Generation and Activity of a Humanized Monoclonal Antibody That Selectively Neutralizes the Epidermal Growth Factor Receptor Ligands Transforming Growth Factor-α and Epiregulin

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ABSTRACT

At least seven distinct epidermal growth factor (EGF) ligands bind to and activate the EGF receptor (EGFR). This activation plays an important role in the embryo and in the maintenance of adult tissues. Importantly, pharmacologic EGFR inhibition also plays a critical role in the pathophysiology of diverse disease states, especially cancer. The roles of specific EGFR ligands are poorly defined in these disease states. Accumulating evidence suggests a role for transforming growth factor α (TGFα) in skin, lung, and kidney disease. To explore the role of Tgfa, we generated a monoclonal antibody (mAb41) that binds to and neutralizes human Tgfa with high affinity (K_D = 36.5 pM). The antibody also binds human epiregulin (Ereg) (K_D = 346.6 pM) and inhibits ligand induced myofibroblast cell proliferation (IC50 values of 0.52 and 1.12 nM for human Tgfa and Ereg, respectively). In vivo, a single administration of the antibody to pregnant mice (30 mg/kg s.c. at day 14 after plug) or weekly administration to neonate mice (20 mg/kg s.c. for 4 weeks) phenocopy Tgfa knockout mice with curly whiskers, stunted growth, and expansion of the hypertrophic zone of growth plate cartilage. Humanization of this monoclonal antibody to a human IgG4 antibody (LY3016859) enables clinical development. Importantly, administration of the humanized antibody to cynomolgus monkeys is absent of the skin toxicity observed with current EGFR inhibitors used clinically and no other pathologies were noted, indicating that neutralization of Tgfa could provide a relatively safe profile as it advances in clinical development.

Introduction

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that signals after ligand binding and homodimerization, or heterodimerization with other ErbB family members (Schlessinger, 2002). This signaling event is accompanied by auto- and cross-phosphorylation with recruitment of accessory proteins and subsequent downstream signaling through multiple pathways including the phospholipase C, extracellular signal-regulated kinase sub-group of the mitogen-activated protein kinases (ERK/MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and p38 pathways (Cohen et al., 1980; Yarden and Sliwkowski, 2001; Oda et al., 2005). EGFR signaling affects diverse cellular processes, including cell survival, proliferation, migration, and differentiation (Carpenter, 1983; Schlessinger et al., 1983). EGFR deficient mice die shortly after birth and show retarded growth, heart and lung defects, and eye and skin abnormalities (Luetteke et al., 1994; Hansen et al., 1997; Chen et al., 2000).

Ligands that bind and activate the EGFR include epidermal growth factor (EGF), transforming growth factor-α (TGFα), heparin-binding epidermal-like growth factor (HB-EGF), epi-

regulin (Ereg), betacellulin (Btc), amphiregulin (Areg) and epigen (Epgn) (Harris et al., 2003; Schneider and Wolf, 2009). All seven proteins are structurally related by a shared sequence of 50–60 amino acids designated the EGF domain, which contains six conserved cysteine residues (Davis, 1990). The EGFR ligands are synthesized as type I transmembrane proteins that are proteolytically processed to release the EGF domain, which may then signal in an autocrine, paracrine, or endocrine manner (Sunnarborg et al., 2002; Harris et al., 2003).

Dysregulation of EGFR signaling contributes to cancer (Normanno et al., 2006) as the clinical use of EGFR inhibitors has demonstrated improved outcomes for patients with
cancer. Clinical use of pharmacologic EGFR inhibitors in patients with cancer also supports its roles in normal adult tissues, most notably skin where their use is accompanied by a rash (Rhee et al., 2005). All of the current drugs that target the EGFR for cancer are associated with a cutaneous purulent skin rash predominantly on the face, neck, shoulder, and back, occurring in up to 85% of patients (Li and Perez-Soler, 2009). The EGFR pathway has also been implicated but less well-studied in other diseases, such as atherosclerosis (Nakata et al., 1996; Reape et al., 1997), hypertension (Kagiyama et al., 2002; Flamant et al., 2003), left ventricular hypertrophy (Asakura et al., 2002), arterial remodeling (Taylor et al., 1999), psoriasis (Sergi et al., 2000; Overbeck and Griesinger, 2012), arthritis (Hallbeck et al., 2005; Swanson et al., 2012), chronic obstructive pulmonary disease (COPD) (Cockayne et al., 2012), asthma (Zhen et al., 2007), and chronic kidney disease (CKD) (Terzi et al., 2000; Lautrette et al., 2005; Liu et al., 2012).

