualquiera de las reivindicaciones 1 a 11, donde los anticuerpos comprenden una región constante de cadena ligera κ .
13. La composición de cualquiera de las reivindicaciones 1 a 12, donde los anticuerpos comprenden una región constante de cadena pesada IgG1.
14. Un método para producir anticuerpos anti- FGFR2IIIb afucosilados, dicho método comprende la preparación de células huésped que comprenden el ácido nucleico que codifica el anticuerpo anti- FGFR2IIIb de cualquiera de las reivindicaciones 1 a 13, el cultivo de las células huésped en condiciones adecuadas para expresar el ácido nucleico que codifica el anticuerpo anti- FGFR2IIIb, y la recuperación de los anticuerpos anti- FGFR2IIIb producidos por las células huésped.
15. El método de la reivindicación 14, en el que el cultivo se realiza en condiciones adecuadas para la producción de anticuerpos anti- FGFR2IIIb afucosilados.
16. El método de cualquiera de las reivindicaciones 14 o 15, donde al menos 95% de los anticuerpos anti-FGFR2IIIb producido por la célula anfitriona están afucosilados.
17. El método de la reivindicación 16 donde la fucosa es indetectable en los anticuerpos anti-FGFR2IIIb producidos por la célula anfitriona.
18. El método de la reivindicación 16 o 17, en donde la fucosa es detectada por un método que comprende HPLC, electroforesis capilar, o espectrometría de masas MALDI-TOF.
19. Una composición farmacéutica que comprende la composición de cualquiera de las reivindicaciones 1 a 13 y un portador farmacéuticamente aceptable.

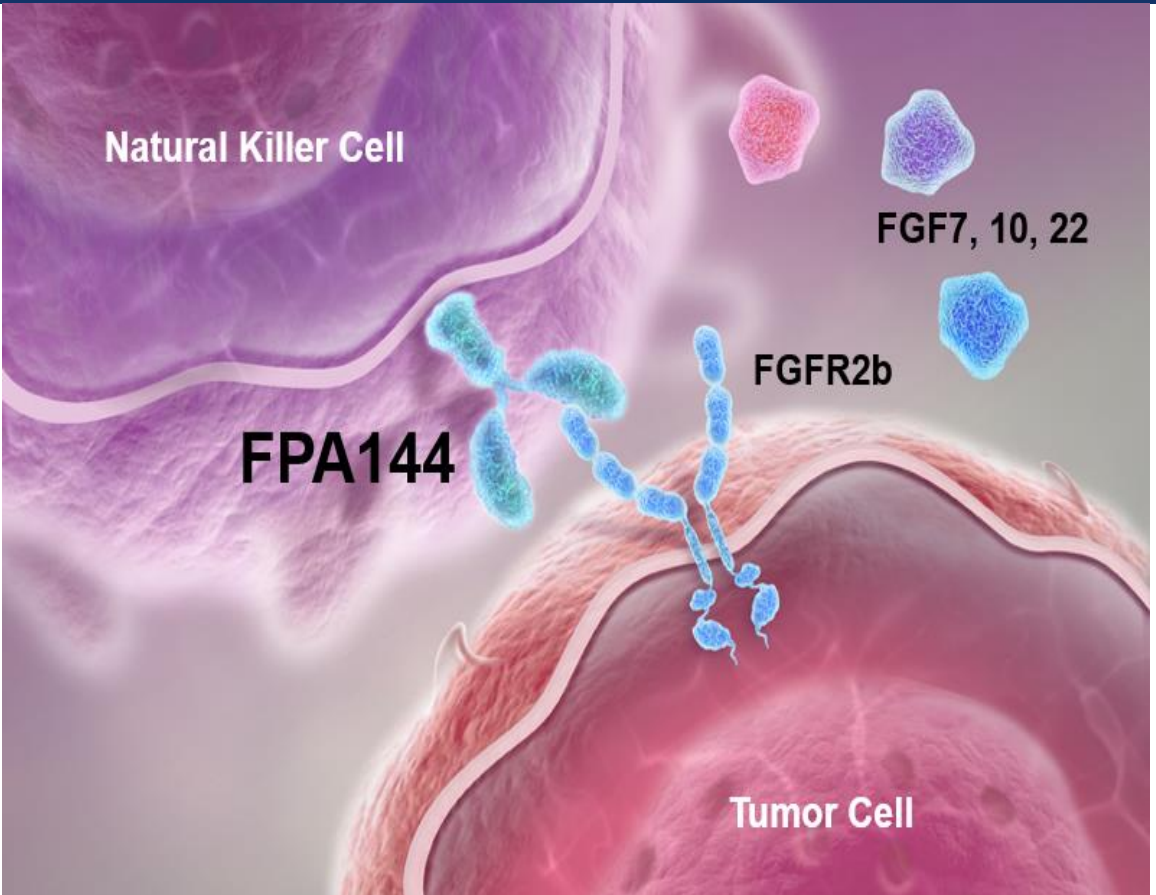
Updated Antitumor Activity and Safety of FPA144, an ADCC-enhanced, FGFR2b Isoform-specific Monoclonal Antibody, in Patients with FGFR2b+ Gastric Cancer

#4067

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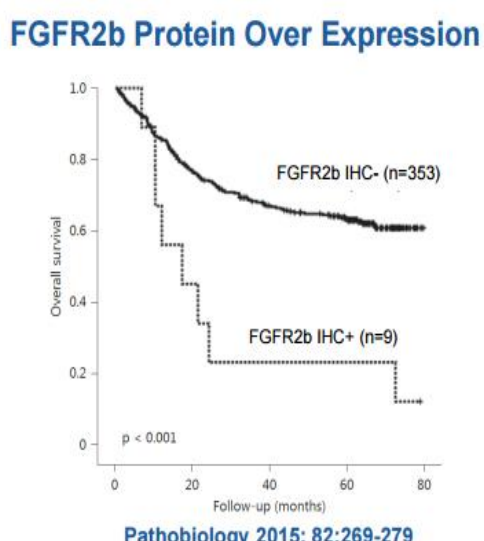
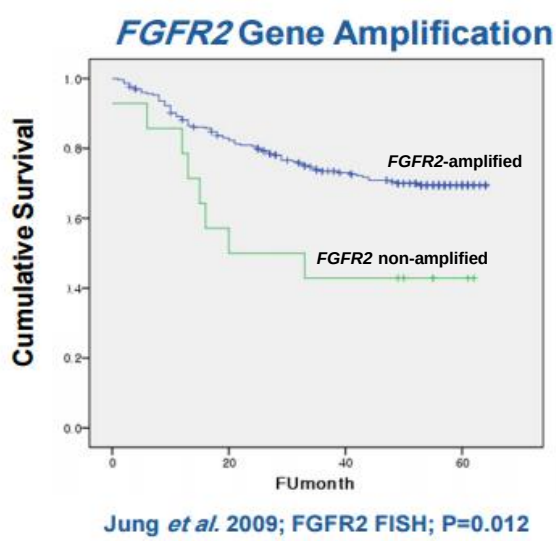
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Background on FPA144



- FPA144, an afucosylated, humanized IgG1 monoclonal antibody, selectively binds the b isoform of FGFR2
- FPA144 therapy is designed to deliver 2 distinct anti-tumor effects:
 - FPA144 inhibits ligand binding to FGFR2b and blocks receptor activation and downstream signaling (Gemo, 2014)
 - FPA144 is glycoengineered for enhanced antibody-dependent cell-mediated cytotoxicity (ADCC)
- Isoform specificity of FPA144 may improve tolerability
- FPA144 is a potential therapy for patients whose gastric or bladder cancers overexpress FGFR2b or amplify the *FGFR2* gene

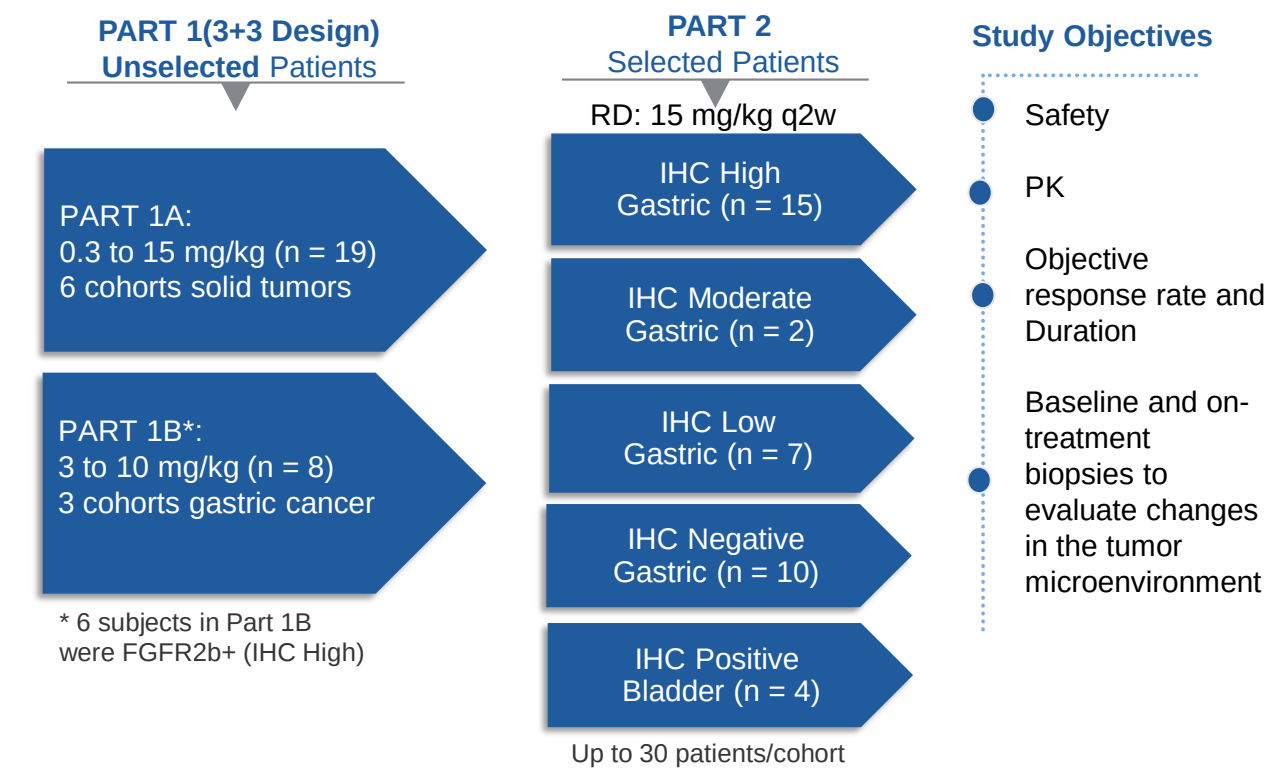
FGFR2b Overexpression and *FGFR2* Gene Amplification are Associated With Poor Prognosis



Patients with gastric or gastroesophageal cancer (GE Cancer) for whom FPA144 is a potential therapy can be identified by 3 methods:

- Tissue for *FGFR2* gene amplification by FISH
- Tissue for FGFR2b overexpression by IHC
- Blood for *FGFR2* gene amplification by circulating DNA (ctDNA)

Clinical Study Design Overview (NCT 02318329)



- Initially, patient selection for PART 2 required both tissue IHC and FISH amplification testing (n = 12), but FISH was discontinued after demonstrating high correlation with IHC results (100%, n = 12).
- High correlation between FGFR2b protein overexpression (IHC) and *FGFR2* gene amplification (FISH) was also supported by external data (Ahn S., et. al., Mod Pathol. 2016)

Study Population as of Data Cut-off March 20, 2017

Table 1				
Patient Baseline Characteristics	All Patients N = 64	All Gastric Cancer* N = 41	IHC High Gastric Cancer N = 21	IHC Negative* Gastric Cancer N = 11
Median Age (yrs) (min, max)	58 (29, 86)	55 (29, 77)	46 (29, 67)	57 (44, 77)
Gender Male N (%)	36 (56.3%)	21 (51.2%)	7 (33.3%)	6 (54.5%)
Race N (%)				
White	27 (42.2%)	7 (17.1%)	3 (14.3%)	3 (27.3%)
Asian	35 (54.7%)	32 (78.0%)	16 (76.2%)	8 (72.7%)
ECOG N (%)				
0	20 (31.3%)	13 (31.7%)	4 (19.0%)	4 (36.4%)
1	44 (68.8%)	28 (68.3%)	17 (81.0%)	7 (63.6%)
Prior Therapies Median (N) (min, max)	3 (1, 8)	3 (1, 6)	3 (1, 6)	4 (2, 5)

* Includes both FGFR2b+ and FGFR2b- gastric cancer patients (with 15 patients meeting the criteria of IHC High, 2 as IHC Moderate, 7 as IHC Low, and 11 as IHC Negative or with an unknown FGFR2b status).
* IHC Negative Gastric Cancer population includes patients with unknown FGFR2b status (n = 2) from Part 1A.

Overall Safety Summary

- No DLTs during dose escalation (MTD not reached)
- No grade 4 or higher treatment-related AEs
- 16 reported SAEs, five treatment-related (across 4 patients):
 - Grade 2 ulcerative keratitis
 - Grade 3 hypersensitivity infusion reaction
 - Grade 3 nausea and vomiting in a single patient
 - Grade 2 ocular limbic stem cell deficiency* (drug discontinued)
- No hyperphosphatemia or retinal toxicity

*Reported after the data cut-off date

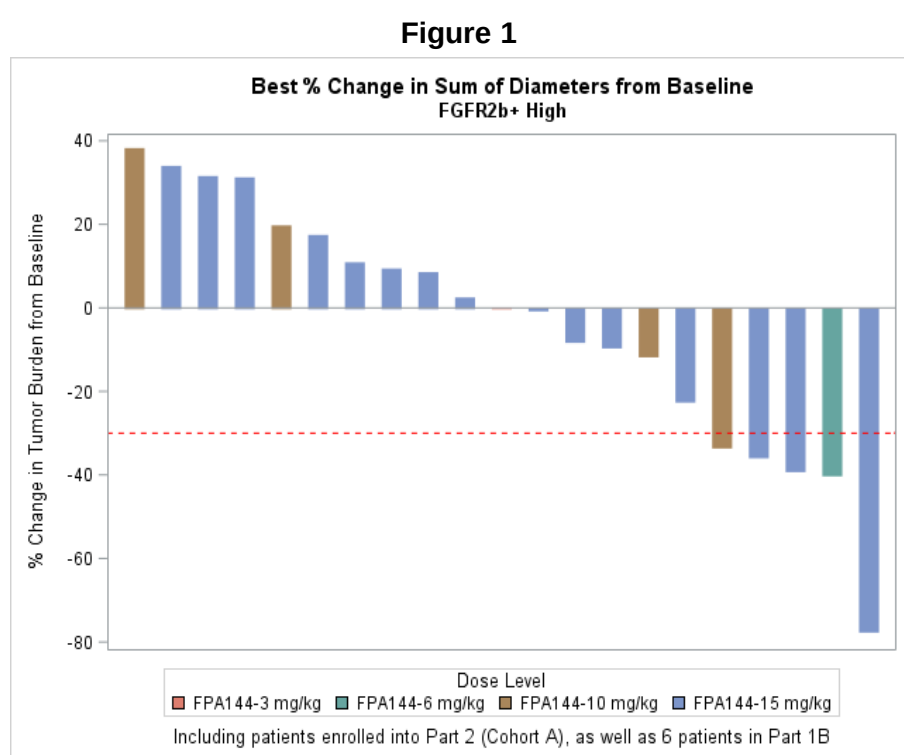
Ocular-related Toxicity

- In repeat-dose toxicology studies, FPA144-treated rats and cynomolgus monkeys displayed a dose-dependent thinning of the corneal epithelium
- Based on these findings, comprehensive ophthalmologic exams, including serial slit lamp examinations, were included in FPA144-001 clinical trial to monitor for ocular-related toxicities
- 23.4% (15/64) of patients who received FPA144 reported one or more ocular-related toxicities (all grade 2 or less)
 - The most common ($\geq 5\%$) ocular-related events include dry eye (10 patients 15.6%) and increased lacrimation (4 patients 6.3%)
 - 2 symptomatic cases associated with corneal-related toxicity (Table 3)

Table 3: Cases of Symptomatic Corneal-related Toxicity

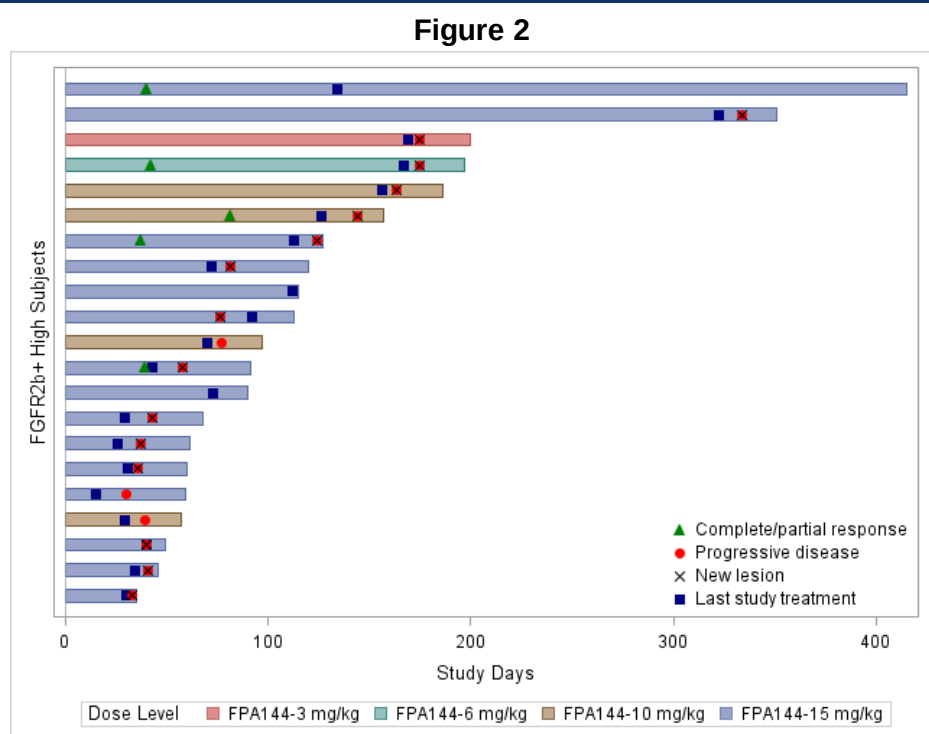
Ocular-related AE	Study Day Onset of Event	Dose	Action	Outcome
Grade 2 Ulcerative Keratitis	Day 100	15 mg/kg	Moxifloxacin administration and FPA144 interruption for 1 dose	Resolution of event. Patient continued treatment with no further ocular complaints.
Grade 2 Limbic Stem Cell Deficiency	Day 448	10 mg/kg	FPA144 permanently discontinued	Within two months of last FPA144 administration, improvement of limbic stem cell deficiency and near complete symptomatic resolution.

Best Response in FGFR2b+ (IHC High) Gastric Cancer Patients



- 5 Partial Responses (4 confirmed, 1 unconfirmed) in 21 patients. Objective Response Rate (ORR): 19.0% (5.4%, 41.9%)
- Median number of prior therapies = ~3
- Of the 4 confirmed partial responders, one received pembrolizumab, another received ramucirumab, and a third received onartuzumab, prior to FPA144 therapy.
- Disease Control Rate at 6 weeks: 57.1% (34.0%, 78.2%)
- No objective tumor responses observed in gastric cancer patients with Moderate or Low IHC status as of March 20, 2017

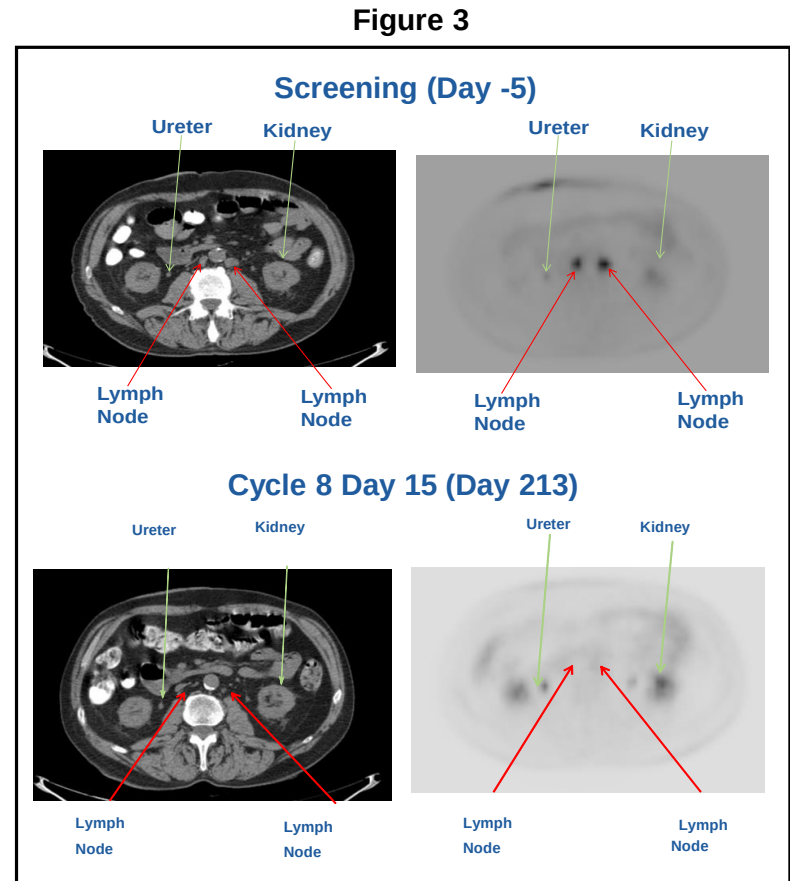
Duration of Treatment in FGFR2b+ (IHC High) Gastric Cancer Patients



- Median Duration of Response (weeks) = 15.4 (9.1, 19.1)
- Median PFS (weeks): 11.0 (5.7, 20.6)

FGFR2b in Urothelial Bladder Cancer (UBC)

- In the dose escalation part 1A, a patient with bladder cancer (IHC 2+) treated with 3mg/kg of FPA144 had a complete response and continues on study for 706 days (Figure 5).
- This observation provided the rationale to explore the use of FPA144 in bladder cancer
- The prevalence of FGFR2b overexpression by IHC in bladder cancer is ~11% in primary tumors and ~14% in metastases
- Based on prevalence and encouraging response, we are currently enrolling FGFR2b-selected bladder cancer patients in Part 2:
 - 4 patients enrolled to date (on treatment 0.1-10 weeks)
 - Too early to evaluate efficacy
- FGF7 (a ligand that binds to FGFR2b) overexpression in bladder cancer correlates with reduced survival



Conclusions

- FPA144 was well tolerated in doses tested up to 15 mg/kg in patients with advanced solid tumors, including patients with gastric cancer
 - No hyperphosphatemia reported
 - Ocular adverse events were all $\leq$ Grade 2. No retinal toxicity reported.
- Confirmed radiographic responses of 19.0% (95% CI: 5.4%, 41.9%) with median duration of response of 15.4 weeks (9.1, 19.1)
- FPA144 efficacy data compares similarly with approved targeted agents in late line gastric cancer which demonstrated low response rates as monotherapy and subsequently demonstrated greater benefit when combined with standard chemotherapy in the front line setting
 - Internal non-clinical data (not shown) demonstrates additive benefit of FPA144 to platinum and 5-FU chemotherapy
- Toxicities observed in this study (n = 64) suggest no overlapping toxicities with platinum and 5-FU chemotherapy
- Data supports further evaluation of the combination of FPA144 with chemotherapy or immunotherapy in FGFR2b-selected gastric and bladder cancer

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Dll1- and Dll4-Mediated Notch Signaling Are Required for Homeostasis of Intestinal Stem Cells

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BACKGROUND & AIMS: Ablation of Notch signaling within the intestinal epithelium results in loss of proliferating crypt progenitors due to their conversion into postmitotic secretory cells. We aimed to confirm that Notch was active in stem cells (SCs), investigate consequences of loss of Notch signaling within the intestinal SC compartment, and identify the physiologic ligands of Notch in mouse intestine. Furthermore, we investigated whether the induction of goblet cell differentiation that results from loss of Notch requires the transcription factor Krüppel-like factor 4 (Klf4). **METHODS:** Transgenic mice that carried a reporter of Notch1 activation were used for lineage tracing experiments. The *in vivo* functions of the Notch ligands Jagged1 (Jag1), Delta-like1 (Dll1), Delta-like4 (Dll4), and the transcription factor Klf4 were assessed in mice with inducible, gut-specific gene targeting (Vil-Cre-ERT²). **RESULTS:** Notch1 signaling was found to be activated in intestinal SCs. Although deletion of Jag1 or Dll4 did not perturb the intestinal epithelium, inactivation of Dll1 resulted in a moderate increase in number of goblet cells without noticeable effects of progenitor proliferation. However, simultaneous inactivation of Dll1 and Dll4 resulted in the complete conversion of proliferating progenitors into postmitotic goblet cells, concomitant with loss of SCs (Olfm4⁺, Lgr5⁺, and Ascl2⁺). Klf4 inactivation did not interfere with goblet cell differentiation in adult wild-type or in Notch pathway-deficient gut. **CONCLUSIONS:** Notch signaling in SCs and progenitors is activated by Dll1 and Dll4 ligands and is required for maintenance of intestinal progenitor and SCs. Klf4 is dispensable for goblet cell differentiation in intestines of adult Notch-deficient mice.