Less is known regarding which specific EGFR ligands are involved in these pathophysiologic disease states. There is evidence that Tgfa may play a role in the pathophysiology of skin, lung, and kidney disease. Tgfa protein levels are elevated in human psoriatic skin (Sergi et al., 2000), and targeted overexpression of Tgfa in mouse skin results in thickened epidermis, hair loss and a leukocytic infiltration and granular layer loss characteristic of human psoriasis (Vassar and Fuchs, 1991). Serum Tgfa levels are also elevated in patients with COPD and are significantly associated with disease severity (Cockayne et al., 2012), whereas the conditional overexpression of Tgfa in the adult mouse lung results in progressive pulmonary fibrosis (Hardie et al., 2004).

More recently, in an animal model of chronic kidney disease, renal Tgfa protein levels were shown to be markedly increased preceding the development of renal lesions (Laouari et al., 2011), and progressive renal disease was markedly attenuated in mice genetically deficient for Tgfa (Laouari et al., 2012).

Current pharmacologic agents that target EGFR might be tested in clinical trials for these diseases although the skin toxicity associated with these drugs is less acceptable in these patients versus cancer patients. An alternative strategy is to target specific ligands to circumvent skin toxicity. To address this, we generated and characterized the activity of a humanized monoclonal antibody LY3016859, which binds and neutralizes the human EGFR ligands Tgfa and Ereg.

Materials and Methods

Cell Lines and Reagents. The HT-29 and PA317 LXS16E6E7 cell lines were purchased from the American Type Culture Collection (Manassas, VA). The mouse myofibroblast cell line MFC.7 was derived from the outgrowth of primary fibroblast cells of a male C57Bl/6J day-21 after unilateral ureteral obstruction surgical mouse model. The primary cells were immortalized with a retrovirus carrying the E6E7 genes of the human papilloma virus and the G418 selectable marker using standard retroviral transduction methods. Retrovirus was obtained from the PA317 LXS16E6E7 cell line, and G418 was purchased from Invitrogen (Carlsbad, CA). Retrovirus-transduced cells were selected in 400 μg/ml G418 for 2 weeks, and then individual cells were plated into 96-well microplates to obtain clonal lines. Clonal lines were characterized for expression of the myofibroblast marker alpha smooth muscle actin and vimentin with commercially available primers and reagents purchased from Affymetrix (Santa Clara, CA) by the TaqMan method (Applied Biosystems, Foster City, CA). Tissue culture media, fetal bovine serum (FBS), antibiotics, bovine serum albumin (BSA), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO). Culture media, fetal bovine serum (FBS), antibiotics, bovine serum albumin (BSA), and phosphate-buffered saline (PBS) were purchased from Invitrogen. All mouse and human EGFR ligands were purchased from R&D Systems (Minneapolis, MN), with the exception of rat (mouse) Tgfa (Phoenix Pharmaceuticals, Burlingame, CA), mouse Egr (Bioscience, San Diego, CA), and mouse IgG4 antibody LY3016859. Additional mutations in the CDRs, leading to the humanized monoclonal IgG4 antibody LY3016859, which binds and neutralizes the human EGFR ligands Tgfa and Ereg.

Antibody Affinity Optimization and Humanization. Based on affinity and the ability to block cell proliferation, a single clonal Fab from the immunization was chosen for affinity maturation. Fab libraries were constructed and were screened for binding to biotinylated mouse/rat Tgfa (the EGFR domain for mouse and rat Tgfa are identical in sequence) by the filter lift method (Watkins, 2002). Single positive clones were isolated and sequenced. Fab was expressed and confirmed to bind to mouse/rat and human Tgfa. Affinity maturation was done as described in Pancook et al. (2001) and yielded an affinity optimized Fab that was converted into monoclonal antibody (mAb41). The mouse mAb41 antibody was chosen for humanization based on its performance in the in vitro myofibroblast proliferation assay and demonstrated in vivo efficacy in mice. The complementary domain regions (CDRs) of mAb41 antibody were transferred into human germline frameworks without additional mutations in the CDRs, leading to the humanized monoclonal IgG4 antibody LY3016859.