Keywords: GI Development; Knockout Mice; Intestinal Stem Cells; Gene Regulation.

hormone-secreting enteroendocrine cells. Although most differentiated cell types reside within the villus, Paneth cells are found at the bottom of the crypt. They are in close contact with the leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) and olfactomedin-4 (Olfm4) expressing intestinal stem cells (SCs).^{1,2} These SCs, also known as crypt base columnar cells, proliferate constantly¹ and give rise to transient amplifying cells, which localize within the crypt compartment just above the Paneth cells. Transient amplifying cells migrate upward and differentiate at the crypt-villus boundary into one of the 4 known cell types of the small intestine. These regeneration and differentiation processes have to be under stringent control to ensure proper homeostasis.

The Notch cascade mediates cell-to-cell signaling and has been shown to be essential for the maintenance of the proliferative crypt compartment, as well as for the formation of absorptive enterocytes (reviewed in van der Flier and Clevers³). The mammalian Notch receptor-ligand family consists of 4 single transmembrane Notch receptors (Notch1–4) and 5 single transmembrane ligands (Delta-like1, 3, and 4 and Jagged1 and 2 [Dll1, 3, and 4 and Jag1 and 2]).⁴ Ligand-receptor interactions between neighboring cells lead to sequential proteolytic cleavages of the receptor, liberating its intracellular domain (NICD). Once released, NICD translocates to the nucleus and heterodimerizes with the transcription factor CSL (also known as RBP-J in the mouse), inducing transcription of downstream target genes. Inhibition of Notch signaling in the intestinal epithelium using conditional gene targeting of RBP-J or by pharmacologic γ -secretase inhibitors, which block the release of NICD, results in the loss of the proliferative crypt compartment and conversion of crypt progenitors into secretory cells.⁵ We recently showed that Notch1 and Notch2 receptors

Abbreviations used in this paper: DBZ, dibenzazepine; Dll, Delta-like; Jag, Jagged; Klf4, Krüppel-like factor 4; NICD, Notch1 intracellular domain; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; SC, stem cell.

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The epithelium of the small intestine consists of 4 principal cell types: absorptive enterocytes, mucus-secreting goblet cells, antimicrobial Paneth cells, and

act redundantly in mediating Notch signaling in the intestine.⁶ Only simultaneous conditional gene inactivation or antibody-mediated inhibition of both receptors resulted in the complete conversion of the crypt progenitors into postmitotic goblet cells.^{6,7} Taken together, these results suggest that Notch signaling is not only a gatekeeper for proliferating crypt progenitors but is also involved in controlling the balance between secretory and absorptive cell types. However, whether the role Notch plays in SC maintenance reflects direct activation of Notch signaling in intestinal SCs is currently unknown.

Cell fate decisions and differentiation processes within the intestinal epithelium involve the Notch target gene *Hes1*, which represses the basic helix-loop-helix transcription factor *Math1*.⁸ The intestine of *Hes1*-deficient mice contains increased Paneth, goblet, and enteroendocrine cell numbers and has a decrease in absorptive cells,^{8,9} whereas *Math1*-mutant animals have lost most secretory cells and are only colonized by enterocytes.^{10,11} These results suggest that *Hes1*-mediated repression of *Math1* contributes to the regulation of the secretory versus absorptive lineage decision. Another important transcription factor shown to be repressed via Notch-mediated *Hes1* expression is the zinc finger transcription factor Krüppel-like factor 4 (*Klf4*).^{12–14} *Klf4*-deficient mice (postnatal day 1) showed a 90% decrease of goblet cell numbers within the colon, indicating that *Klf4* is important for goblet cell differentiation during fetal life.^{15,16} The fact that blocking Notch signaling correlates with increased *Klf4* messenger RNA expression and results in goblet cell metaplasia, combined with *in vitro* studies showing that Notch or *Hes1* can repress *Klf4* promoter-driven transcription, resulted in a model in which Notch signaling suppresses the goblet cell fate through *Hes1*-mediated repression of *Klf4*.^{12–14}

Here, we identify the physiologic Notch ligands of the murine intestine and show the importance of the Notch pathway for proper intestinal SC homeostasis. Furthermore, our results show that *Klf4* is dispensable for goblet cell differentiation in adult intestines of Notch mutant mice, complementing recent observations that *Math1* acts downstream of Notch in driving goblet cell fate.¹¹

Materials and Methods

Animals

All animal work was conducted according to Swiss national guidelines. This study has been reviewed and approved by the Service Vétérinaire Cantonal of Etat de Vaud. All NIP1::CreERT² study-related animals were housed in the Washington University mouse facility and were approved by the Animal Studies Committee of Washington University.

The Cre-ERT² recombinase activity was induced by injecting 2- to 3-week-old mice with tamoxifen (10 mg/kg body weight in corn oil; Sigma-Aldrich, Buchs, Switzer-

land) intraperitoneally for 5 consecutive days. The γ -secretase inhibitor dibenzazepine (DBZ; 20 μ mol/kg body weight in 0.5% wt/vol hydroxypropyl methylcellulose, 0.1% wt/vol NP-40 in water; Calbiochem-Merk, Zug, Switzerland) was injected intraperitoneally for 4 consecutive days. Mice were analyzed 2 weeks after the last injection or 3 to 5 days after the last injection in case of lethal phenotypes (Dl1-Dl4^{vil-Cre-ERT2}, RBP-J^{vil-Cre-ERT2}, RBP-J - *Klf4*^{vil-Cre-ERT2}, and DBZ-treated mice).

The targeting and screening of NIP1::CreERT² ES cells have been described,¹⁷ and detailed analysis of the animals will be described elsewhere. The NIP1::CreERT² reporter was activated by injecting 1-month-old mice with tamoxifen (220 mg/kg) for 3 consecutive days. Mice were analyzed 8 months after the last injection.

Tissue sample preparation, immunohistochemistry, *in situ* hybridization, Southern blot, and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) are described in Supplementary Materials and Methods.

Results

Notch1 Activation Occurs in Adult Intestinal Stem Cells

Notch signaling in the intestine plays an important role as a gatekeeper of progenitor cells and also as a regulator of the absorptive/secretory lineage.^{5,6} To gain further insight whether Notch signaling might also occur at the level of adult intestinal SCs, we generated a tamoxifen-inducible Notch1 activation-dependent reporter knock-in mouse line, NIP1::CreERT² (Figure 1A).¹⁷ The intracellular domain of the targeted Notch1 allele was replaced with a complementary DNA encoding a 6 $\times$ myc-tagged CreERT² (6mtCreERT²). Binding of Notch ligands to the NIP1::CreERT² will trigger the release of the 6mtCreERT² from the membrane, but only in the presence of tamoxifen can CreERT² enter the nucleus. Within the nucleus, CreERT² will mediate the excision of a floxed stop cassette in reporter strains such as Rosa26R or the Rosa26-EYFP mice and permanently label the cells and all their offspring with LacZ or EYFP, respectively.

To ascertain that adult intestinal SCs were labeled at the time of activation, we examined the intestine of NIP1::CreERT² Rosa LacZ or EYFP mice 8 months after tamoxifen administration for 3 consecutive days. Labeled villi were maintained along the entire intestine, with more labeled villi visible in the duodenum and jejunum (Figure 1B) similar to NIP::CRE mice.¹⁸ Entire crypt-villus units were clearly labeled (Figure 1C), and double immunofluorescence for EYFP and cell type-specific markers identified all the differentiated intestinal cell types: goblet cells (EYFP⁺, DBA⁺ [Dolichos biflorus agglutinin]; Figure 1D), Paneth cells (EYFP⁺, lysozyme⁺; Figure 1E), and enteroendocrine cells (EYFP⁺, chromogranin⁺; Figure 1F). The *in vivo* lineage tracing shows that Notch1 activation occurs within the adult intestinal SCs under physiologic condition; however, the physiologic ligands

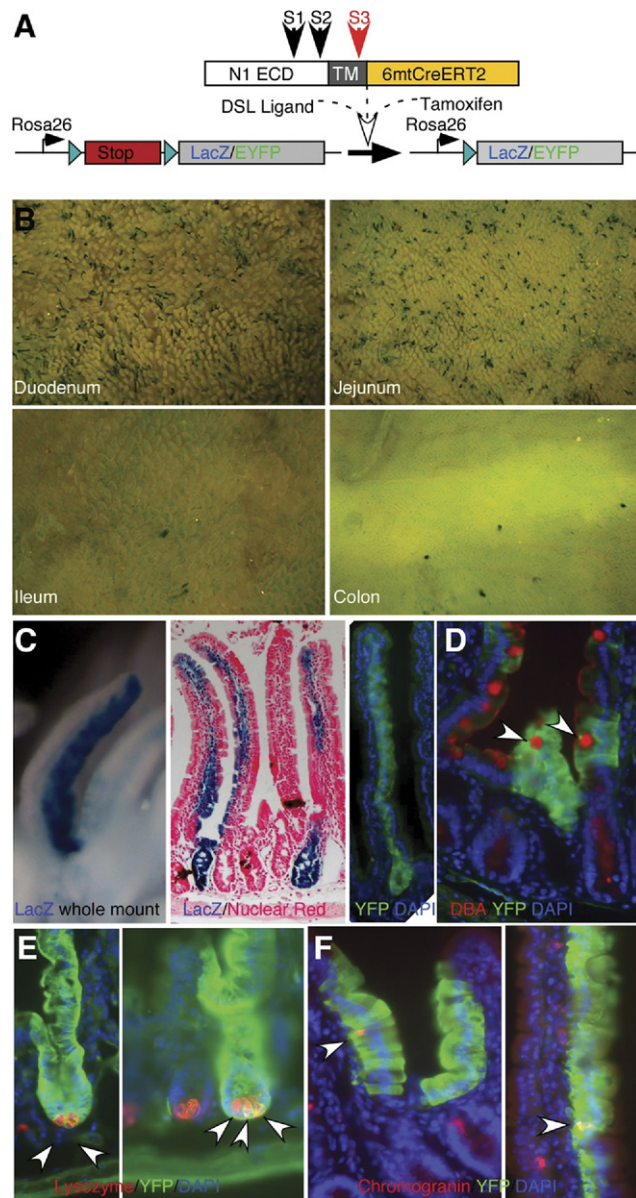


Figure 1. Notch1 is activated in intestinal stem cells. (A) Schematic illustration of NIP1::CreERT² knock-in reporter mice (see text for details). N1 ECD, Notch1 extracellular domain; TM, Notch1 transmembrane domain. (B and C) LacZ whole mount staining and EYFP expression in different intestinal segments 8 months after reporter activation. Note fully labeled crypt-villus unit from both the Rosa LacZ and EYFP reporters. (D–F) Double staining of sections derived from the small intestine of NIP1::CreERT² Rosa EYFP reporter mice for the (D) goblet cell marker DBA, (E) Paneth cell marker lysozyme, and (F) enteroendocrine marker chromogranin. Double-positive cells are highlighted with arrowheads.

triggering Notch signaling within the intestine remain to be identified.

Dll1 Deficiency Induces Increased Goblet Cell Numbers

In situ hybridization-based expression studies as well as LacZ knock-in alleles for Dll1 and Dll4 point to Jag1, Dll1, and Dll4 as potential candidates for the phys-

iological Notch1 and Notch2 ligands in the intestine.^{19–21} We investigated the role of each ligand in intestinal homeostasis using an inducible loss-of-function approach. Specifically, mice carrying floxed alleles for the Dll1,²² Dll4,²³ and Jag1²⁴ genes were intercrossed with transgenic mice expressing Cre-ERT² under the control of the villin promoter (vil-Cre-ERT²).²⁵ Two- to 3-week-old Dll1^{flox/flox} vil-Cre-ERT² (Dll1^{vil-Cre-ERT2}), Dll4^{flox/flox} vil-Cre-ERT² (Dll4^{vil-Cre-ERT2}), and Jag1^{flox/flox} vil-Cre-ERT² (Jag1^{vil-Cre-ERT2}), as well as corresponding littermate controls lacking the vil-Cre-ERT² transgene (control), were injected with tamoxifen on 5 consecutive days and analyzed 2 weeks after the last injection. Successful gene ablation here and in subsequent experiments was confirmed by Southern blot analysis of genomic DNA derived from epithelial cells of the small intestine and colon (Supplementary Figure 1) and/or combined with qRT-PCR for messenger RNA expression of the targeted ligands (Figure 2C and 3D).

Loss of Notch signaling within the small intestine resulted in goblet cell metaplasia accompanied by a loss of proliferating progenitors.^{5,6} Therefore, we performed histologic analysis combined with Alcian blue and Ki67 staining of individual mice gene targeted for each ligand. The small intestine and colon of Dll4 and Jag1 mutant mice revealed no significant differences in morphology and goblet cell number (Figure 2A and B and Supplementary Figure 2). Moreover, the proliferative crypt compartment appeared to be intact (Figure 2A). Lysozyme and synaptophysin antibody staining of intestinal sections derived from Dll4, Dll1, and Jag1 mutant mice identified no change within the Paneth and enteroendocrine cell compartments relative to controls (Supplementary Figure 3). In contrast, inactivation of Dll1 within the small intestine resulted in an average increase of 83% of goblet cells ($P < .0001$) (Figure 2B), reminiscent of Notch1 loss.^{7,18} Despite the increase of goblet cell numbers in Dll1^{vil-Cre-ERT2} animals, there was no significant change of Ki67⁺ crypt progenitor cells compared with control mice.

Taken together, these results suggest that inactivation of individual ligands is not sufficient to recapitulate the phenotypes observed in mice in which Notch signaling was completely abrogated.^{5,6} Nevertheless, inactivation of the Dll1 gene showed a moderate increase in goblet cell numbers, indicating that Dll1 might be one of several physiologic Notch ligands in the crypt compartment.

Dll1 and Dll4 Cooperate to Maintain the Crypt Progenitor Compartment

To investigate whether functional redundancy existed at the level of the ligands as well as the receptors, we generated compound conditional knockout mice of Dll1 with other ligands (Dll1-Jag1^{vil-Cre-ERT2} and Dll1-Dll4^{vil-Cre-ERT2}) to assess functional redundancy. Simultaneous inactivation of both Jag1 and Dll1 within

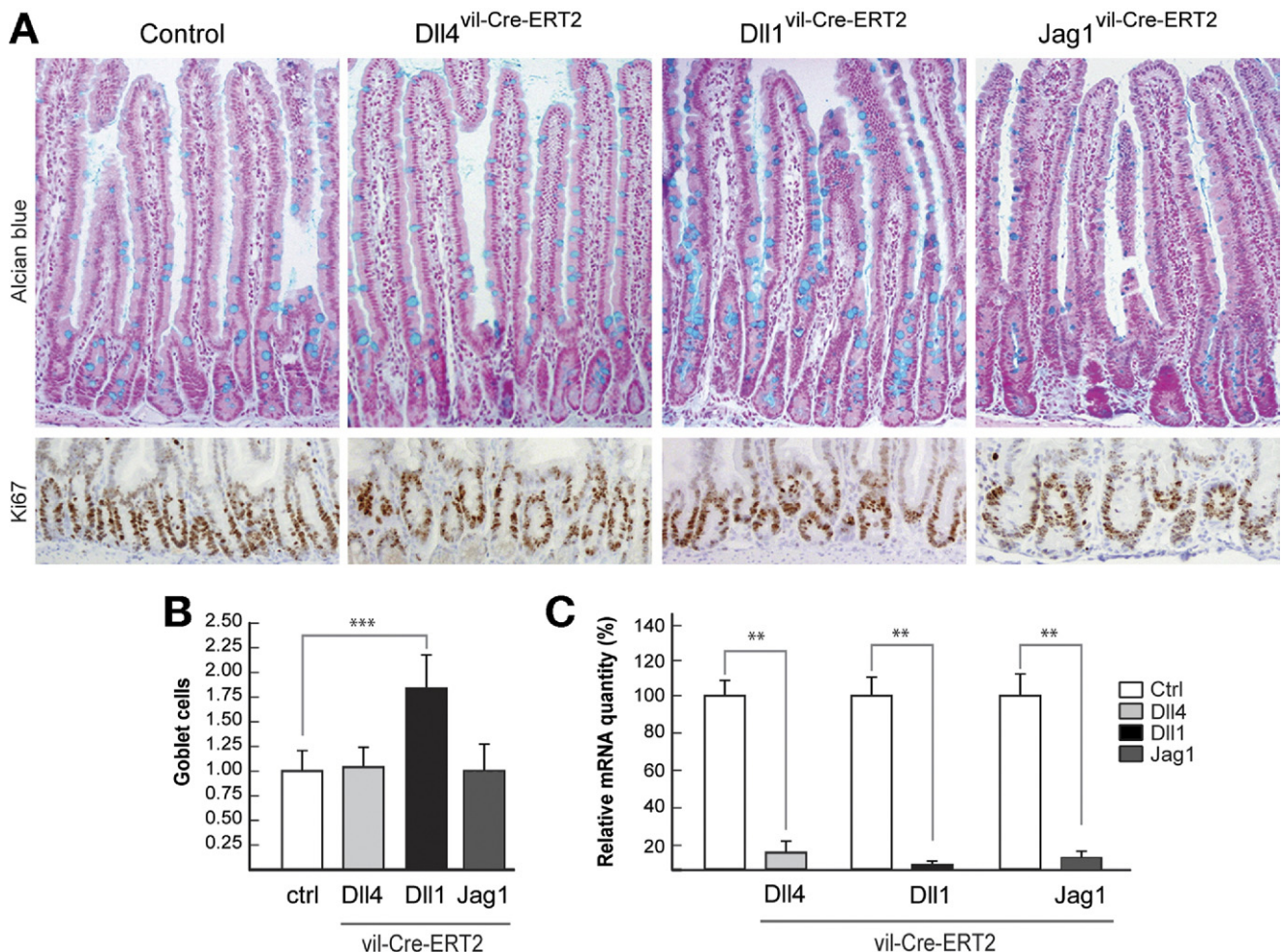


Figure 2. Increased goblet cell differentiation in Dll1-deficient intestine. (A) Alcian blue (top panel) and Ki67 (bottom panel) staining of representative sections from the proximal small intestine of control, Dll4^{vil-Cre-ERT2}, Dll1^{vil-Cre-ERT2}, and Jag1^{vil-Cre-ERT2} mice. (B) Quantification of goblet cells in control and the indicated mutant mice. The number of Alcian blue–positive goblet cells per crypt-villus unit was counted and expressed as relative values to littermate controls (1.00). Dll1 mutant mice show a 1.83-fold increase ($***P < .0001$) in goblet cell number. (C) Relative messenger RNA quantification of Dll4, Dll1, and Jag1, derived from control and single mutant mice ($**P < .01$).

the small intestine did not differ from loss of Dll1 alone (compare Figure 3A and B with Figure 2A). Furthermore, the large intestine of these double mutant mice was comparable to control animals (Figure 3C). Thus, under physiologic conditions, Jag1 does not contribute to Notch activation in the gut.

In contrast, Dll1-Dll4 double knockout mice showed a rapid and progressive loss of weight, accompanied by cachexia and poor grooming, leading to death within 4 to 6 days after the last tamoxifen injection. Histologic examination of the small intestine revealed a complete conversion of crypt progenitors into goblet cells as well as loss of Ki67⁺ cells, indicating loss of the proliferative crypt compartment (Figure 3A and B). Similarly, in the colon, we observed reduced numbers of Ki67⁺ cells and a corresponding increase of goblet cell numbers reaching the apical epithelium (Figure 3C). Active Notch1 signaling within the crypt compartment of the small intestine was investigated by NICD1 immuno-

staining, Hes1 in situ hybridization, and qRT-PCR for Math1. Dll1-Dll4 double-deficient and RBP-J mutant intestinal crypts did not show any NICD1 staining and were also negative for Hes1 messenger RNA expression (Figure 4A and B). In contrast, Math1 messenger RNA expression, which is normally repressed by Hes1 in the crypt compartment, was strongly up-regulated in both Notch mutant animals compared with control mice (Figure 4C). Taken together, these data show that Notch signaling is abrogated in Dll1-Dll4 double-mutant mice and that these animals phenocopy RBP-J and Notch1-Notch2 mutant mice.^{5,6}

Similarly to RBP-J-deficient mice, the Paneth and enteroendocrine cell compartments were not significantly affected in the Dll1-Dll4-deficient intestine (Supplementary Figure 3). This may be explained in terms of the relatively long lifetimes of these secretory cell types; given that the intestinal tissues of this mice were fixed only 3 to 5 days after the final injection of tamoxifen, there will not

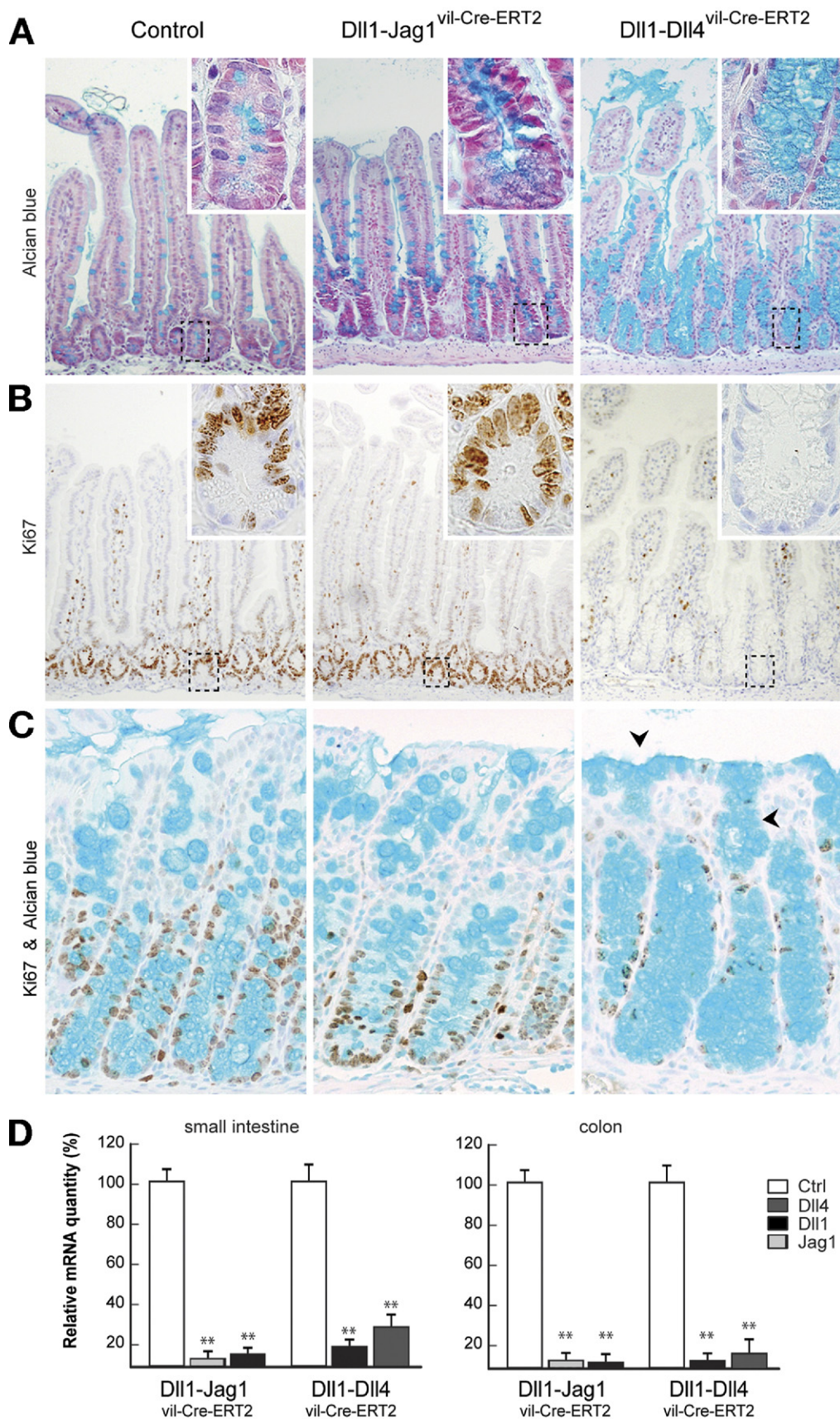


Figure 3. Complete conversion of crypt progenitors into postmitotic goblet cells in DII1-DII4 double mutant mice. (A) Alcian blue and (B) Ki67 antibody staining of representative sections from the small intestine of control, DII1-Jag1^{vil-Cre-ERT2}, and DII1-DII4^{vil-Cre-ERT2} mice. The DII1-Jag1 double-mutant mice show an increase in goblet cell numbers similar to single DII1 mutant mice (1.88; $P < .0001$), while the proliferative crypt compartment of DII1-DII4^{vil-Cre-ERT2} is completely converted into postmitotic goblet cells. *Insets* in panels show high magnification (400 $\times$) of the outlined crypt regions. (C) Alcian blue and Ki67 staining of colonic crypts revealed a similar phenotype. Not only the crypt but also the intercrypt epithelium is converted into goblet cells (arrowheads). (D) Quantitative messenger RNA analysis from epithelial cells of the small intestine and colon confirmed efficient gene inactivation (** $P < .01$).

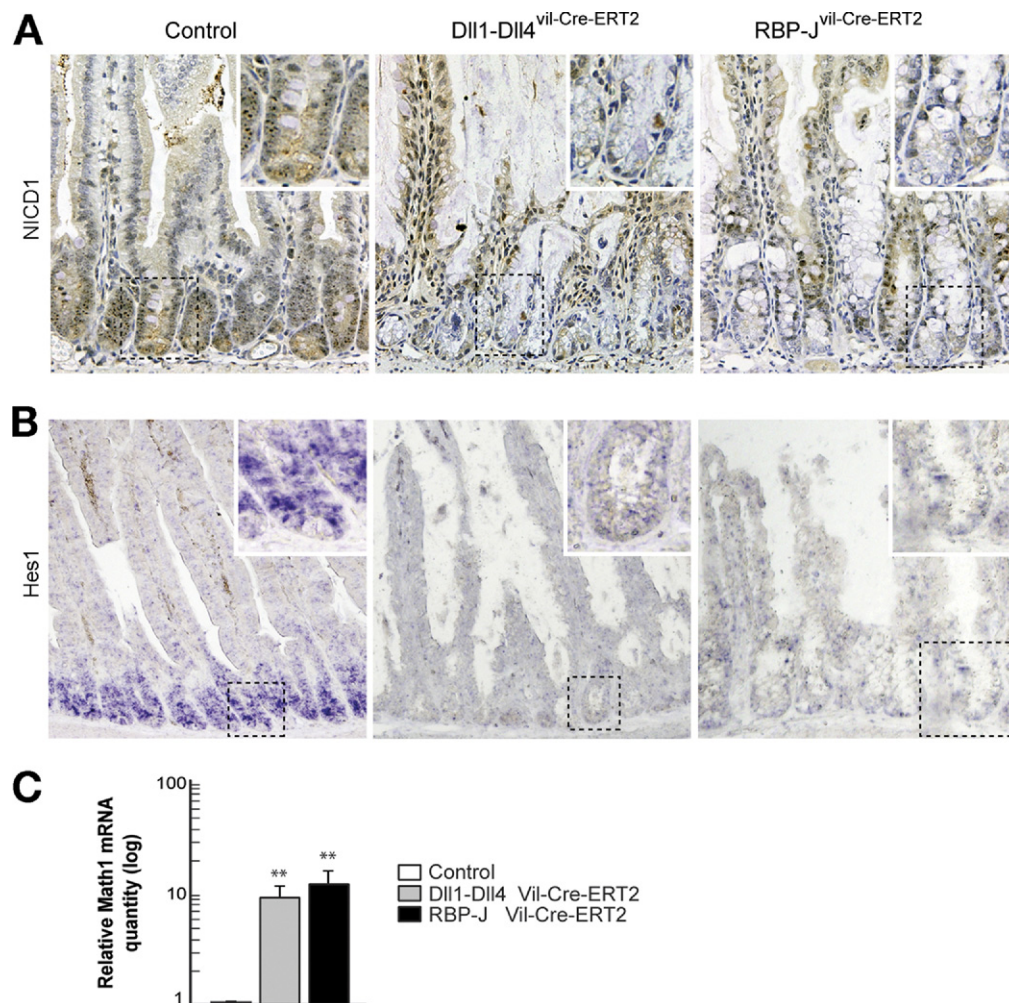


Figure 4. Notch signaling is disrupted in the small intestine of Dll1-Dll4 and RBP-J mutant mice. (A) Active Notch signaling was visualized with anti-NICD1 antibody staining on representative sections from control, Dll1-Dll4⁻, and RBP-J⁻ deficient intestines. Disruption of Notch signaling was confirmed by the absence of nuclear staining in the crypt compartment of Dll1-Dll4⁻ and RBP-J⁻ deficient mice. (B) In situ hybridization of the Notch target gene Hes1 on sections from indicated mice, and (C) quantification of Math1 messenger RNA level derived from epithelial cells of control, Dll1-Dll4⁻, and RBP-J mutant mice (***P* < .01).

have been sufficient time for the adjustment of Paneth and enteroendocrine cell numbers according to their production rate.²⁶ These results identified Dll1 and Dll4 as the physiologic ligands mediating Notch signaling in the crypt compartment of the small intestine and colon. Thus, the presence of both Dll1 and Dll4 ligands is essential for maintenance of the proliferative crypt compartment.

Loss of Lgr5⁺ Intestinal Stem Cells in Notch Mutant Mice

Earlier we showed that Notch1 was active in intestinal SCs. To address whether loss of Notch signaling affects intestinal SCs in addition to crypt progenitor cells, we investigated the consequences of inactivating different combinations of Notch ligands in the SC compartment of the small intestine. Lineage tracing experiments for both Bmi1 and Lgr5 identified SCs that are capable of replenishing all cell types of the small intestine.^{1,27} Bmi1 expression marks the so-called +4 position, which was proposed to be the location of the SCs due to their ability to retain DNA labels.²⁸ In contrast, Lgr5⁺ cells are cycling, and although they occasion-

ally localize to the +4 position, it is currently not clear whether Bmi1⁺ and Lgr5⁺ cells represent distinct, overlapping, or identical SC populations within the small intestine. Olfm4 was recently identified as being a specific and robust marker of mouse and human Lgr5-type SCs in the small intestine.³ We therefore analyzed control, Dll4, Dll1, Jag1, Dll1-Jag1, and Dll1-Dll4 mutant mice for the presence or absence of Lgr5-type SCs using Olfm4 in situ hybridization. As expected, the intestine of control, Dll4, Dll1, Jag1, and Dll1-Jag1 mutant mice showed the presence of normal numbers of Olfm4⁺ intestinal SCs at the bottom of the crypt (Supplementary Figure 4). In contrast, the intestine of Dll1-Dll4⁻ and RBP-J⁻ deficient mice lacked Olfm4⁺ SCs in crypts where gene inactivation was complete (Figure 5). We further confirmed that loss of Notch signaling is not only affecting Olfm4 expression on SCs, but the SC compartment itself by analyzing 2 Wnt targets/SC markers: Lgr5¹ and Ascl2.²⁹ Expression of both genes was ablated in Dll1-Dll4⁻ and RBP-J⁻ deficient small intestine (Figure 5). The concomitant loss of Olfm4, Lgr5, and Ascl2 expression, the labeling of SCs with NIP1::CreER^{T2}, and the complete loss

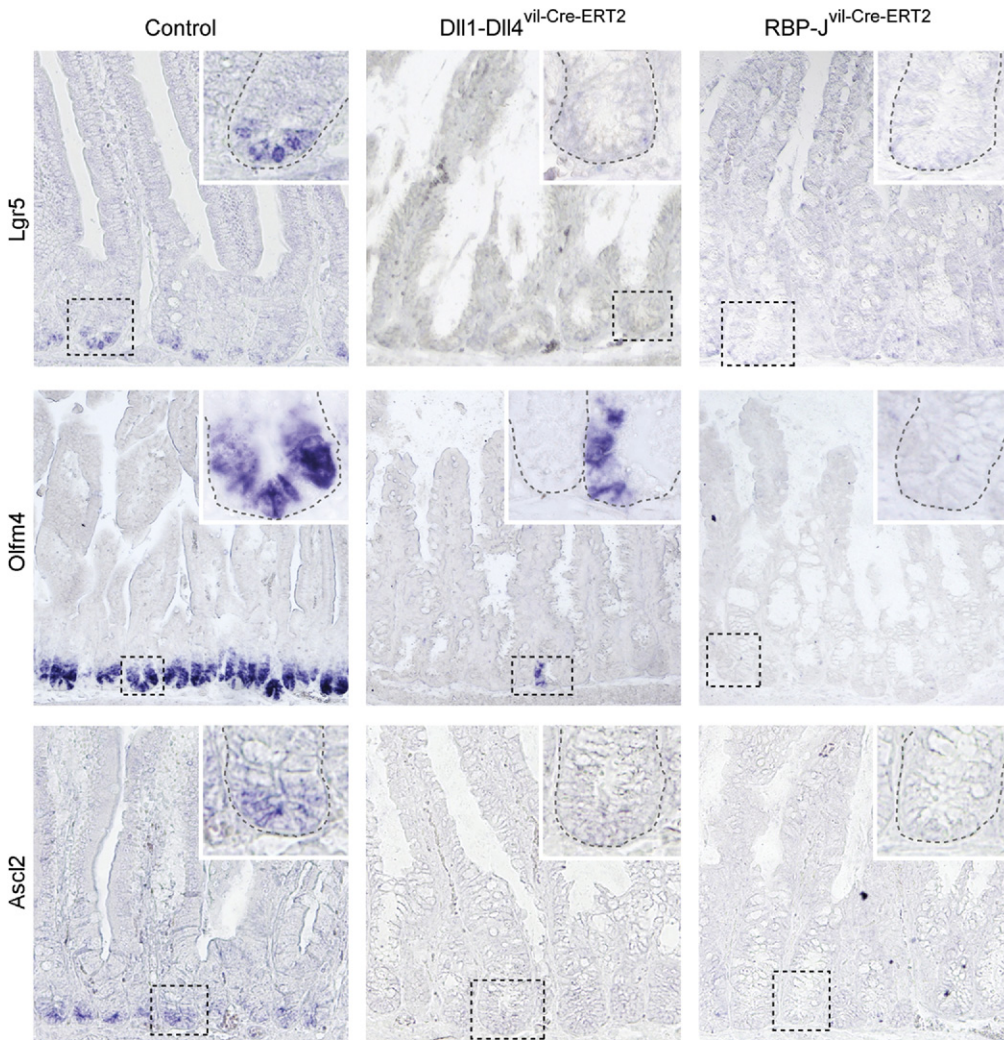


Figure 5. Loss of intestinal stem cell markers in Notch signaling-deficient *Dll1-Dll4* and *RBP-J* mutant mice. In situ hybridization analysis for SC markers *Lgr5*, *Olfm4*, and *Ascl2* on sections of the small intestine. Simultaneous inactivation of *Dll1* and *Dll4* or *RBP-J* resulted in the loss of expression of all 3 SC markers described previously. *Insets* show high magnification (400 $\times$) of outlined crypt regions. Note that for *Olfm4* in situ hybridization on *Dll1-Dll4* mutant mice, a region with a few positive SCs (presumably none deleted) was chosen on purpose to indicate successful probe labeling.

of Ki67⁺ cells within the crypt (Figure 3B) indicates that cycling Lgr5⁺ SCs convert into postmitotic goblet cells when Notch signaling is lost. Taken together, these results indicate that Notch signaling is directly involved in the maintenance of SCs within the small intestine.

Goblet Cell Metaplasia in Adult Notch Signaling Deficient Mice Is Not Driven by *Klf4* Up-regulation

Finally, we wanted to investigate the function of a candidate playing a role in driving goblet cell metaplasia following loss of Notch signaling. Several reports suggest that the zinc finger transcription factor *Klf4* acts as a master regulator for goblet cell differentiation during fetal life. *Klf4* is up-regulated as a consequence of Notch deficiency and is thereby thought to drive goblet cell metaplasia.¹²⁻¹⁴ Because *Klf4*-null mice die shortly after birth, we intercrossed mice carrying floxed alleles of the *Klf4* gene³⁰ with *vil-Cre-ERT2* transgenic mice. Two-week-old *Klf4*^{flx/flx}*vil-Cre-ERT2* (hereafter *Klf4*^{vil-Cre-ERT2}) and corresponding littermate controls lacking the *vil-Cre-ERT2* transgene (control) were

analyzed 2 weeks after the last tamoxifen injection. Successful inactivation of the *Klf4* gene was confirmed by Southern blot analysis and/or by qRT-PCR. Surprisingly, histologic analysis with Alcian blue staining did not reveal any differences in goblet cell numbers between control and *Klf4* mutant animals in either the small intestine or the colon (Supplementary Figure 5). Proliferation of crypt progenitors and Paneth and enteroendocrine cell compartments within the small intestine were also similar between the 2 groups of mice (Supplementary Figure 5).

Although under homeostatic conditions *Klf4* seems to be dispensable for goblet cell fate specification and differentiation within the adult intestine, it was still conceivable that reactivation of *Klf4* expression in the crypt compartment after loss of Notch signaling might trigger goblet cell differentiation. If this hypothesis is correct, one would expect that loss of *Klf4* in Notch-deficient intestine should reduce or suppress the goblet cell metaplasia. We investigated this possibility by generating double-mutant mice intercrossing *RBP-J*^{flx/flx} mice with *Klf4*

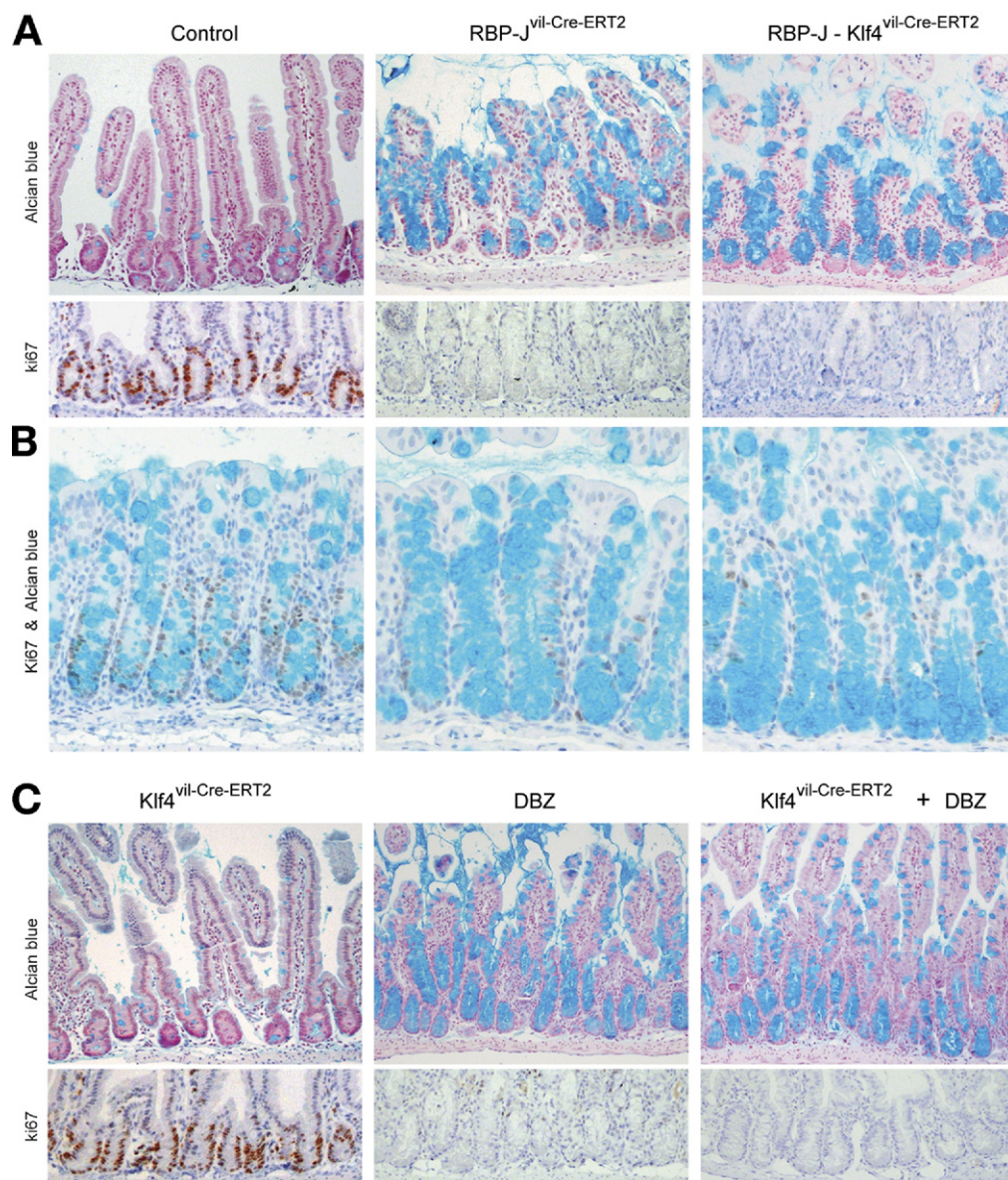


Figure 6. Klf4 is dispensable for goblet cell metaplasia induced by loss of Notch signaling. (A and B) Alcian blue (top panels) and Ki67 (bottom panels) staining on sections derived from the (A) proximal intestine and (B) colon of the indicated mice. Note the complete loss of the proliferative crypt compartment and the complete conversion into goblet cells in RBP-J and RBP-J - Klf4 mutant intestine. (C) Pharmaceutical inhibition of Notch signaling using the γ -secretase inhibitor DBZ after gut-specific inactivation of Klf4 results in the loss of proliferating crypt progenitors due to their conversion into postmitotic goblet cells.

mutant animals (RBP-J - Klf4^{vil-Cre-ERT2}). Gene inactivation in double-mutant mice and control RBP-J^{vil-Cre-ERT2} mice was induced and analyzed 4 days after inactivation. Interestingly, the phenotype of the small intestine and colon of RBP-J - Klf4^{vil-Cre-ERT2} double-deficient mice was indistinguishable from RBP-J^{vil-Cre-ERT2} animals (Figure 6A–C), suggesting that Klf4 does not play a regulatory role in goblet cell metaplasia or in loss of proliferation within the crypt. This observation was further confirmed by analyzing intestine of Klf4^{vil-Cre-ERT2} and control mice that were treated with the pharmacologic Notch inhibitor DBZ. Both control and Klf4 mutant DBZ-treated animals displayed goblet cell metaplasia and loss of the proliferative crypt compartment (Figure 6C). These findings show that Klf4 is not a mediator of goblet cell differentiation induced by loss of Notch signaling in the adult intestine.

Discussion

It is now well established that both Wnt and Notch signaling are essential for the maintenance of the proliferative crypt compartment. Both pathways are also involved in cellular differentiation processes of the intestinal epithelium.³ Loss of Notch signaling due to genetic inactivation of the RBP-J gene, or as a consequence of using pharmacologic γ -secretase inhibitors or blocking antibodies, results in the loss of proliferating crypt progenitors, which convert into postmitotic goblet cells.^{5,7} In recent years, aberrant Notch signaling has been linked to numerous diseases, in particular to cancer, which led to increased attention of the Notch cascade as a potential therapeutic target. Although γ -secretase inhibitors are currently considered and tested in clinical trials for several conditions, the intestinal toxicity due to their indis-

criminate blockade of Notch receptors in the gut, with consequent loss of SCs, represents a serious restriction.³¹ New therapeutic strategies, such as the development of inhibitory antibodies against individual Notch receptors and/or ligands, are currently being developed and tested in preclinical models.^{7,32–34} It is therefore important to understand which components of the Notch cascade mediate crypt progenitor maintenance and how their inhibition results in goblet cell metaplasia. In this context, we previously established that the Notch1 and Notch2 receptors function redundantly in the small intestine, because only the concomitant genetic ablation of both receptors results in goblet cell metaplasia and loss of crypt progenitors.⁶ These genetic results were recently confirmed, showing that antibody-mediated inhibition of Notch1 and Notch2 causes severe intestinal toxicity while selective blocking of Notch1 inhibited tumor growth in preclinical models without major gut toxicity.⁷

In this study, we identified Dll1 and Dll4 as being the key physiologic ligands mediating Notch signaling in the intestinal epithelium of the mouse. Intestine-specific inactivation of individual ligands such as Dll4 and Jag1 did not show any obvious phenotype and loss of Dll1 mimicked loss of Notch1 by producing a moderate increase in goblet cell numbers without noticeable perturbation of the proliferative compartment. These results suggested a functional redundancy among the ligands similar to the one observed within the receptors. Just as loss of Notch1 was not fully compensated by Notch2, loss of Dll1 is not fully compensated by Dll4. In contrast, loss of Dll4 is completely counterbalanced by Dll1, indicating that Dll1 contributes more to Notch1 signaling in the intestinal epithelium than Dll4. It is currently unclear whether these differences between Dll1 and Dll4 are mediated by different expression patterns within the crypt compartment or by differences in their ability to bind different Notch receptors, as has been shown within the hematopoietic system.³⁵

Jag1 has been shown to be downstream of Wnt signaling in multiple organs, including the intestine.³⁶ Moreover, deletion of a single Jag1 allele in APC^{Min/+} mice resulted in reduced tumor growth, and human adenomas derived from patients with familial adenomatous polyposis showed high levels of Jag1 that correlated with activated Notch1 and Notch2 in tumor cells containing nuclear β -catenin.³⁶ Although Jag1 is indeed a target gene of β -catenin-mediated Wnt signaling, and Wnt signaling is essential for the maintenance of the crypt compartment,³ Jag1 is not a functionally important Notch ligand in the mouse intestine. We find that Dll4, and not Jag1, is acting redundantly with Dll1. Human colorectal tumors, colorectal cancer cell lines, and adenomas of APC^{Min/+} mice all exhibit increased levels of β -catenin-mediated Wnt signaling compared with their normal intestinal epithelium. It is therefore possible that elevated Jag1 expression under pathologic conditions could con-

tribute to disease progression, whereas the physiologic levels of Jag1 in the crypt compartment are not functionally significant.

Dll4 is best known for its essential role during development of embryonic vasculature and arteriogenesis.^{37,38} The finding that Dll4 is preferentially expressed in tumor vasculature as opposed to normal blood vessels³⁹ rendered Dll4 an attractive therapeutic target for tumor angiogenesis. Studies targeting blood vessel formation using blocking antibodies to Dll4 revealed substantial tumor growth reduction in cancer cell line-based xenograft models.^{33,34} Unlike blocking Notch signaling using a γ -secretase inhibitor, neutralizing anti-Dll4 antibodies had no measurable effect on goblet cell differentiation and/or proliferation of crypt progenitor cells.³³ This led to the interpretation that Dll4-mediated Notch signaling would be largely restricted to the vascular compartment.³³ Although our genetic studies confirm that ablation of Dll4 in the intestinal epithelium does not perturb proliferation or goblet cell differentiation in the crypt, they show that Dll4-mediated Notch signaling is indeed occurring in the intestine and that the absence of a measurable phenotype in Dll4 mutant mice is due to redundant Dll1-mediated Notch signaling.

To date, Notch signaling has been mostly described to function at the level of intestinal progenitors but not necessarily at the SC level. Label-retaining assays combined with irradiation-induced regeneration of the intestinal epithelium positioned SCs at the +4 position, just above the Paneth cells.²⁸ Recent in vivo lineage tracing experiments revealed that slow cycling Bmi1⁺ cells localize to the +4 position and give rise to all 4 epithelial lineages of the gut,²⁷ suggesting that these cells represent SCs. Crypt base columnar cells were identified as another pool of SCs based on Lgr5 expression and lineage tracing experiments. In contrast to the +4 cells, Lgr5⁺ cells are actively cycling, long lived, and also give rise to all 4 epithelial lineages.¹ Moreover, a single Lgr5⁺ cell has the potential to form a self-renewing mini-gut in culture.⁴⁰ Whether these 2 pools of SCs represent separate entities or whether one is the descendent of the other needs further investigation. Independent of these issues, our Dll1-Dll4 and RBP-J mutant mice combined with the lineage tracing experiments for Notch1 show that Notch signaling is essential for the maintenance of the Lgr5⁺ SCs and that intestinal SCs have active Notch1 signaling. Because Notch1 and Notch2 have redundant functions within the small intestine, it is conceivable that SCs may also have active Notch2 signaling. Future studies are needed to verify this hypothesis. Which cell provides Dll1 and Dll4 ligands to trigger Notch signaling in stem and progenitor cells is currently not well defined. In the absence of effective anti-Dll1 and Dll4 antibodies, in situ hybridization and Dll1^{lacZ/+} and Dll4^{lacZ/+} knock-in mice identify individual cells within the crypt and secretory cells as ligand-presenting cells.^{19–21,41}

The gastrointestinal toxicity of γ -secretase inhibitors, resulting from loss of SCs/progenitors, which limits the clinical utility of these drugs, is avoided in mice in which Math1 is conditionally inactivated in the intestine, but at the expense of loss of secretory cells.^{11,42} This shows the absolute requirement of Math1 for conversion of SCs to secretory cells in crypts deficient in Notch signaling. Multiple studies have suggested that goblet cell metaplasia in γ -secretase inhibitor-treated mice is mediated by derepression of Klf4,^{12–14} positioning Klf4 downstream of Math1. However, our genetic loss-of-function analysis failed to support such a role for Klf4 in the intestine and shows that Klf4 is dispensable for this process.

In conclusion, our results identified Dll1 and Dll4 as the physiologically relevant Notch1 and Notch2 ligands within the small intestine of the mouse. These 2 ligands cooperate and exhibit a partial functional redundancy, with Dll1 contributing more to Notch signaling than Dll4 in the intestinal epithelium. Collectively, our experiments reveal that intestinal SCs activate Notch1, that Notch signaling is required for SC maintenance, and that Klf4 is not an essential mediator of goblet cell differentiation.

Supplementary Material

Note: To access the supplementary materials accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.01.005.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Tissue Sample Preparation, Immunohistochemistry, and In Situ Hybridization

Intestinal samples were flushed gently with cold phosphate-buffered saline and fixed overnight in 4% paraformaldehyde at 4°C or in formalin at room temperature. Samples were then dehydrated, embedded in paraffin, and sectioned at 4 μ m. After dewaxing and rehydration, endogenous peroxidase activity was quenched (5 minutes, 3% H₂O₂) and antigen retrieval was performed (20 minutes at 95°C in 10 mmol/L sodium citrate buffer, pH 6). Paneth and enteroendocrine cells were stained with a rabbit anti-lysozyme (1:500 dilution overnight at 4°C; Dako, Baar, Switzerland) and rabbit anti-synaptophysin antibody (1:100 dilution overnight at 4°C; Dako) or an anti-NICD1 antibody (1:500 Abcam 8925, Cambridge, United Kingdom), all diluted in phosphate-buffered saline containing 1% bovine serum albumin. Sections were then incubated with species-specific horseradish peroxidase-labeled secondary antibody, and staining was revealed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). Actively proliferating cells were stained with mouse anti-Ki67 antibody (1:100 dilution, 1 hour at room temperature; Novocastra, Nunningen, Switzerland). Double immunofluorescence staining experiments were performed on frozen sections with the primary antibodies chicken anti-EYFP (1:1000 dilution; Aves Labs, Tigard, OR), rhodamine-labeled Dolichos biflorus agglutinin (DBA) (1:500 dilution; Vector Laboratories, Burlingame, CA), rabbit anti-lysozyme (1:500 dilution; Invitrogen, Luzern, Switzerland), and rabbit anti-chromogranin (1:500 dilution; Abcam, Luzern, Switzerland) and visualized with donkey anti-chicken DyLight488 and donkey anti-rabbit DyLight549 secondary antibodies (1:500 dilution; Jackson ImmunoResearch, West Grove, PA). Goblet cells were stained with Alcian blue (pH 2.5; Sigma) and counterstained with Nuclear Fast Red. For immunostained sections, the following counterstains were used: Alcian blue and Nuclear Fast Red for Paneth cells, Alcian blue and Mayer's hematoxylin for enteroendocrine cells, and Ki67 antibody staining. Immunostained sections were then mounted. The numbers of goblet, Paneth, and enteroendocrine cells are shown as percentage relative to littermate controls. At least 3 mice per genotype were analyzed, selecting 100 crypt/villus units from the proximal and distal intestine per mouse. Statistical significance was verified with a Mann-Whitney *U* 2-tailed test. X-gal staining was performed as described,¹ and sections were counterstained with Nuclear Fast Red. For in situ hybridization, intestines were flushed with ice-cold phosphate-buffered saline and processed as described.² The in situ probes utilized were obtained from the IMAGE consortium or

newly designed. Probes from complementary DNA of mouse *Olfm4*, *Lgr5*, and *Ascl2* were generated by in vitro transcription with a DIG RNA Labeling Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Intestinal Crypt Cell Isolation, Southern Blot, and qRT-PCR Analysis

A fraction enriched for intestinal crypt cells was obtained using the following isolation protocol. Briefly, 5- to 10-mm pieces of freshly isolated intestines were incubated for 8 minutes in phosphate-buffered saline/30 mmol/L EDTA at 37°C and then vigorously shaken for 1 minute. The small pieces of intestine were then reincubated in fresh phosphate-buffered saline/30 mmol/L EDTA solution, and the detached cells were collected and kept on ice. The procedure was sequentially repeated 4 to 5 times with a short incubation time (4–5 minutes). The last 2 fractions are highly enriched in crypt intestinal cells and were used for genomic DNA isolation (Proteinase K, phenol/chloroform purification) and RNA isolation (TRIzol; Invitrogen).

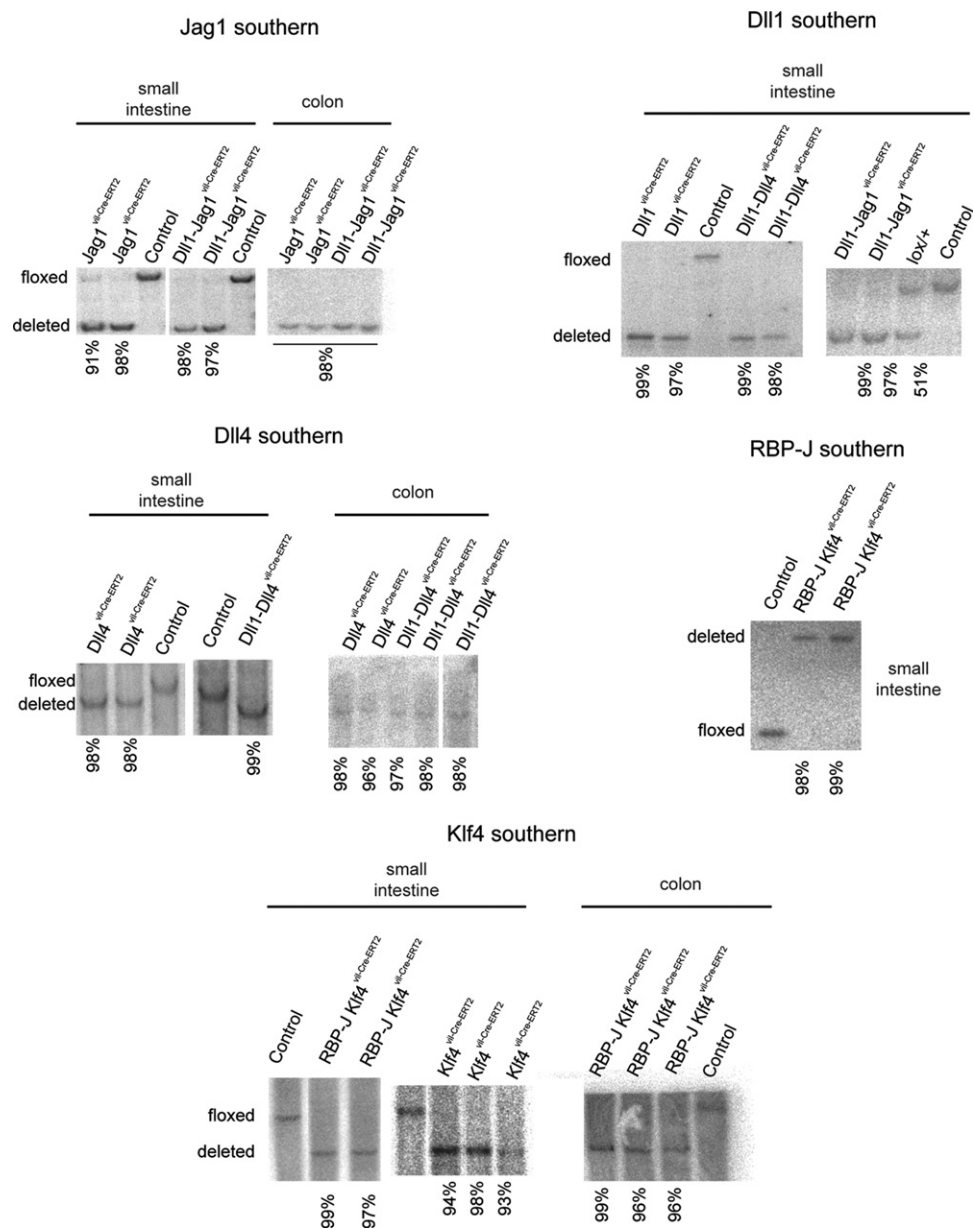
The Southern blot analysis strategy was as described previously for *Dll1*,³ *Dll4*,⁴ *Jag1*,⁵ *Klf4*,⁶ and *RBP-J*.⁷ Briefly, 20 μ g of genomic DNA was digested with the appropriate restriction enzyme, denatured, transferred to a nylon membrane, and incubated overnight at 65°C with the specific P³² radiolabeled probe. The labeled membrane was exposed on a Phosphor imager plate and scanned with a Fuji Film BAS-1000 scanner (Dielsdorf, Switzerland). Gene deletion was quantified with the Aida image analyzer software (Fuji Film).

qRT-PCR was performed on complementary DNA obtained from the reverse transcription of 1 μ g of total RNA derived from intestinal crypt cells (SuperScript II and random hexamers primers; Invitrogen). Gene expression was assessed using the Light Cycler Carousel PCR machine (Roche Diagnostics) following the manufacturer's instructions. Relative levels of gene expression were normalized to *Gapdh* or *Actb* gene expression using the 2^{- $\Delta\Delta$ CT} method. Gene specific primer sequences are available on request. Three mice at least per genotype were analyzed. Statistical significance was verified with a Mann-Whitney *U* 2-tailed test.

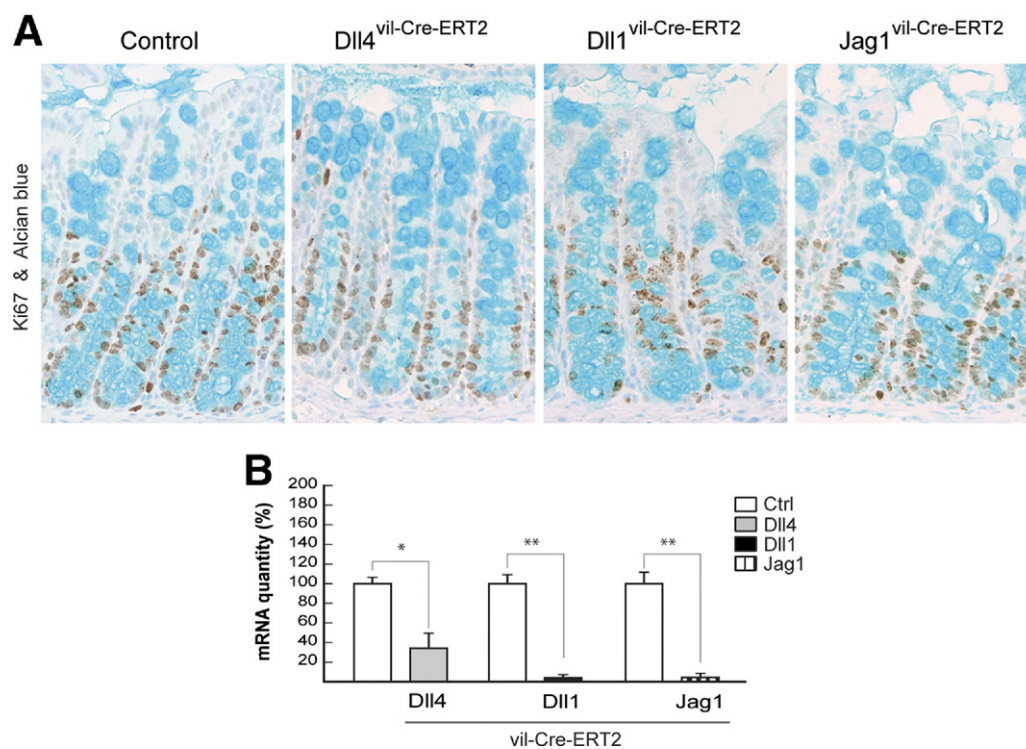
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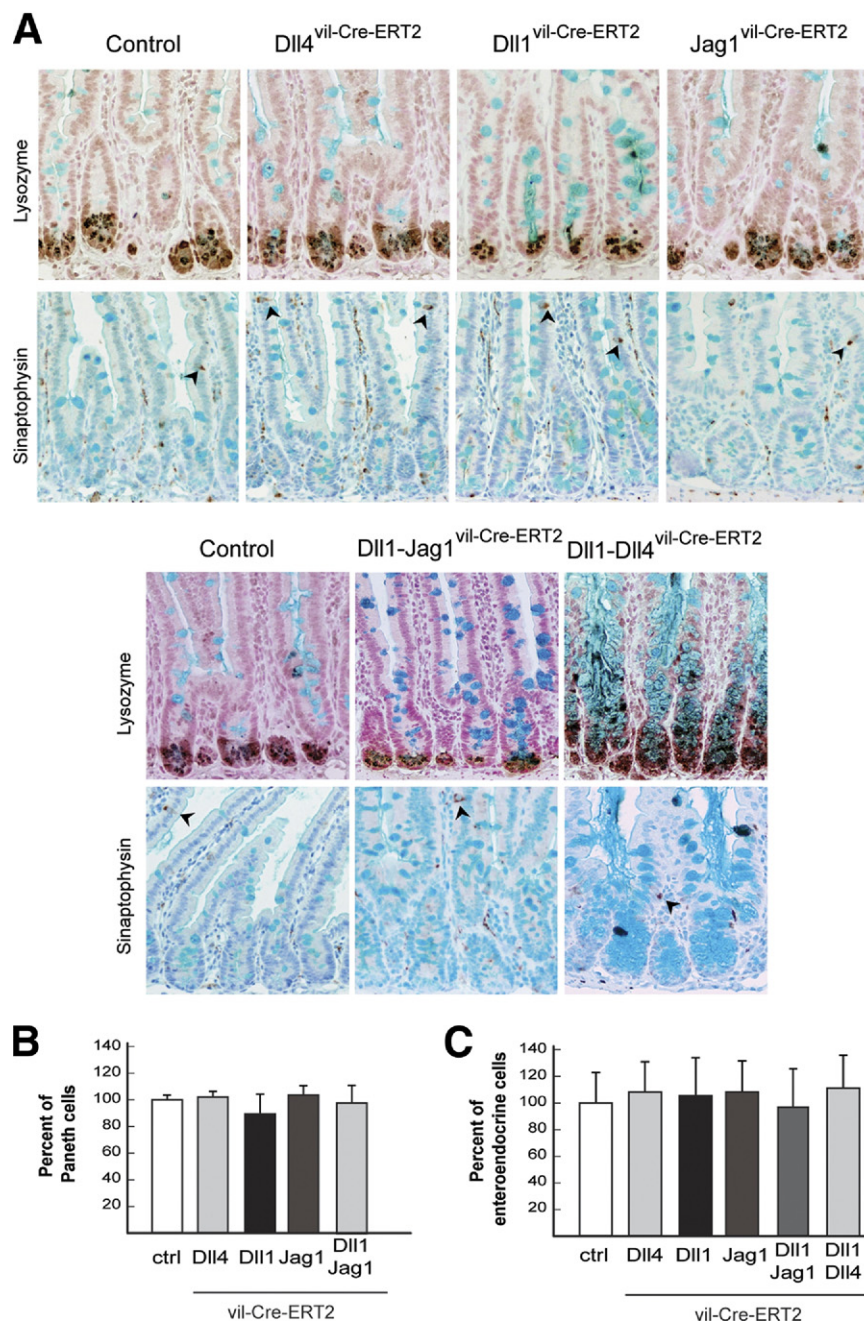
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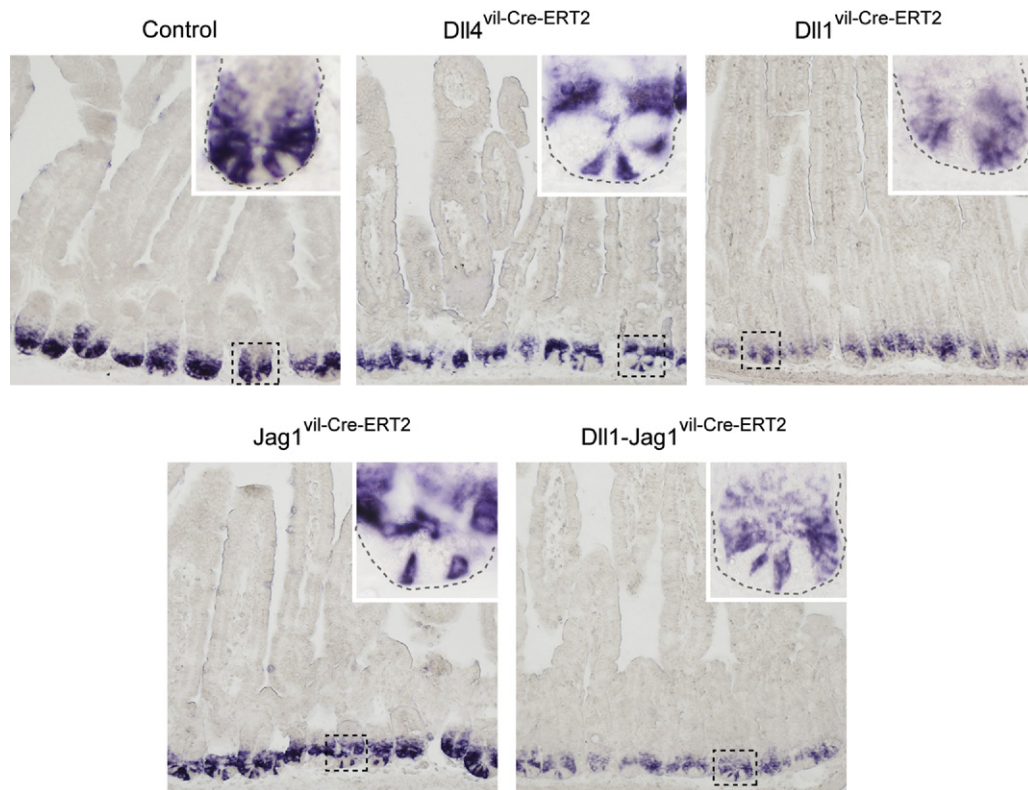
Supplementary Figure 1. Southern blot analysis of genomic DNA derived from epithelial cells of the small intestine or colon of the indicated mice. See Materials and Methods for details.



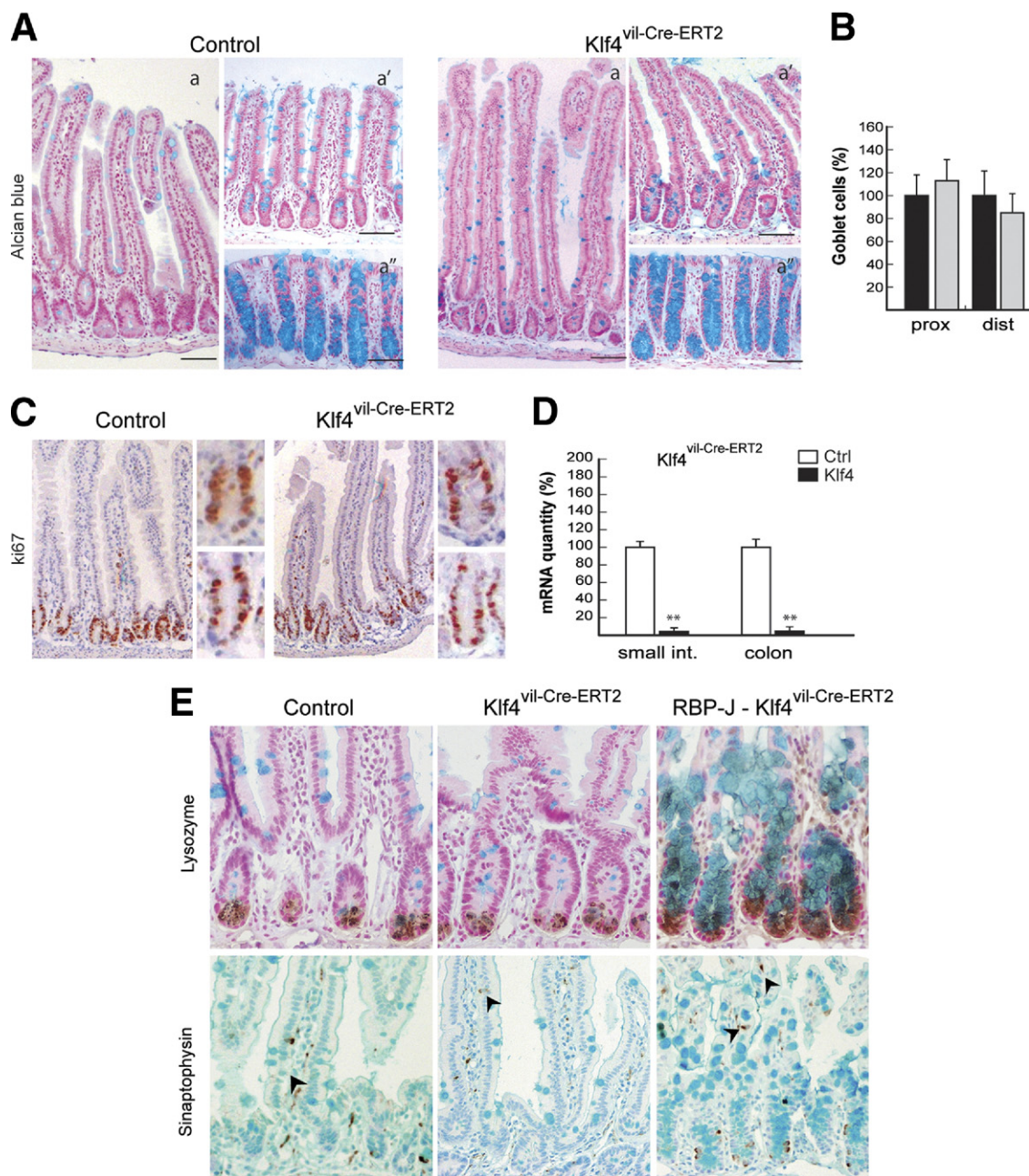
Supplementary Figure 2. (A) Representative colon sections of control, DII4^{vil-Cre-ERT2}, DII1^{vil-Cre-ERT2}, and Jag1^{vil-Cre-ERT2} mice. Goblet cells and proliferating crypt progenitors were visualized by Alcian blue and Ki67 double staining. No major differences were noted between control and the ligand mutant mice. (B) Relative messenger RNA quantification of DII4, DII1, and Jag1, derived from epithelial cells of control and single ligand deficient mice, confirmed efficient gene deletion (* $P < .05$, ** $P < .01$).



Supplementary Figure 3. (A) Paneth cells and enteroendocrine cells were visualized performing anti-lysozyme and anti-synaptophysin antibody staining on sections derived from the proximal intestine of control, $DII4^{vil-Cre-ERT2}$, $DII1^{vil-Cre-ERT2}$, $Jag1^{vil-Cre-ERT2}$, $DII1-Jag1^{vil-Cre-ERT2}$, and $DII1-DII4^{vil-Cre-ERT2}$ mice, respectively. The same sections were counterstained with Alcian blue to visualize goblet cells at the same time. *Arrowheads* indicate enteroendocrine cells. (B and C) Quantification of Paneth and enteroendocrine cells in indicated mutant mice. Numbers of Paneth cells per crypt or enteroendocrine cells per crypt/villus unit were counted and expressed as percentage of littermate control mice (100%). See Materials and Methods for details.



Supplementary Figure 4. In situ hybridization analysis for the SC marker Olfm4 on sections derived from the small intestine of control and Dll4, Dll1, Jag1, and Dll1-Jag1 mutant mice. *Insets in panels* show high magnification (400×) of the outlined crypt regions. No significant difference in Olfm4 expression was observed.



Supplementary Figure 5. Normal goblet cell numbers in the adult intestine of Klf4 mutant mice. (A) Alcian blue–positive goblet cell staining on (a) proximal, (a') distal small intestine, and (a'') colon sections from control and Klf4 mutant mice. (B) Goblet cell number quantification of control (gray bar) and Klf4 (black bar) small intestine mutant mice. Relative percentage to littermate control intestines (100%). (C) Actively proliferating cells from control and Klf4 mutant proximal intestine were stained with anti-Ki67 antibody. (D) Quantification of Klf4 messenger RNA levels (black bar) derived from the small intestine or colonic crypt of Klf4 deficient and control (100%) mice (** $P < .01$). (E) Paneth and enteroendocrine cells were visualized by lysozyme and synaptophysin staining on sections derived from control, Klf4^{vil-Cre-ERT2}, and RBP-J - Klf4^{vil-Cre-ERT2} mice. The same sections were counterstained with Alcian blue to visualize goblet cells at the same time. Arrowheads indicate enteroendocrine cells.

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Adicionalmente, me permito resaltar que, de acuerdo a lo indicado en la GUÍA PARA EXAMEN DE SOLICITUDES DE PATENTE Y MODELO DE UTILIDAD de su despacho, “*si, por ejemplo, la característica técnica esencial de la composición es el compuesto activo, el examinador no deberá requerir que se mencionen también otras características estructurales.*”, por lo cual, es pertinente destacar que la composición de la presente solicitud tiene como característica técnica esencial el compuesto activo, es decir, el anticuerpo como se define en la reivindicación 1, en consecuencia, el examinador no debería requerir la definición mediante los excipientes y adyuvantes y sus proporciones.

Con base en lo anterior, es indiscutible que la reivindicación 1 es completamente clara y define la composición de manera adecuada.

Por otra parte, respetuosamente me permito disentir de la objeción sobre las reivindicaciones 1 y 17 (ahora reivindicaciones 1 y 16), puesto que el término “pluralidad” se encuentra definido en la descripción como sigue:

“Cuando se hace referencia a una composición que comprende una pluralidad de anticuerpos, se considera que los anticuerpos estarán afucosilados si <5% de los anticuerpos en la composición comprenden fucosa en Asn297.”.

(Página 23, líneas 5 – 8).

Adicionalmente, el término “al menos” se encuentra antecediendo el porcentaje de afucosilación, es decir “al menos el 95%” y, de acuerdo a esto, una persona versada en la materia reconocería que este término se refiere de manera clara a un rango porcentual del 95 – 100%.

Por lo tanto, es evidente que dichos términos son completamente claros y son suficientemente precisos para definir el grado de afucosilación del anticuerpo que hace parte de la composición de la invención de la presente solicitud.

Por otra parte, me permito señalar que la anterior reivindicación 8 fue cancelada del pliego reivindicatorio, de manera que se considera superada la objeción por falta de concisión con la reivindicación 1 y por falta de claridad sobre esta.

Finalmente, de manera respetuosa, me permito expresar nuestro desacuerdo con la objeción sobre las anteriores reivindicaciones 15 – 19 (ahora reivindicaciones 14 – 18), puesto que el método que allí se define es completamente claro y se define mediante sus características técnicas esenciales, es decir, su secuencia de etapas técnicas que se resumen en:

- i. la preparación de células huésped que comprenden el ácido nucleico que codifica el anticuerpo anti- FGFR2IIIb de cualquiera de las reivindicaciones 1 a 13,
- ii. el cultivo de las células huésped en condiciones adecuadas para expresar el ácido nucleico que codifica el anticuerpo anti- FGFR2IIIb, y
- iii. la recuperación de los anticuerpos anti- FGFR2IIIb producidos por las células huésped.

Además, es evidente que un versado en la materia, con base en su conocimiento técnico en el área, estaría en la capacidad de seleccionar las condiciones por las cuales es posible preparar y cultivar las células huésped y recuperar los anticuerpos producidos; es por esto, que no se considera necesaria la inclusión de condiciones específicas dentro de las características técnicas del método reclamado.

Por lo anterior, las reivindicaciones 14 – 18 son completamente claras y definen el método de manera adecuada.

Dibujos y secuencias

De manera respetuosa, me permito señalar que las secuencias fueron presentadas inicialmente de acuerdo con el estándar ST 25, sin embargo, debido a la configuración del sistema online de SIPI, no es posible presentar el formato digital .txt, puesto que se reconocen formatos digitales .pdf. No obstante, me permito anexar el correspondiente listado de secuencias en el formato digital de acuerdo con el estándar ST 25.

Nivel inventivo

Respetuosamente, me permito disentir de la objeción por falta de nivel inventivo en la reivindicación 1, puesto que la composición que aquí se reclama posee amplias ventajas inesperadas que no son obvias en vista de la combinación de los documentos D1 y D2 y, D1 y D3.

En primera medida, si bien coincidimos con la apreciación del examinador relacionada con que la diferencia entre la composición de la reivindicación 1 y el anticuerpo divulgado en el documento D1 consiste en que el anticuerpo de la reivindicación 1 se encuentra afucosilado; de manera respetuosa disintimos de la apreciación de que la afucosilación sólo tiene como resultado un incremento en la actividad ADCC del anticuerpo, de acuerdo a los argumentos que expondremos a continuación.

Como se expone en la Declaración del inventor que aquí se anexa, una persona versada en la materia entendería que el documento D1 enseña anticuerpos monoclonales anti-FGFR2 para el tratamiento de tumores sólidos (D1, columnas 12 – 13 y ejemplo 4). En contraste, las enseñanzas de los documentos D2 y D3 son, de hecho, limitadas a datos de anticuerpos afucosilados para sólo tres dianas en el contexto de desórdenes autoinmunes o cánceres de origen inmune:

“La citotoxicidad ADCC en tumores sólidos no es bien entendida y los mecanismos fundamentales pueden ser diferentes. En la referencia Yamane, ambos ejemplos, KW-0761 y MEDI-563/BIW-8405, se dirigen a indicaciones inmunes, con KW-0761 dirigido a linfoma cutáneo de células T y MEDI-563/BIW-8405 dirigido a eosinófilos en el asma. Además, anticuerpos anti-CD20 del tipo referido en D3 (un ejemplo de cuál es obinutuzumab, el primer anticuerpo aprobado con ADCC incrementada vía afucosilación), también se dirigen a cánceres de origen inmune, linfomas y leucemias.”.

(Párrafo 7 de la Declaración del inventor).

De manera más específica, las enseñanzas del documento D2 se basan en dos ejemplos de anticuerpos para el tratamiento de cánceres de origen inmune. Más concretamente, MEDI-563 (benralizumab) en la actualidad está indicado para el asma y COPD y se dirige a un receptor en los eosinófilos; KW-0761 (mogamulizumab) se dirige a un receptor en las células T y ha sido estudiado en contra de las leucemias y linfomas de

las células T. Similarmente, el documento D3 trata de un anticuerpo anti-CD20, el cual también se utiliza para el tratamiento de linfomas.

De acuerdo a lo anterior, es evidente que el campo técnico de la presente invención es diferente al campo técnico de los documentos D2 y D3, puesto que, pese a que se trata de anticuerpos útiles en el tratamiento del cáncer, el campo técnico específico es diferente en cuanto al tipo de cáncer, como se explicó en los párrafos previos. En este sentido, un versado en la materia, no estaría motivado a utilizar las enseñanzas de los documentos D2 y D3 y realizar cambios en los anticuerpos revelados en el documento D1 con el fin de incrementar la actividad ADCC específica o modificar la farmacocinética y toxicidad de dichos anticuerpos, puesto que las dianas farmacológicas son diferentes y por tanto, la actividad específica podría ser diferente, por lo que, el versado en la materia no podría predecir o tener altas expectativas de incrementar la actividad ADCC con base en los documentos D2 y D3, puesto que estos demuestran un incremento en la actividad, pero relacionada con otras dianas farmacológicas.

Contrario a las afirmaciones del examinador, el documento D2 no sugiere que el anticuerpo no fucosilado que aquí se reclama podría presentar una actividad ADCC específica en tumores sólidos mejorada, puesto que, el documento D2 indica que los anticuerpos afucosilados pueden tener una afinidad de unión más alta por FcγRIIIa y que esta afinidad más alta puede llevar a una eficacia incrementada en pacientes con cánceres de origen inmune y otras enfermedades con señalización inmune defectuosa (como el asma); por lo cual, un versado en la materia no habría predicho a partir del documento D2 si esta afinidad o eficacia incrementada pudiera haber sido observada en la aplicación de la composición mencionada anteriormente.

Específicamente, una persona versada en la materia, con base en los documentos D1 – D3, no habría predicho cómo remover la fucosa, por ejemplo, en la posición Asn 297, de los anticuerpos del documento D1 porque dicha remoción, de hecho, impactaría la eficacia general de los anticuerpos en la composición de la reivindicación 1 y la reivindicación 33 (renumerada como reivindicación 18) cuando se usan para las preparaciones de un medicamento para el tratamiento del cáncer.

En este sentido, el inventor de la presente, indica que:

“Por lo tanto, en mi opinión, una persona versada en la materia con base en los documentos D1 y D2/D3 no podría predecir cómo, removiendo la fucosa en la posición Asn297, podría impactar en la eficacia general de los anticuerpos reclamados en esta aplicación”.

(Declaración del inventor, párrafo 8).

Por lo tanto, además de tener en cuenta que se desea incrementar la actividad ADCC de los anticuerpos del documento D1, se debe tener en cuenta que dicho efecto no afecte la eficacia y seguridad de los anticuerpos, información que no se proporciona ni sugiere en los documentos D2 y D3, lo que corresponde a una evidencia más de la actividad inventiva de la invención de la presente solicitud.

Además, de acuerdo a la GUÍA PARA EXAMEN DE SOLICITUDES DE PATENTE Y MODELO DE UTILIDAD de su despacho, si después de examinar el nivel inventivo de una invención (es decir que dicha invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica) persistiera la duda de la obviedad de esta, se podrían tener en cuenta los indicios de nivel inventivo como lo es un efecto técnico proporcionado por la invención.

En este sentido, si aún, teniendo en cuenta los argumentos presentados anteriormente respecto a la no obviedad en el hallazgo de la presente invención, el examinador considera que se requiere entrar a estudiar los indicios de nivel inventivo, me permito resaltar los efectos técnicos que se proporcionan mediante la presente invención y que, en vista del arte previo, no habrían podido ser fácilmente u obviamente predichos.

Como se puede evidenciar en la declaración del inventor que aquí se anexa, existen otros factores inciertos en vista de los documentos D1 – D3 respecto a las propiedades de los anticuerpos reclamados y su uso en el tratamiento de cánceres que sobreexpresan FGFR2, como cáncer gástrico, dichas incertidumbres se relacionan con la toxicidad y farmacocinética de los anticuerpos que aquí se reclaman.

Problemas de toxicidad impredecibles

Primero, es importante tener en cuenta que un anticuerpo afucosilado anti-FGFR2IIIb podría tener una toxicidad más alta que un anticuerpo no modificado. Como se menciona en el documento D2, los anticuerpos afucosilados pueden competir fuertemente con anticuerpos IgG nativos por la unión a receptores Fc-gamma en células natural killer (NK) (D2, página 232, párrafo puente entre la primera y segunda columna). Esto, a su vez, puede inducir a las células NK a atacar a las células portadoras del antígeno contra el que se pretende que se unan los anticuerpos, independientemente de si esas células son células tumorales o normales.

Como indica el Dr. Pierce en su declaración, un anticuerpo afucosilado podría inducir a las células NK a matar no sólo células tumorales sino también células normales que expresen el antígeno diana del anticuerpo:

“Otra posible consecuencia no intencionada de la glicoingeniería de un anticuerpo para aumentar ADCC es la toxicidad incrementada. (También revisado por Reusch & Tejada en la página 5). Un anticuerpo afucosilado podría inducir a las células NK a matar células normales que expresan la diana del antígeno del anticuerpo. En contraste, esas células normales podrían ser evitadas por un anticuerpo que no ha sido modificado por glicoingeniería. Estas células asesinas podrían llevar a una toxicidad peor y hacer que el anticuerpo afucosilado no fuera adecuado para su uso en pacientes. La toxicidad de un anticuerpo afucosilado anti-FGFR2IIIb no puede ser extrapolada de estudios de otros anticuerpos afucosilados, puesto que el perfil de expresión de los antígenos diana individuales es único para cada antígeno. Por lo tanto, una persona versada en la materia no habría predicho el impacto de la afucosilación de los anticuerpos reclamados en su toxicidad”.

(Declaración del inventor, párrafo 12).

Es por esto, que el examinador falla en extrapolar los resultados obtenidos en los documentos D2 y D3, puesto que no tiene en cuenta que el perfil de expresión de cada antígeno es diferente y, por lo tanto, los diferentes anticuerpos para cada antígeno no tendrán el mismo efecto tras un cambio similar.

Ahora bien. debido a la alta afinidad de los anticuerpos afucosilados por FcγRIIIa, los anticuerpos afucosilados deberían ser más eficientes en matar las células mediadas por NK que sus anticuerpos fucosilados parentales (es decir, aquellos que no han sido modificados). La eficacia incrementada en la muerte celular también podría conducir a un aumento en la muerte de las células normales que expresan FGFR2IIIb, incluidas las células que expresan el antígeno diana a niveles bajos. (D2 página 232, segunda columna, primer párrafo completo). En varios tejidos, matar las células normales que expresan un antígeno diana puede causar toxicidad no deseada. Por esta razón, la toxicidad de un anticuerpo afucosilado anti-FGFR2IIIb no puede extrapolarse a partir de estudios de otros anticuerpos afucosilados para diferentes antígenos diana, como en el documento D3, por ejemplo, porque la toxicidad resultante depende en parte del patrón de expresión del antígeno diana individual y, por lo tanto, del tipo de células normales que podrían eliminarse debido al anticuerpo, como se mencionó anteriormente en el extracto del párrafo 12 de la declaración del inventor.

Como se conoce en el campo técnico, el factor FGFR2IIIb no solo está sobreexpresado por ciertas células tumorales, sino que también se expresa en el epitelio colorrectal normal (*Yoshino et al., Figuras 2A y 3A, refiriéndose a FGFR2IIIb por su nombre alternativo, KGFR*).



Figura 2A – Yoshino *et al.*

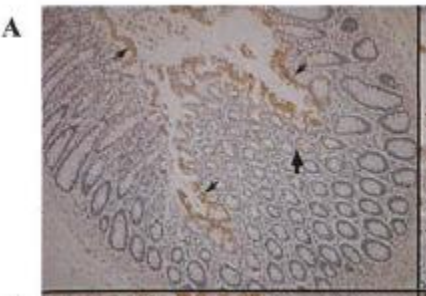


Figura 3A – Yoshino *et al.*

Dicho documento en las figuras 2A y 3A, muestra un análisis inmunohistoquímico de KGFR en el cual se observó inmunorreactividad en la superficie de células epiteliales, por lo tanto, se evidencia que dicho factor se expresa en células no tumorales.

Adicionalmente, se cree que el factor FGFR2IIIb juega un papel importante en la diferenciación de las células epiteliales colorrectales normales (*Yoshino et al.*, página 247, resumen.) Por lo tanto, teniendo en cuenta este documento, un versado en la materia sabría que un anticuerpo anti-FGFR2IIIb afucosilado podría destruir las células epiteliales colorrectales normales que expresan FGFR2IIIb, dando como resultado toxicidad severa.

Además, es ampliamente conocido que los anticuerpos y otros agentes que se unen a las dianas en el epitelio gastrointestinal normal pueden causar toxicidad intestinal severa (*Pellegrinet et al., Gastroenterology 140: 1230-1240 (2011)*, donde se analiza la toxicidad intestinal severa asociada con la inhibición de la señalización de Notch en el párrafo que cubre las páginas 1237-1238).

Por lo tanto, en concordancia con la apreciación del inventor, el impacto de la afucosilación de un anticuerpo FGFR2IIIb sobre la toxicidad no habría sido predecible en vista de la expresión de FGFR2IIIb en las células normales mencionadas anteriormente. (Declaración del inventor, párrafo 12), puesto que, más específicamente, un anticuerpo afucosilado anti-FGFR2IIIb podría inducir a células NK a matar células epiteliales colorrectales normales y, en consecuencia, la toxicidad desfavorable resultante podría hacer que dicho anticuerpo afucosilado fuera inadecuado para el uso en pacientes en comparación con su homólogo normalmente fucosilado (Declaración del inventor, párrafo 12).

Sin embargo, para explorar esta posibilidad, el solicitante Five Prime Therapeutics patrocinó los estudios de toxicología en los que se ensayó un anticuerpo afucosilado anti-FGFR2IIIb que tiene las secuencias reivindicadas de HVR, dominio variable y cadena pesada/ligera frente a su correspondiente forma fucosilada tanto en ratas como en monos cynomolgus (Declaración del inventor, párrafo 13).

De manera inesperada, no se observó aumento de la toxicidad en los animales tratados con la forma afucosilada del anticuerpo anti-FGFR2IIIb en comparación con la forma fucosilada. Por lo que, evidentemente este resultado no se hubiera predicho por las razones discutidas anteriormente.

Además, el anticuerpo afucosilado anti-FGFR2IIIb que tiene las secuencias de la HVR, el dominio variable, y cadena pesada y ligera de las reivindicaciones 1 a 7 se encuentra actualmente en ensayos clínicos de Fase 1. Este anticuerpo demostró un perfil de seguridad aceptable cuando se administró a pacientes con tumores sólidos avanzados, incluidos cáncer gástrico y de vejiga. Estos ensayos iniciales mostraron resultados prometedores en estos tipos de cáncer, que son difíciles de tratar y tienen bajas tasas de respuesta con las terapias actualmente aprobadas. (Declaración del inventor, párrafo 14).

En estos estudios se determinó que la dosis recomendada en este ensayo fuera de 15 mg/kg cada dos semanas (*Catenacci et al.*), la cual es significativamente más alta que la dosis típica de terapias con anticuerpos fucosilados actualmente comercializadas, que están en el rango de 1-10 mg/kg (D2 página 232, segunda columna, primer párrafo completo). Sin embargo, el anticuerpo afucosilado anti-FGFR2IIIb fue bien tolerado en ensayos clínicos y tiene la ventaja de ser administrado una vez cada dos semanas.

Tomados en conjunto, estos resultados muestran que el perfil de toxicidad de los anticuerpos anti-FGFR2IIIb afucosilados utilizados en la composición reivindicada no pudieron haber sido predichos o extrapolados de los resultados observados para anticuerpos contra objetivos diferentes en otras condiciones de cáncer. Además, los datos de los ensayos de Fase 1 muestran datos que prometen que las dosis toleradas de anticuerpos pueden usarse para proporcionar respuesta cuando se usan en sujetos humanos.

Por lo que, en conclusión, en este anticuerpo en específico, la toxicidad no se ve aumentada por la afucosilación contrario a lo que podría esperarse, lo cual es un indicio de que la presente invención posee nivel inventivo.

Farmacocinética impredecible

Otra propiedad de un anticuerpo anti-FGFR2IIIb afucosilado que no se habría predicho es su tasa de eliminación. Según lo discutido por el Dr. Pierce, un anticuerpo afucosilado (trastuzumab) dirigido a una diana diferente (Her2) mostró una velocidad de aclaramiento más rápida en experimentos farmacocinéticos preclínicos que un anticuerpo no modificado. (Declaración del inventor, párrafo 9).

Teniendo en cuenta estos datos, el Dr. Pierce señala que una persona normalmente versada en la materia podría haber predicho que las versiones afucosiladas de los anticuerpos de D1 también habrían alterado la farmacocinética, específicamente una velocidad de eliminación más rápida (Declaración del inventor, párrafo 9). Por lo que se resalta que, una eliminación más rápida podría afectar negativamente la eficacia de los anticuerpos al reducir la biodisponibilidad del anticuerpo (Declaración del inventor, párrafo 9).

Para explorar esta posibilidad en los anticuerpos de la presente invención, el solicitante Five Prime Therapeutics patrocinó estudios farmacocinéticos en monos cynomolgus para evaluar las tasas de eliminación de anticuerpos anti-FGFR2IIIb fucosilados y afucosilados. (Declaración del inventor, párrafos 10 y 11.) El aclaramiento del anticuerpo afucosilado no fue diferente del aclaramiento del anticuerpo fucosilado. Como podría entender un versado en la materia, este resultado no se esperaba a la vista de los datos anteriores sobre el anticuerpo anti-Her2 trastuzumab (Declaración del inventor, párrafo 9), puesto que, como se había visto en estudios previos, se esperaba que la tasa de aclaramiento fuera mayor y, en consecuencia, se afectara la biodisponibilidad del anticuerpo. Por lo cual, es importante considerar que, en la presente invención, tras la modificación por aglicosilación, en específico, por aglucosilación, se evidenció que el anticuerpo no presentó este inconveniente.

Teniendo en cuenta lo anterior, una persona versada en la materia no habría llegado a la conclusión de que afucosilar los anticuerpos de D1 habría mejorado sus propiedades; adicionalmente, no existe evidencia o sugerencia en los documentos D2 y D3 que indique dicha mejora. Por el contrario, los efectos de tal modificación habrían sido inciertos o se esperaría que no tuvieran resultados positivos. En consecuencia, la combinación de los documentos D1 y D2 y, D1 y D3 no sugiere que afucosilar los anticuerpos del documento D1 pudiera ser beneficioso o podría conducir a resultados predecibles y mucho menos en la mejora de sus propiedades.

En resumen, la declaración del Dr. Pierce proporciona evidencia de nuevos efectos técnicos no conocidos en el campo técnico específico. Esta declaración proporciona datos que muestran que los anticuerpos, de acuerdo con las presentes reivindicaciones, tienen farmacocinética y toxicidad inesperadamente similares en monos cynomolgus y ratas a sus correspondientes formas fucosiladas. (Declaración del inventor, párrafos 10 – 13).

Como señala el Dr. Pierce, ninguno de esos resultados se esperaba antes de realizar los experimentos (Declaración del inventor, párrafos 10 – 13). Además, se encontró que un anticuerpo anti-FGFR2IIIb afucosilado, de acuerdo con las reivindicaciones que aquí se presentan, tiene un perfil de seguridad aceptable en ensayos clínicos de Fase I.

Por todas estas razones, los anticuerpos reivindicados tienen nivel inventivo sobre las combinaciones de los documentos D1 y D2, así como de los documentos D1 y D3.

Teniendo en cuenta lo anterior, es evidente que el examinador falla en extrapolar los resultados obtenidos en los documentos D2 y D3 a los anticuerpos obtenidos en el documento D1, puesto que no tiene en cuenta que cualquier cambio realizado en anticuerpos diferentes y que se dirigen a distintas dianas farmacológicas tiene un efecto impredecible en vista del conocimiento técnico en el campo. Por lo cual, es evidente que la presente invención es inventiva.

En vista de lo anteriormente expuesto, respetuosamente solicito el retiro de las objeciones de la presente solicitud y la concesión del privilegio solicitado, ya que en opinión del solicitante cumple con todos los requisitos establecidos en la Decisión 486.

Anexos

- Nuevo pliego reivindicatorio.
- Listado de secuencias.
- Declaración del inventor.
- Currículo del inventor.
- Artículo: Yoshino et. al. *“Keratinocyte growth factor receptor expression in normal colorectal epithelial cells and differentiated type of colorectal cancer”*.
- Artículo: Reusch et. al. *“Fc glycans of therapeutic antibodies as critical quality attributes”*.
- Artículo: Pelegrinet et. al. *“Dll1- and Dll4-Mediated Notch Signaling Are Required for Homeostasis of Intestinal Stem Cells”*.

- Artículo: Catenacci et al. *“Updated Antitumor Activity and Safety of FPA144, an ADCC-enhanced, FGFR2b Isoform-specific Monoclonal Antibody, in Patients with FGFR2b+ Gastric Cancer”*.
- Artículo: Juntilla et al. *“Superior In vivo efficacy of Afucosylated Trastuzumab in the Treatment of HER2-amplified Breast Cancer”*.

Atentamente,

JUAN PABLO CADENA SARMIENTO

T.P. No. 57.492 del C.S.J.

C.C. 80.410.337 de Usaquén

Calle 70 A No. 4 – 41

REIVINDICACIONES

1. Una composición que comprende una pluralidad de anticuerpos anti-FGFR2IIIb, donde cada anticuerpo anti-FGFR2IIIb comprende regiones variables de cadena pesada y cadena ligera, donde la región variable de cadena pesada comprende:
 - (i) HVR-H1 que comprende la secuencia de aminoácidos de la SEQ ID NO: 6;
 - (ii) HVR-H2 que comprende la secuencia de aminoácidos de la SEQ ID NO: 7; y
 - (iii) HVR-H3 que comprende la secuencia de aminoácidos de la SEQ ID NO: 8;y la región variable de cadena ligera comprende:
 - (iv) HVR-L1 que comprende la secuencia de aminoácidos de la SEQ ID NO: 9;
 - (v) HVR-L2 que comprende la secuencia de aminoácidos de la SEQ ID NO: 10; y
 - (vi) HVR-L3 que comprende la secuencia de aminoácidos de la SEQ ID NO: 11;donde al menos 95% de los anticuerpos anti-FGFR2IIIb en la composición están afucosilados.
2. La composición de la reivindicación 1, donde el dominio variable de cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 4.
3. La composición de la reivindicación 1 o la reivindicación 2, donde el dominio variable de cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 5.
4. La composición de la reivindicación 1, donde el dominio variable de cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 4 y el dominio variable de cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 5.
5. La composición de cualquiera de las reivindicaciones 1 a 4, donde la cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 2.
6. La composición de cualquiera de las reivindicaciones 1 a 5, donde la cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 3.
7. La composición de la reivindicación 1, donde la cadena pesada comprende la secuencia de aminoácidos de la SEQ ID NO: 2 y la cadena ligera comprende la secuencia de aminoácidos de la SEQ ID NO: 3.
8. La composición de cualquiera de las reivindicaciones 1 a 7, donde los anticuerpos son anticuerpos monoclonales.

9. La composición de cualquiera de las reivindicaciones 1 a 8, donde los anticuerpos son anticuerpos quiméricos.
10. La composición de cualquiera de las reivindicaciones 1 a 9, donde los anticuerpos son anticuerpos humanizados.
11. La composición de cualquiera de las reivindicaciones 1 a 10, donde los anticuerpos carecen de fucosa en Asn297.
12. La composición de cualquiera de las reivindicaciones 1 a 11, donde los anticuerpos comprenden una región constante de cadena ligera κ .
13. La composición de cualquiera de las reivindicaciones 1 a 12, donde los anticuerpos comprenden una región constante de cadena pesada IgG1.
14. Un método para producir anticuerpos anti- FGFR2IIIb afucosilados, dicho método comprende la preparación de células huésped que comprenden el ácido nucleico que codifica el anticuerpo anti- FGFR2IIIb de cualquiera de las reivindicaciones 1 a 13, el cultivo de las células huésped en condiciones adecuadas para expresar el ácido nucleico que codifica el anticuerpo anti- FGFR2IIIb, y la recuperación de los anticuerpos anti- FGFR2IIIb producidos por las células huésped.
15. El método de la reivindicación 14, en el que el cultivo se realiza en condiciones adecuadas para la producción de anticuerpos anti- FGFR2IIIb afucosilados.
16. El método de cualquiera de las reivindicaciones 14 o 15, donde al menos 95% de los anticuerpos anti-FGFR2IIIb producido por la célula anfitriona están afucosilados.
17. El método de la reivindicación 16 donde la fucosa es indetectable en los anticuerpos anti-FGFR2IIIb producidos por la célula anfitriona.
18. El método de la reivindicación 16 o 17, en donde la fucosa es detectada por un método que comprende HPLC, electroforesis capilar, o espectrometría de masas MALDI-TOF.
19. Una composición farmacéutica que comprende la composición de cualquiera de las reivindicaciones 1 a 13 y un portador farmacéuticamente aceptable.

Review

Fc glycans of therapeutic antibodies as critical quality attributes

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Abstract

Critical quality attributes (CQA) are physical, chemical, biological or microbiological properties or characteristics that must be within an appropriate limit, range or distribution to ensure the desired product quality, safety and efficacy. For monoclonal antibody therapeutics that rely on fraction crystallizable (Fc)-mediated effector function for their clinical activity, the terminal sugars of Fc glycans have been shown to be critical for safety or efficacy. Different glycosylation variants have also been shown to influence the pharmacodynamic and pharmacokinetic behavior while other Fc glycan structural elements may be involved in adverse immune reactions. This review focuses on the role of Fc glycans as CQAs. Fc glycan information from the published literature is summarized and evaluated for impact on patient safety, immunogenicity, bioactivity and pharmacodynamics/pharmacokinetics.

Key words: biologic activity, critical quality attribute, Fc glycans, immunogenicity, therapeutic antibody**Introduction**

Therapeutic monoclonal antibodies recognizing cell surface expressed antigens, can engage Fcγ receptors (FcγR) on effector cells (monocytes, macrophages, natural killer (NK) cells, neutrophils, eosinophils and dendritic cells), or bind to Complement 1q (C1q), and elicit immune effector functions such as antibody-dependent cellular phagocytosis (ADCP), antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Ravetch 1997). Additionally, direct apoptosis may be enhanced by effector cell dependent crosslinking (Ravetch 1997). The family of FcγR mediating these activities includes FcγRI, FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb [60–62]. These receptors differ in their antibody binding affinities [FcγRI binds with higher affinity to immunoglobulin G (IgG) than FcγRII and FcγRIII] and can be characterized as either activating (FcγRI, FcγRIIa and FcγRIII) or inhibitory (FcγRIIb).

Antibodies (also commonly referred to as immunoglobulins, or IgG) consist of two heavy chains and two light chains. Each heavy chain contains three domains, CH1, CH2 and CH3 with a hinge region between CH1 and CH2. The heavy chain contains biantennary N-glycan structures linked to Asn297 in the CH2 domain (Jefferis

2009a). The two glycan chains on each of the CH2 domains are often different and contribute to the asymmetrical binding of the fraction crystallizable (Fc) to the Fc-receptors (Ferrara et al. 2006; Masuda et al. 2007; Mimura et al. 2007; Jefferis 2012). Fc glycosylation also stabilizes the Fc structure (Bowden et al. 2012). N-glycosylation is one of the most important post-translational modifications and often results in a remarkable heterogeneity of protein glycoforms (Jefferis 2005; Beck et al. 2008). Depending on the recombinant expression system, this may be a complex type, hybrid type or high-mannose type structure (Durocher and Butler 2009; Jefferis 2009a). The use of mammalian expression systems generally results in complex type biantennary oligosaccharides in the Fc portions. These glycans may have a core fucose and a bisecting N-acetylglucosamine. Moreover, they can vary in terminal galactose and sialic acid content (see Figure 1 and Table I) (Parekh et al. 1985; Jefferis 2005; Durocher and Butler 2009; Jefferis 2009a). In addition, some IgGs contain additional N-glycans in the variable regions of the fragment antigen binding (Fab) portion. Fab N-glycans have been described to differ from the oligosaccharides of the Fc region in that they are generally more highly galactosylated and are more extensively decorated with sialic acids. About 15–20% of IgG are glycosylated in the Fab part with no impact

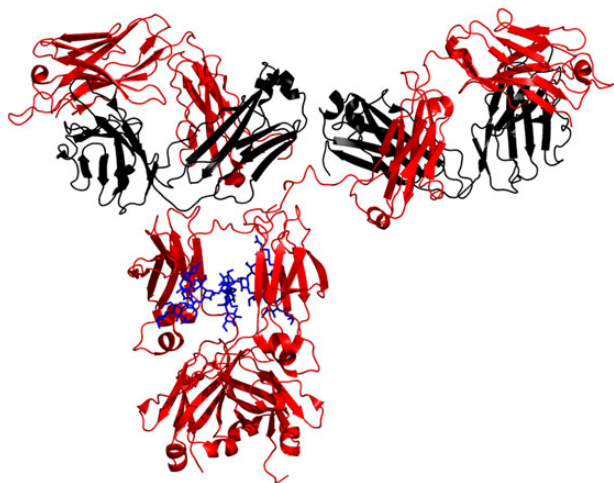


Fig. 1. Ribbon model of an IgG1 (red: heavy chain; black: light chain; blue: glycans) (drawn with pyMol).

to structure (Jefferis 2005; Holland et al. 2006; Huang et al. 2006; Lim et al. 2008). However, Fab glycosylation is not within the scope of this review.

Where the mechanism of action (MoA) involves immune-mediated effector function, these activities can be greatly influenced by the Fc glycosylation pattern. For example, a high degree of galactosylation can promote activation of the complement system in vitro by increasing C1q binding and CDC and could have modest effects on ADCC (Hodoniczky et al. 2005; Nimmerjahn et al. 2007; Houde et al. 2010). It is also well known that decreases in core-fucose levels lead to a pronounced increase in ADCC via increased affinity of IgG1 for FcγRIIIa on immune cells (Okazaki et al. 2004; Jiang et al. 2011). Recent findings indicate that the lack of core fucose also promotes ADCP mediated by FcγRIIIa-positive monocytes and macrophages (Golay et al. 2013; Herter et al. 2014). Differences in glycosylation patterns can also lead to differences in the pharmacodynamic and pharmacokinetic behavior (Newkirk et al. 1996; Goetze et al. 2011). In addition, IgG glycan structural elements such as α1,3-bound galactose and N-glycolylneuraminic acid (NGNA) may be involved in adverse immune reactions (Zhu and Hurst 2002; Chung et al. 2008).

Table I. Glycostructures commonly found in the Fc portion of a therapeutic antibody

Name and composition	Classification	Structure	Exemplary relative abundance for a therapeutic antibody ^a
G0F [H3N4F1]	Complex, fucosylated		35.5
G1F [H4N4F1]	Complex, fucosylated		43.4
G2F [H5N4F1]	Complex, fucosylated		9.5
G1FS [H4N4F1S1]	Complex, fucosylated		0.2
G2S1F [H5N4F1S1]	Complex, fucosylated		0.7
G2S2F [H5N4F1S2]	Complex, fucosylated		0.1
G0 [H3N4]	Complex, nonfucosylated		4.6
G1 [H4N4]	Complex, nonfucosylated		3.3
G2 [H5N4]	Complex, nonfucosylated		0.3
G0F-N [H3N3F1]	Hybrid (monoantennary)		0.5
G0-N [H3N3]	Hybrid (monoantennary)		0.4
M5 [H5N2]	High mannose		1.5
M6 [H6N2]	High mannose		0.1

■, N-acetylglucosamine; ●, mannose; ●, galactose; ▲, fucose; ◆, N-acetylneuraminic acid.

^aData from Reusch et al. (2015).

In some cases, a correlation has been established between FcγRIIIa phenotype and the clinical response, suggesting that ADCC activity could contribute to clinical efficacy (Cartron et al. 2002; Musolino et al. 2008; Bibeau et al. 2009). These results prompted the idea to use glycoengineering as a means of improving efficacy (Beck et al. 2008). Glycoengineering is a process used to manufacture antibodies lacking core fucose, which results in significantly higher binding affinity of human IgG1 antibodies to human FcγRIIIa, and consequently greatly enhanced ADCC activity. Shinkawa et al. (2003) showed a 50-fold increase in ADCC by IgG1s containing a high proportion of afucosylated Fc glycans. Highly afucosylated antibodies can be generated by modifying the oligosaccharide biosynthesis activities in various ways. For example, overexpression of *N-acetylglucosamine-transferase III* (GnTIII) in the Golgi apparatus of the production cell line generates bisected oligosaccharide structures associated with the Fc constant region of the antibody and suppresses fucosylation. In such expression systems, the level of GnTIII expression correlates with the generation of afucosylated IgG1 glycoforms and resulting enhanced ADCC activity (Ferrara et al. 2006). Highly afucosylated antibodies can also be produced using a fucosyltransferase-deficient producer Chinese hamster ovary (CHO) cell line. In this technology, both FUT8 alleles are disrupted by homologous recombination, resulting in completely afucosylated recombinant IgGs (Mori et al. 2004; Matsumiya et al. 2007).

A critical quality attribute (CQA) is defined as a physical, chemical, biological or microbiological property or characteristic that should be within an appropriate limit, range or distribution to ensure the desired product quality, safety and efficacy (Shinkawa et al. 2003). Identification of CQAs is a component of the quality by design (QbD) approach to product development, along with identification of the process parameters that affect these CQAs, the design and control of manufacturing via in-process and quality control testing of critical quality and performance attributes of raw and in-process materials and processes that ensure manufacturing consistency. When the mode of action of an antibody involves effector function, glycosylation represents one of the main sources of heterogeneity with potential impact to safety and efficacy, making it a critical manufacturing parameter to monitor. Therefore, a thorough characterization of carbohydrate content, the structure of the carbohydrate chain, the oligosaccharides and glycosylation sites present on the antibody is critical (Shinkawa et al. 2003).

This review focuses on product-specific clinical and nonclinical information, analytical and biological characterization, general and platform antibody knowledge and published literature.

Results

CQAs

The Annex to ICH Q8 defines CQAs as physical, chemical, biological or microbiological properties or characteristics that should be within an appropriate limit, range or distribution to ensure the desired product quality, safety/immunogenicity, efficacy and pharmacodynamics/pharmacokinetics (Table II).

Safety/immunogenicity

The criticality assessment of a CQA with respect to safety and immunogenicity cannot be performed independent of qualitative and quantitative analysis of the various glycoforms and should take into account nonclinical, e.g. investigational new drug-enabling tox studies, and clinical experience.

Table II. Impact of Fc glycans on safety/immunogenicity, biologic activity/efficacy and clearance (PK/PD)

Glycan species	Safety/immunogenicity	Biologic activity/efficacy	Clearance (PK/PD)
Galactose	Unknown	+	Unknown
α1,3-galactose	--	Unknown	Unknown
Fucose	(-)	++	Unknown
Bisecting GlcNAc	(-)	+	Unknown
High mannose	Unknown	+	--
NANA	Unknown	(-)	+
NGNA	--	(-)	+
β1,2-Xylose/ α1,3-Fucose	--	Unknown	Unknown
NGHC	Unknown	-	(-)

+ Positive impact; - negative impact; ++ high positive impact; -- high negative impact; (+/-) potential impact.

Safety is assessed based on nonclinical observations most often from studies on primates such as cynomolgus monkey, as well as observed clinical adverse events (AEs). These AEs can be target-related or nontarget-related, i.e. “off-target effects”, anti-therapeutic antibody (ATA) related or ATA-independent.

AEs include infusion-related reactions (IRR), injection site reactions, hypersensitivity, anaphylaxis, rash, neutropenia, thrombocytopenia and so on. IRR may be linked to IgG Fc glycosylation, as certain glycoforms can mediate complement activation. Furthermore, Fc glycans that affect Fc effector functions may impact on safety either by increasing otherwise low cell killing potential or through secondary effects such as cytokine release triggered by the activation of effector cells (Baldo 2013).

Biologic activity (efficacy)

While the glycosylation pattern is not known to affect the interaction of an antibody with its target, it can greatly influence effector functions by modulating binding to FcγRy on immune cells (Lund et al. 1995; Daeron 1997; Ravetch 1997; Gerber and Mosser 2001; Ravetch and Bolland 2001; Sondermann et al. 2001; Radaev and Sun 2002; Nimmerjahn and Ravetch 2006). Therefore, it is important to consider the contribution of Fc effector function to the MoA as part of the CQA assessment. This holds true independent of the classification of therapeutic antibodies based on their putative mechanisms of action (Class I MoA: cell-bound antigen with depletion; Class II MoA: cell-bound antigen with functional blocking; Class III MoA: soluble antigen with blocking) (Jiang et al. 2011).

Clearance (PK/PD)

The neonatal Fc receptor (FcRn) plays a role in adult salvage of IgG. FcRn in the acidic endosomes bind to IgG internalized through transcytosis. The IgG is recycled to the cell surface and is released at the pH of blood (so it is prevented from lysosomal degradation). While aglycosylation has profound impacts to effector function, the interaction of IgG-Fc with FcRn is believed to be independent of Fc glycosylation (Jefferis 2012). Changes of the levels of the different glycoforms of the antibody as a component of circulation time are interpreted as arising from differences in clearance rates. In principle, this could be due to differences in binding to the FcRn (Ghetie and Ward 2000; Akilesh et al. 2007; Roopenian and Akilesh 2007; Kuo et al. 2009; Tesar and Bjorkman 2010) or to differences in the clearing rate mediated

by C-type lectins such as dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) or nonclassical Fc-binding receptors including mannose-binding lectin 2 (MBL2), Dectin 1 and the macrophage mannose receptor (MMR) (Wileman et al. 1986; Weis et al. 1998; Dong et al. 1999; Lee et al. 2002; Allavena et al. 2004).

In general, the influence of Fc glycans on pharmacokinetics/pharmacodynamics (PK/PD) is not well understood and the literature concerning the effects of Fc glycosylation variability on PK properties is ambiguous (Deng et al. 2012). While some reports show no impact to PK for different glycospecies (Harris 2005; Huang et al. 2006; Jones et al. 2007; Millward et al. 2008; Chen et al. 2009), others report changes in PK (Wright and Morrison 1994; Newkirk et al. 1996; Kanda et al. 2007).

In contrast, Leabman et al. performed a comprehensive analysis regarding the impact of glycosylation on PK in cynomolgous monkey. This assessment includes data from aglycosylated antibodies generated via engineering (N297A, N297G) or by production in *Escherichia coli*, an antibody with a (L234A/L235A) LALA mutation as well as glycoengineered antibodies. The antibodies targeted different antigen types, including highly expressed multi-transmembrane receptors, soluble cytokines, cell surface proteins and ligands. The results of this comprehensive study demonstrated that antibodies with differences in glycosylation that significantly alter FcγRIIIa binding show no differences in PK (Leabman et al. 2013). The inclusion of a broad range of antibody targets in this study increases the likelihood that these findings are broadly applicable. Several studies have shown that glycoproteins, i.e. IgG-Fc fusion proteins with terminal N-acetylglucosamine residues, are thought to be cleared by means of the mannose receptor. However, this clearance was mediated by N-acetylglucosamine in the receptor region and the Fc glycan ratio did not change (Jones et al. 2007; Keck et al. 2008).

Fc-glycans may not be accessible to asialoglycoprotein or mannose receptors that could mediate antibody clearance. Tao and Morrison (1989) showed that the serum half-life of aglycosylated IgG-Gln [IgG1 with one N-acetyl-glucosamine (GlcNAc)] in mice remains the same as for wild-type IgG1. However, Dong et al. (1999) found an increase in binding and uptake of agalactosyl IgG by mannose receptor on macrophages and dendritic cells.

Impact of Fc glycans

α1,3-Galactose

IgG glycan structural elements such as α1,3-bound galactose may be involved in adverse immune reactions. In humans, anti-α1,3-galactose IgG constitutes as much as 1% of circulating IgG. This glycovariant is unique among endogenous human antibodies because of its atypically high concentration in serum (30–100 µg/mL) and its presence in all humans (Galili et al. 1984).

Therapeutic monoclonal antibodies produced in murine myeloid cell lines like SP2/0 or NSO contain α1,3-gal structures on their Fc glycans (Larsen et al. 1989; Sheeley et al. 1997; Chung et al. 2008). It is generally accepted that CHO cells lack the biosynthetic machinery to synthesize glycoproteins with α1,3-gal moieties (Jenkins et al. 1996). Bosques et al. determined that CHO 1,3-α-galactosyltransferase-1 is active and detected α1,3-gal on two of their products; however, these products were CTLA4-Fc fusion proteins, and the α1,3-gal was on the CTLA4 portion. The authors indicated that this may be a phenomenon resulting during single cell cloning of the production cell line, and that this does not occur in all CHO cells (Bosques et al. 2010).

There are several reports discussing the role of α1,3-gal epitope in xenotransplantation and on glycoproteins (Borrebaeck et al. 1993; Galili 2001; Deglon et al. 2003). The most relevant case is that of cetuximab, a chimeric IgG1 monoclonal antibody (mAb) against the epidermal growth factor receptor that is approved for use in colorectal cancer and squamous-cell carcinoma of the head and neck. While cetuximab is glycosylated on both its Fc and Fab domains, the α1,3-gal structures are found only on the Fab portion (Qian et al. 2007). This may be related to the sequence in the CDR and the fact that cetuximab is produced using SP2/0 cells.

Chung et al. reported a high prevalence of hypersensitivity reactions to cetuximab in some regions of the United States. In most of the subjects who exhibited a hypersensitivity reaction, IgE antibodies specific for the galactose-α-1,3-galactose on cetuximab were present in serum prior to therapy (Chung et al. 2008).

In contrast, van Lammerts et al. reported that anti-α1,3-gal IgE from allergic patients do not bind α1,3-galactosylated glycans on intact therapeutic antibody Fc domains. The authors showed that cetuximab was bound by α-Gal-specific IgE antibodies in the serum of patients and that this binding is restricted to the Fab domain. Cetuximab Fc domains were not bound even though they contained detectable amounts of α1,3-gal. This is believed to be due to the inaccessibility of the α-Gal moiety in the Fc domain, since binding is observed due to glycan exposure following proteolytic digest. The authors observed an increased affinity of α1,3-gal IgE antibodies for glycostructures on the Fab domain bearing two α1,3-gal moieties suggesting that therapeutic mAbs produced in rodent cell lines, and only glycosylated in their Fc domains, are not recognized by α1,3-gal-specific IgE antibodies (Lammerts van Bueren et al. 2011). In conclusion, based on the literature, α1,3-gal in Fc-glycans may be a CQA concerning safety/immunogenicity. It should be closely monitored if Sp2/0 or NS0 cells are used for production of therapeutic antibodies.

β1,2-xylose and α1,3-fucose

Plants are attractive hosts for the manufacture of recombinant protein therapeutics as they are relatively inexpensive systems that can be readily scaled up. However, plant-derived monoclonal antibodies contain complex N-glycans containing β(1,2)-xylose and α1,3-fucose residues not present in humans and are therefore regarded as “carbohydrate cross-reactive determinants” in IgE from sera of allergic patients (Tekoah et al. 2004). The glycan-specific IgE show 23% prevalence and have been found at levels up to 71% in individuals with multiple pollen sensitivity. However, the clinical relevance of these reactive IgE remains unclear. Strasser et al. used RNAi to successfully silence XylIT, the gene coding for β1,2-xylotransferase, in tomato plants and demonstrated a patient-specific reduction in IgE reactivity in their studies (Paulus et al. 2011). *Arabidopsis thaliana* knockouts of the XylIT (encoding β1,2-xylotransferase) and FucT (encoding α1,3 fucosyl transferase) genes have also been generated that are viable with no obvious phenotype. This represents a path forward in the production of protein biotherapeutics with human type N-glycosylation (Strasser et al. 2004). In conclusion if plants are used to produce therapeutic antibodies and the transferases are not silenced, β1,2-xylose and α1,3-fucose should be taken into account as CQAs.

N-glycolylneuraminic acid

Glycans of therapeutic proteins that are produced in SP2/0, NSO and, to a much lesser extent, in CHO cell lines are often modified with the non-human sialic acid N-glycolylneuraminic acid (Neu5Gc; NGNA)

(Hokke et al. 1990; Noguchi et al. 1996). Humans do not synthesize NGNA as the gene encoding CMP-N-acetylneuraminic acid hydroxylase, which produces CMP-NGNA from CMP-N-acetylneuraminic acid (NANA), is mutated (Varki 2007). It was previously thought that healthy humans did not react to NGNA; however, more recent findings suggest that all humans have anti-NGNA antibodies, some at levels up to 0.1–0.2% of circulating IgG. This may be due to dietary exposure, and the subsequent incorporation of NGNA into human proteins (Tangvoranuntakul et al. 2003; Padler-Karavani et al. 2008). Padler-Karavani et al. (2008) suggested that the ongoing antigen–antibody reaction may generate chronic inflammation, possibly contributing to the high frequency of diet-related carcinoma and other diseases in humans.

It has been demonstrated that many humans produce antibodies against oligosaccharide structures with terminal NGNA (Zhu and Hurst 2002). The potential impact of NGNA was evaluated by Durocher and Butler (2009) who indicated that this immunogenicity can reduce efficacy due to rapid clearance of the biotherapeutic, or alternatively, prevent drug re-administration due to an undesirable immune response. As these ATAs represent the first obstacle to xenotransplantation in humans, they are clinically significant from a safety perspective (Milland and Sandrin 2006; Higgins 2010).

Biotherapeutics produced in human cell lines may also become contaminated with NGNA that is incorporated from animal-derived culture medium materials (Ghaderi et al. 2010). NGNA in cells is recycled in lysosomes and incorporated into glycoproteins (Bardor et al. 2005).

In conclusion, NGNA on Fc-glycans is potentially immunogenic and for this reason is most probably a CQA.

Terminal sialic acid

The anti-inflammatory activity of immunoglobulin has repeatedly been linked to Fc sialylation, which may lead to decreased ADCC activity and, in some cases, even affect target binding (Fukushima and Takasaki 1993; Kaneko et al. 2006; Scallon et al. 2007; Anthony, Nimmerjahn, et al. 2008; Anthony, Wermeling et al. 2008; Nimmerjahn and Ravetch 2008). In general, the amount of sialylated Fc glycans of therapeutic antibodies is very low (Jefferis 2006).

In conclusion if there is a significant amount of sialylated glycans, and ADCC is part of the MoA of the therapeutic antibody, the influence of sialylation on FcγRIIIa binding and ADCC should be determined in vitro as part of the CQA assessment.

α1,6-core fucose

Changes in the levels of afucosylated glycostructures on therapeutic antibodies may be of concern from a safety perspective. Toxicity due to off-target binding may also be a concern for antibodies with higher ADCC. Jiang et al. indicate that increased afucosylation leading to increased effector function is a potential safety concern for monoclonal antibodies with moderate or low effector function (Jiang et al. 2011).

Increased crosslinking of activating FcγRs due to higher levels of afucose may also be a safety concern due to the additional release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α and IFNγ.

The lack of core fucose (i.e. the α1,6-linked fucose on the GlcNAc residue involved in the amide bond with the asparagine of the N-glycosylation site) on the carbohydrate moiety linked to the Fc region of an IgG molecule leads to a pronounced increase in ADCC via increased affinity for the FcγRIIIa expressed on immune cells such as NK cells and macrophages (Rothman et al. 1989; Umana et al. 1999;

Shields et al. 2002; Niwa et al. 2004; Okazaki et al. 2004; Yamane-Ohnuki et al. 2004; Peipp et al. 2008; Chung et al. 2012). Additionally, the absence of core fucose leads to increased Fc-dependent binding to FcγRIII positive nonclassical/intermediate monocytes and macrophages, which translates into increased ADCC (Herter et al. 2014). In contrast, binding to FcγRI and II-positive and FcγRIII-negative classical monocytes, as well as ADCC-mediated by these cells, remain unchanged, irrespective of fucosylation levels (Herter et al. 2014).

While afucosylation increases ADCC in glycoengineered antibodies, the impact on ADCC due to small variations in afucosylation levels resulting from standard CHO-based manufacturing processes remains to be assessed. Finally, increased afucosylation has not been demonstrated to have any impact on CDC activity.

Studies by Junttila et al. highlighted the impact of afucosylation on pharmacological properties and half-life of an IgG1, relative to a wild-type control. In these studies, afucosylated trastuzumab displayed moderately altered pharmacokinetic dispositions compared with trastuzumab, slightly faster elimination from the circulation, and a modest reduction in half-life (Junttila et al. 2010). The authors hypothesized that the faster clearance may result from the differential bio-distribution that leads to the enrichment of antibodies to immune effector cell-rich organs due to the increased FcγRIII affinity. In conclusion concerning the scientific literature there is no clear evidence that afucosylated Fc glycans might be of concern for safety.

There is clear evidence that afucosylated Fc glycans increase ADCC and if ADCC is part of the MoA, afucosylated Fc-glycans are always likely to be a CQA concerning efficacy.

Bisecting GlcNAc

CHO cells lack the gene encoding GnTIII. Therefore antibodies produced in CHO cells lack detectable bisecting GlcNAc glycostructures (Durocher and Butler 2009). GnTIII overexpressed in antibody-producing cells catalyzes the addition of GlcNAc to N-linked oligosaccharides. The addition of bisecting GlcNAc inhibits core-fucosylation and conversion of hybrid to complex glycans (Ferrara et al. 2006).

In general, glycostructures containing bisecting GlcNAc are abundant in human polyclonal IgG and are not considered a safety concern (Jefferis 2009a). Recently, Ritamo et al. (2013) showed that 7% of the Fc-glycans on intravenous IgG (IVIG) contain glycan structures with bisecting GlcNAc (analyzed at the glycopeptide level) (Ritamo et al. 2013). Therefore, it is unlikely that ATAs are produced against the bisecting GlcNAc epitope. In summary, bisecting GlcNAc structures are not a safety issue.

Galactose

Highly galactosylated glycostructures on therapeutic antibodies may be of concern as they increase in vitro C1q-binding and CDC activity (Boyd et al. 1995; Tsuchiya et al. 1989). Increased galactosylation of CHO-derived antibodies has been shown to increase FcγRII and FcγRIIIa binding as measured using surface plasmon resonance (Ritamo et al. 2013). Additionally, it has been demonstrated that the increased galactosylation enhances ADCC activity (Jiang et al. 2011). However, the impact of galactosylation is subtle compared with the impact of afucosylation (Ritamo et al. 2013).

Several reports indicate that a high degree of galactosylation promotes increased binding to C1q leading to enhanced activation of the complement system and CDC activity (Hodoniczky et al. 2005; Jefferis 2009a; Nimmerjahn et al. 2007). Similar effects have been observed for the glycoengineered therapeutic antibody obinutuzumab

in vitro, although CDC is not considered to be a relevant in vivo activity. Several reports state that the extent of terminal galactosylation does not affect ADCC in standard IgG1 antibodies (Boyd et al. 1995; Shinkawa et al. 2003; Hodoniczky et al. 2005). Another report found that terminal galactosylation has a positive effect on FcγRIIIa binding (Houde et al. 2010). We have recently demonstrated enhanced ADCC upon enzymatic hypergalactosylation of four different monoclonal antibodies produced using standard CHO manufacturing processes (Tejada et al. submitted; Thomann et al. 2015). We also showed that elevated levels of terminal galactose have no effect on ADCC of two different glycoengineered therapeutic antibodies (Thomann et al. 2015), indicating that the extent to which terminal galactose modulates ADCC depends on the background level of afucosylation. Lastly, we quantitatively compared the effects of galactosylation and afucosylation in the context of glycan heterogeneity to demonstrate that while galactose can influence ADCC activity, afucosylation remains the primary driver of this activity.

Karsten et al. (2012) recently reported an anti-inflammatory activity of IgG1 mediated by Fc galactosylation and association of FcγRIIb and dectin-1. The authors concluded that high N-glycan galactosylation of IgG1 molecules promotes cooperative signaling of the FcγRIIb with dectin-1, resulting in an inhibitory signaling pathway that blocks proinflammatory effector functions.

In conclusion if CDC is part of the MoA for a therapeutic antibody the impact of galactosylation levels reflecting the manufacturing experience should be considered as it relates to efficacy during CQA assessment. If ADCC is part of the MoA of the therapeutic antibody, the impact of galactosylation should be determined by in vitro studies as part of CQA assessment.

High mannose glycostructures

No data or direct evidence in the literature supports the immunogenicity of high mannose glycan structures (Pacis et al. 2011). Studies by Zhou et al. reported enhanced FcγRIIIa binding and ADCC in antibodies with oligomannose-type glycans generated using kifunensine (Yu et al. 2012). Kanda et al. (2007) generated core fucose-lacking human IgG1 antibodies with three different N-linked Fc oligosaccharides, namely, a high-mannose, hybrid and complex type, using the same producing clone. They demonstrated that the mannosylated glycostructures could induce ADCC via FcγRIIIa binding but to a lesser extent than the hybrid and complex structures. The same holds true for CDC and C1q binding. More recently, Yu et al. (2012) confirmed that high mannose glycoforms exhibited higher FcγRIIIa binding and ADCC activity as well as decreased C1q binding and CDC activity.

In general, core fucose is absent in the highly mannosylated glycoforms generated. Therefore, it remains unclear if the observed increases in FcγRIIIa binding and ADCC activity are due to the presence of multiple mannose moieties, or the absence of core fucose. The reduced C1q binding and CDC activity observed in these studies may also reflect the lack of terminal galactose in these highly mannosylated forms.

Recent studies show clear evidence for selective clearance of oligomannose forms of IgG1 and IgG2 most likely via a mannose receptor-mediated mechanism (Goetze et al. 2011; Alessandri et al. 2012; Yu et al. 2012). Goetze et al. (2011) used IgG1 and IgG2 in human studies to demonstrate that Man5 isoforms were preferentially removed from circulation, independent of the route of administration. In conclusion if there is a significant amount of mannosylated glycans and if ADCC is part of the mode of action of a therapeutic antibody, mannosylation may be a CQA concerning efficacy. Additionally, there is clear

evidence that oligomannose bearing IgGs are faster cleared than others and therefore may be a CQA concerning PK/PD.

Nonglycosylated heavy chain

Jung et al. (2011) concluded that the unmasking of the region around Asn297 does not result in the formation of a neo-epitope. At present, no adverse immunogenicity effects associated with high levels of nonglycosylated heavy chain (NGHC) have been reported in any clinical study. Furthermore, antibodies with high levels of NGHC have significantly reduced bioactivity and effector function. Therefore, NGHC is likely not a concern from a safety or immunogenicity perspective. Aglycosylation of Asn297 completely abolishes binding to FcγRIII and reduces the binding affinity to C1q 10-fold (Tao and Morrison 1989; Mimura et al. 2000). The reduced binding affinity of aglycosylated antibodies abrogates both ADCC and CDC activities (Tao and Morrison 1989; Lund et al. 1996; Sazinsky et al. 2008; Jefferis 2009b; Jung et al. 2010, 2011). Ha et al. isolated hemi-glycosylated mAb (mAb with only one heavy chain being glycosylated and the other one being aglycosylated). It was separated from fully glycosylated and aglycosylated forms using cation-exchange chromatography (Ha et al. 2011). These studies demonstrated that the hemi-glycosylated mAb had decreased binding to all FcγR including both activating and inhibiting receptors. The hemi-glycosylated form also showed decreased ADCC using peripheral blood mononuclear cells as effector cells. The studies also showed a modest, but statistically significant, decrease in C1q binding for the hemi-glycosylated mAb. Shatz et al. (2013) generated heterodimers with different glycan structures and showed that removal of fucose from only one chain of a fully glycosylated heterodimer is sufficient to fully restore ADCC activity relative to a wild-type molecule. However, reduced potencies were observed for hemi-glycosylated or hemi-glycosylated-hemi-afucosylated heterodimers, relative to wild-type or a fully glycosylated heterodimer. These studies showed that afucosylation has a significant impact on the ADCC activity of the hemi-glycosylated species; however, the impact of afucosylation is far greater when both heavy chains are glycosylated. Aglycosylated antibodies have been shown to exhibit conformational changes, decreased thermal stability, increased tendency to aggregate and loss of effector functions (Ghirlando et al. 1999; Hristodorov et al. 2013). Evidence regarding the impact of aglycosylation on PK is inconclusive as there are studies demonstrating decreased and normal half-life. For example, comparisons of recombinant chimeric or mouse monoclonal antibodies against N297A mutant versions in mouse or rat show reduced half-lives for the mutant aglycosylated antibodies compared with their wild-type counterparts (Wawrzynczak et al. 1989, 1992).

In contrast, Hristodorov et al. (2013) generated six different wild-type antibodies and compared them to aglycosylated versions, generated via N297A mutation. The aglycosylated antibodies were found to be less stable and displayed a propensity to aggregate when subjected to low pH reflecting viral inactivation conditions common in the antibody manufacturing process. The PK properties of both wild-type and aglycosylated antibodies were determined to be nearly identical in rat studies. Tao and Morrison (1989) used a chimeric mouse-human IgG and found a shorter half-life for aglycosylated IgG3 compared with that of wild-type IgG3 in mice, while no change was observed for aglycosylated IgG1 compared with wild-type IgG1 (Tao and Morrison 1989). Jung et al. (2011) state that aglycosylated antibodies display PK that are at least comparable to that of “normal” IgG therapeutics.

In conclusion, the literature is not quite clear here and if there is a significant amount of NGHC the influence on product quality should be assessed by in vitro studies to decide if it is a CQA or not.

Discussion

The QbD paradigm requires an in-depth understanding of (i) the mechanism(s) of action of the biotherapeutic, which are associated with the desired clinical activity, (ii) the properties (CQA) of the biotherapeutic that are associated with the desired clinical activity, (iii) the process parameters that impact the CQA and (iv) the control of the manufacturing process through a timely analysis and monitoring of the CQA, along with appropriate control of process components such as raw and in-process materials. This ensures that the product with the desired qualities is achieved consistently over time.

For mAb therapeutics specifically, glycosylation is one of the most relevant post-translational modifications in the manufacturing process. This results in remarkable heterogeneity of the antibody glycoforms. The diverse set of hosts used by industry in the manufacture of monoclonal antibodies also increases this glyco-heterogeneity, which can be critical to antibodies associated with MoAs such as CDC, ADCC and ADCP.

Different glycovariants such as high mannose, have been reported to reduce the serum half-life of therapeutic antibodies while other Fc glycan structural elements such as α 1,3-bound galactose and NGNA may be involved in adverse immune reactions. Both should be considered in terms of efficacy and safety, respectively.

The intimate relationship established between Fc glycosylation of the mechanisms associated with the clinical activity of therapeutic antibodies makes the thorough analysis and characterization of the glycosylation-specific CQA critical to the appropriate assessment of the impact that changes observed in specific glycovariants may have on safety and/or efficacy. This information can appropriately inform manufacturing process development such that these processes are more finely adjusted to deliver the desired Fc glycosylation.

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Conflict of interest statement

None declared.

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; AE, adverse events; ATA, anti-therapeutic antibody; CDC, complement dependent cytotoxicity; CHO, Chinese hamster ovary; CQA, critical quality attribute; C1q, Complement 1q; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin; Fab, fragment antigen binding; Fc, fraction crystalizable; Fc γ R, Fc γ receptors; FcRn, neonatal Fc receptor; GlcNAc, N-acetyl-glucosamine; GnTIII, N-acetylglucosaminyltransferase III; IFN, interferon; IgG, immunoglobulin G; IRR, infusion-related reactions; MBL2, mannose-binding lectin 2; mAb, monoclonal antibody; MMR, macrophage mannose receptor; MoA, mechanism/mode of action; NGHC, nonglycosylated heavy chain; NGNA, N-glycolylneuraminic acid;

NK cells, natural killer cells; PK/PD, pharmacokinetics/pharmacodynamics; QbD, quality by design; TNF, tumor necrosis factor.

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Summary:

- Asset team leader for oncology antibody therapeutics, including immunoncology targets, from preclinical development through global Phase I development, including responsibility for overall strategy, timelines, and budget.
 - Leader for development and implementation of FISH and IHC laboratory developed tests for prospective patient selection
 - Core strengths in nonclinical development
 - Experience in all stages of oncology drug discovery, from hit identification through initial clinical trials, including in vitro assay development, screening, in vivo hit confirmation and validation
 - Strong communication skills, both written and verbal
-

WORK EXPERIENCE:

- **Five Prime Therapeutics, Inc. October 2002- present,**
 - 2016- present Executive Director, Asset Team Leader, Development Sciences
 - 2014- 2016, Director, Project Team Leader, Clinical Development
 - 2012-2014, Associate Director, Discovery Biology, Oncology
 - 2008- 2012, Senior Scientist, Discovery Biology, Oncology
 - 2006- 2008, Scientist III, Discovery Biology
 - 2004-2006, Scientist II, Discovery Biology
 - 2002-2004, Scientist I, Discovery Biology
- Currently asset team leader responsible for FivePrime immune-oncology antibody therapeutics targeted for 2017-2018 INDs
- Previously project team leader for FPA144, through Phase I development responsible for the overall development strategy.
- Translational medicine responsibilities for development of FISH and IHC research use only laboratory developed tests that are used for prospective patient selection.
- Member of joint development committee of another clinical candidate currently in Phase I development.
- Responsible for hit follow-up through preclinical validation of therapeutic efficacy for several lead molecules
- Led in vitro screening projects in oncology designed to identify novel protein therapeutics/ antibody targets. These assays include 3-D colony formation assays on both tumor initiating cells and on traditional cell lines and complex cell signaling assays
- Project team leader responsible for the successful establishment and use of more than 30 patient derived xenograft models of breast, lung and colon tumors in mice including extensive hands on pharmacology experience, primarily in mice.
- Previously, leader of several projects aimed to identify potential protein therapeutics for other therapeutic indications including inflammatory bowel disease, mucositis, radiation proctitis, and other regenerative medicine indications

EDUCATION:

- **Postdoctoral Fellow, 1998-2002**, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC
 - Advisor: Robert J. Lefkowitz, M.D.
 - Research Topic: Mechanisms of G-Protein Coupled Receptor Signaling to MAPK Cascades and Cross Talk with Receptor Tyrosine Kinases
- **Ph.D. Pharmacology/ Toxicology**, University of Arizona, Tucson, AZ
 - Dissertation Adviser: John W. Regan, Ph.D.
 - Dissertation Title: The FP Prostanoid Receptor: Isoforms and Functional Studies
- **B.A. English**, Bates College, Lewiston, ME

SKILLS:

- Leadership of program teams from lead identification through Phase 1 testing
- Extensive animal experience including establishment of both subcutaneous and orthotopic patient derived xenograft models
- Development and execution of complex HTS assays including cell signaling ELISAs and multiplex assays such as luminex
- Expert in cell signaling, especially G-protein coupled receptor and receptor tyrosine kinase signaling
- Experience with many different functional assays including ligand binding, ELISA, traditional second messenger signaling, gene reporter assays, western blotting
- Extensive experience with cell culture, including primary cell cultures, establishment of new tumor initiating cell lines, mouse embryonic stem cells, and mouse embryo fibroblast cell line establishment from genetically modified animals. Transfection experience with a number of systems including electroporation, retroviral and adenoviral based systems
- Protein purification from a number of systems including E. coli and mammalian systems
- Molecular biology experience including subcloning, cDNA and genomic library screening, southern and northern blotting, in vitro transcription and translation
- Excellent writing and computer skills
- Currently stand on both the laboratory operations committee and the laboratory purchasing committee

PUBLICATIONS:

1. Martin NP, Whalen EJ, Zamah MA, **Pierce KL**, Lefkowitz RJ. PKA-mediated phosphorylation of the beta(1)-adrenergic receptor promotes G(s)/G(i) switching. *Cell Signal.* (2004) **16**:1397-403.
2. Tohgo A, Choy EW, Gesty-Palmer D, **Pierce KL**, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM. The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem.* (2003) **278**:6258-67.
- 2: Lefkowitz RJ, **Pierce KL**, Luttrell LM. Dancing with different partners: protein kinase a phosphorylation of seven membrane-spanning receptors regulates their G protein-coupling specificity. *Mol Pharmacol.* (2002) **62**:971-4.

3: **Pierce KL**, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol.* (2002) **3**:639-50. Review.

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5: Anthony TL, Fujino H, **Pierce KL**, Yool AJ, Regan JW. Differential regulation of Ca(2+)-dependent Cl- currents by FP prostanoid receptor isoforms in *Xenopus* oocytes. *Biochem Pharmacol.* (2002) **63**:1797-806.

6: Tohgo A*, **Pierce KL***, Choy EW, Lefkowitz RJ, Luttrell LM. Beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem.* (2002) **277**:9429-36. * These authors contributed equally.

7: **Pierce KL**, Lefkowitz RJ. Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci.* (2001) **2**:727-33. Review.

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9: **Pierce KL**, Tohgo A, Ahn S, Field ME, Luttrell LM, Lefkowitz RJ. Epidermal growth factor (EGF) receptor-dependent ERK activation by G protein-coupled receptors: a co-culture system for identifying intermediates upstream and downstream of heparin-binding EGF shedding. *J Biol Chem.* (2001) **276**:23155-60.

10: Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, **Pierce KL**, Lefkowitz RJ. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci U S A.* (2001) **98**:2449-54.

11: Cao W, Luttrell LM, Medvedev AV, **Pierce KL**, Daniel KW, Dixon TM, Lefkowitz RJ, Collins S. Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. *J Biol Chem.* (2000) **275**:38131-4.

12: Woodward DF, Krauss AH, Chen J, Gil DW, Kedzie KM, Protzman CE, Shi L, Chen R, Krauss HA, Bogardus A, Dinh HT, Wheeler LA, Andrews SW, Burk RM, Gac T, Roof MB, Garst ME, Kaplan LJ, Sachs G, **Pierce KL**, Regan JW, Ross RA, Chan MF. Replacement of the carboxylic acid group of prostaglandin F2 α with a hydroxyl or methoxy substituent provides biologically unique compounds. *Br J Pharmacol.* (2000) **130**:1933-43.

13: Fujino H, **Pierce KL**, Srinivasan D, Protzman CE, Krauss AH, Woodward DF, Regan JW. Delayed reversal of shape change in cells expressing FP(B) prostanoid receptors. Possible role of receptor resensitization. *J Biol Chem.* (2000) **275**:29907-14.

- 14: Anthony TL, **Pierce KL**, Regan JW. Characterization of EP prostanoid receptor subtypes in primary cultures of bovine ciliary epithelial cells by immunofluorescent microscopy and functional studies. *Curr Eye Res.* (2000) 20:394-404.
- 15: Roudabush FL, **Pierce KL**, Maudsley S, Khan KD, Luttrell LM. Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. *J Biol Chem.* (2000) 275:22583-9.
- 16: Maudsley S, **Pierce KL**, Zamah AM, Miller WE, Ahn S, Daaka Y, Lefkowitz RJ, Luttrell LM. The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. *J Biol Chem.* (2000) 275:9572-80.
- 17: **Pierce KL**, Maudsley S, Daaka Y, Luttrell LM, Lefkowitz RJ. Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. *Proc Natl Acad Sci U S A.* (2000) 97:1489-94.
- 18: Fujino H, Srinivasan D, **Pierce KL**, Regan JW. Differential regulation of prostaglandin F(2alpha) receptor isoforms by protein kinase C. *Mol Pharmacol.* (2000) 57:353-8.
- 19: **Pierce KL**, Fujino H, Srinivasan D, Regan JW. Activation of FP prostanoid receptor isoforms leads to Rho-mediated changes in cell morphology and in the cell cytoskeleton. *J Biol Chem.* (1999) 274: 35944-9.
- 20: **Pierce KL**, Regan JW. Prostanoid receptor heterogeneity through alternative mRNA splicing. *Life Sci.* (1998) 62:1479-83. Review.
- 21: Anthony TL, **Pierce KL**, Stamer WD, Regan JW. Prostaglandin F2 alpha receptors in the human trabecular meshwork. *Invest Ophthalmol Vis Sci.* (1998) 39:315-21.
- 22: Pierce KL, Bailey TJ, Hoyer PB, Gil DW, Woodward DF, Regan JW. Cloning of a carboxyl-terminal isoform of the prostanoid FP receptor. *J Biol Chem.* (1997) 272:883-7.
- 23: Graves PE, **Pierce KL**, Bailey TJ, Rueda BR, Gil DW, Woodward DF, Yool AJ, Hoyer PB, Regan JW. Cloning of a receptor for prostaglandin F2 alpha from the ovine corpus luteum. *Endocrinology* (1995) 136:3430-6.
- 24: **Pierce KL**, Gil DW, Woodward DF, Regan JW. Cloning of human prostanoid receptors. *Trends Pharmacol Sci.* (1995) 16:253-6. Review
- 25: Regan JW, **Bailey TJ**, Pepperl DJ, Pierce KL, Bogardus AM, Donello JE, Fairbairn CE, Kedzie KM, Woodward DF, Gil DW. Cloning of a novel human prostaglandin receptor with characteristics of the pharmacologically defined EP2 subtype. *Mol Pharmacol.* (1994) 46:213-20.
- 26: Regan JW, Bailey TJ, Donello JE, Pierce KL, Pepperl DJ, Zhang D, Kedzie KM, Fairbairn CE, Bogardus AM, Woodward DF, et al. Molecular cloning and expression of

human EP3 receptors: evidence of three variants with differing carboxyl termini. *Br J Pharmacol.* (1994)**112**:377-85.

REFERENCES:

References available on request

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In the Matter of EPA 14752705.5 - Declaration of Dr. Kristen Pierce

I, Kristen Pierce, hereby declare as follows:

1. As shown in the attached Curriculum Vitae, I have been employed by the patent applicant, Five Prime Therapeutics, Inc., since 2002 and my current title is Senior Director, Asset Team Leader, Clinical Development. I have a PhD in Pharmacology and Toxicology from the University of Arizona and conducted post-doctoral research at Duke University prior to joining Five Prime Therapeutics. Further details of my experience and publications are provided in the attached CV.
2. I am a co-inventor of the present European patent application, EP 14752705.5.
3. I have reviewed the application text and examination report of the European Patent Office dated 9th March 2017. I have also seen the claims that are being submitted with this Declaration, which I understand relate to use of specifically defined anti-FGFR2IIIb antibodies to treat cancers in which this target is over-expressed. I am informed by Five Prime that the Examiner believes that this claimed invention is obvious in view of WO 2010/054265 ("D1") in the light of either of Yamane-Ohnuki & Satoh (*mABS* 1: 230-236 (2009), referred to as "D2") or Niwa *et al* (*Journal of Immunological Methods* 306: 151-160 (2005), referred to as "D3").
4. D1 describes antibodies with the HVR sequences recited in claim 1 whereas D2 is a review article describing production of afucosylated therapeutic antibodies. D3 is an earlier publication also relating to afucosylated antibodies and is cited as reference 27 by D2. I assume therefore that the authors of D2 took into account the contents of D3 when writing their review. I do not consider that D3 has any significant information, in relation to the present invention, over and above D2, and therefore, my comments in relation to D2 apply equally to D3.
5. The D2 review article notes that, as of 2009, Phase I clinical trials had been performed with two afucosylated antibodies called MEDI-563 and BIW-8405. The article states that "[c]ompared to fucosylated IgGs, non-fucosylated forms exhibit dramatically enhanced ADCC due to the enhancement of FcγRIIIa binding capacity without any detectable change in CDC or antigen binding capability." (D2 at 232, first column of text.) The article also states that "[t]he non-fucosylated antibodies have much higher

binding affinity for FcγRIIIa than fucosylated human serum IgG, which is an essential feature for overcoming the competition of human serum IgG or binding of therapeutic IgG1 to FcγRIIIa on natural killer (NK) cells." (D2 at 232, sentence bridging first and second columns.)

6. A main focus of D1 is on uses of anti-FGFR2 monoclonal antibodies for treating solid tumors. (See D1 at cols. 12-13 and Example 4.) While D2 indicates that afucosylated antibodies may have a higher binding affinity for FcγRIIIa, and that this higher affinity may lead to increased efficacy in patients with cancers of immune origin and other diseases with defective immune signaling (such as asthma), it has not been demonstrated that this increased affinity translates to increased efficacy in patients with solid tumors.
7. ADCC in solid tumors is not well understood and the fundamental mechanisms may be different. In the Yamane reference, both of the examples given, KW-0761 and MEDI-563/BIW-8405, target immune indications, with KW-0761 targeting cutaneous T-cell lymphoma and MEDI-563/BIW-8405, targeting eosinophils in asthma. Likewise, anti-CD20 antibodies of the type referred to in D3 (an example of which is Obinutuzumab, the first approved antibody with enhanced ADCC via afucosylation), also target cancers of immune origin, lymphomas and leukemias.
8. Therefore, in my opinion, a person of ordinary skill in the art reading D1 and D2/D3 could not predict how removing the fucose at Asn297 would impact the overall efficacy of the antibodies claimed in this application.
9. Changing an antibody's sugar content can also alter its pharmacokinetic properties. (Reviewed by D. Reusch & M. Tejada, *Glycobiology* 2015, 1-10, Table 8 and pages 3-5; copy submitted with this Declaration). Producing an afucosylated antibody may alter the clearance of the antibody. For example, in preclinical experiments, it has been demonstrated that afucosylated trastuzumab (an anti-Her2 antibody) has faster clearance than unmodified trastuzumab in mice genetically modified to express the human FcγRIIIa. (See Junttila et al., *Am. Soc. Cancer Res.* 70: 4481-9 (2010); copy submitted with this Declaration.) Thus, a person of ordinary skill in the art might predict that afucosylated versions of the antibodies of D1 will also have altered

pharmacokinetics, specifically a faster clearance. A faster clearance rate could have an impact on the efficacy of the antibodies.

10. For this reason, Five Prime Therapeutics sponsored a pharmacokinetic study in cynomolgus monkeys to test the clearance rates of fucosylated and afucosylated anti-FGFR2IIIb antibodies. We found that the clearance of the afucosylated antibody was not different from the clearance of the fucosylated antibody. This result was not expected in view of the prior data on anti-Her2 antibodies.
11. Specifically, one male and one female monkey were dosed with 10 mg/kg of either a fucosylated anti-FGFR2IIIb antibody or an afucosylated anti-FGFR2IIIb antibody with the claimed HVR and variable domain sequences. The afucosylated and fucosylated forms of the antibody were found to have similar PK profiles with a half-life of 7.4-7.7 days during the first-order elimination phase (occurring within 3 weeks of dosing) followed by an accelerated terminal clearance suggestive of target mediated clearance.
12. Another unintended possible consequence of glycoengineering an antibody for enhanced ADCC is increased toxicity. (Also reviewed by Reusch & Tejada at page 5.) An afucosylated antibody might induce NK cells to kill normal cells expressing the antibody's target antigen. In contrast, these normal cells may be spared by a non-glycoengineered antibody. This cell killing could lead to worse toxicity and make the afucosylated antibody unsuitable for use in patients. The toxicity of an afucosylated anti-FGFR2IIIb antibody cannot be extrapolated from studies of other afucosylated antibodies, since the expression profile of the individual target antigens is unique to each antigen. Thus, a person of ordinary skill could not have predicted the impact of afucosylation of the claimed antibodies on their toxicity.
13. To address this issue, Five Prime Therapeutics sponsored toxicology studies comparing the afucosylated form of an anti-FGFR2IIIb antibody with the claimed HVR and variable domain sequences to the fucosylated form in both rats and cynomolgus monkeys. No increased toxicity was noted in the animals treated with the afucosylated form of the FGFR2IIIb antibody compared to the fucosylated form. This result could not have been predicted in advance for the reasons described in the

preceding paragraph, and thus, was not expected. In these studies, both rats and monkeys were treated with 4 weekly doses of 1.5, 30 or 150 mg/kg of either the fucosylated or the afucosylated FGFR2IIIb antibodies. The animals were sacrificed on day 29. For all animals, regular in-life observations were made, and at necropsy, both macroscopic and microscopic examinations were performed. In all of the studies, the fucosylated and non-fucosylated antiFGFR2IIIb antibodies were well tolerated with no changes in the clinical chemistry, although there were some observations made in one group of treated animals but not another.

14. In light of the positive animal studies referred to above, Five Prime has sponsored a Phase 1 clinical trial with this afucosylated anti-FGFR2IIIb antibody. This trial included a "dose escalation" study, in which the antibody was administered to patients having advanced solid tumors. In additional cohorts, the antibody was administered specifically to gastric cancer patients. Preliminary results showed that 5 gastric cancer patients that tested positive for high expression of FGFR2IIIb showed partial responses. Gastric cancer in particular has low response rates to currently approved treatments. Furthermore, in the dose escalation study, a patient with bladder cancer positive for FGFR2IIIb showed a complete response to the antibody and survival over at least 700 days.
15. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.



Kristen Pierce



Date

Encs:

CV of Dr. Pierce

D. Reusch & M. Tejada, *Glycobiology* 2015

Junttila et al., *Am. Soc. Cancer Res.* 2010