Western Blot Analysis. Electrophoresis of the proteins was performed using precast Novex 4–20% Tris-glycine gels and Novex Tris-glycine SDS running buffer (Invitrogen). Serial dilutions of recombinant human Tgfa (200, 100, 50, 25, or 12.5 ng per lane) were treated with or without NuPAGE 10% Sample Reducing Agent (Invitrogen), or 20 μg of HT-29 cell lysate in reducing buffer was heated and run at 125 V for 90 minutes at room temperature. Proteins were transferred to polyvinylidene difluoride using the iBlot Dry Blotting System with iBlot Transfer Stack, polyvinylidene difluoride, Mini (Invitrogen). The membrane was blocked with pH 8.0 Tris-buffered saline containing 3% nonfat milk (Sigma-Aldrich, St. Louis, MO) for 2 hours at room temperature and then incubated with LY3016859 at 1 μg/ml overnight at 4°C.

After primary incubation, the membrane was washed in wash buffer (Tris-buffered saline + 0.1% Tween 20) and then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-human Fc-specific IgG at 0.1 μg/ml for 30 minutes at room temperature. After removal of the secondary antibody, the membrane was washed several times and then incubated with Super Signal West Pico Chemiluminescent Substrate solution (Thermo Fisher Scientific, Waltham, MA) for 5 minutes. The membrane was placed in a plastic membrane protector in an X-ray film cassette with CL-X Posure Film (Pierce Biotechnology, Rockford, IL) for 30 seconds, and the film was developed using a Konica SRX-101 system (Konica Minolta, Tokyo, Japan).

For phospho-EGFR (pEGFR) and pERK Western blot analysis of HT-29 cells, cells were seeded in six-well plates (100,000 cells/well) in McCoy’s 5A (GIBCO/Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum. After a 2- to 3-day recovery from passage, the cells were seeded in six-well plates containing McCoy’s 5A with no serum for 48 hours.

On the day of the experiment, the cells were treated with mAb41 antibody or control IgG at 30 μg per well. Thirty minutes after antibody addition, the cells were stimulated with tumor necrosis factor α (TNF-α) (3 ng/ml), TGFα (3 ng/ml), Ereg (25 ng/ml), or thrombin (10 U/well). For Tgfa and Ereg proteins, they were preincubated with mAb41 or control IgG for 30 minutes and then...
added to cells. The cells were stimulated for 15 minutes with growth factors and then were lysed in 100 µl of m-PER (mammalian protein extraction reagent) followed by 200 µl of radioimmunoprecipitation assay (RIPA) buffer (both from Thermo Fisher Scientific). The lysis buffers contained protease and phosphatase inhibitors (Halt protease and phosphatase inhibitor; Thermo Fisher Scientific). The remaining adherent cells were scraped; the lysate was collected and centrifuged at 12,000 g for 5 minutes, and the supernatant was collected. Protein in the extract was measured by the BCA protein assay method (Thermo Fisher Scientific), and 20 µg of protein was added per well. 

The plates were incubated in 5% blocking buffer (Bio-Rad Laboratories, Hercules, CA) in 1 × TBS–0.05% Tween 20 for 1 hour at room temperature followed by overnight incubation in 5% BSA in 1 × TBS–0.05% Tween 20 with primary antibody at 4 °C. All primary antibodies were used at a 1:1000 dilution and purchased from Cell Signaling Technologies (Beverly, MA): pEGFR, EGFR, pERK1/2, ERK1/2, and GAPDH. GAPDH and total protein were used as internal loading controls.

Primary antibody was washed three times with 1 × TBS–0.05% Tween 20 followed by a 1-hour incubation with secondary antibody 1:5000 enhanced chemiluminescence anti-rabbit IgG (GE Healthcare Bio-Sciences, Pittsburgh, PA) in blocking buffer. After three washes, detection was performed using SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Fisher Scientific) with image and quantification using an ImageQuant Las4000 (GE Healthcare Bio-Sciences). Data were quantified by normalizing to loading controls and displayed as increases in pEGFR or pERK1/2 relative to total EGFR and ERK1/2, respectively.

Surface Plasmon Resonance Analysis. Biacore T2000 instrument (BIAcore AB, Uppsala, Sweden), reagents, and Biacore T2000 evaluation software version 4.1 were used for the surface plasmon resonance analysis. A CM5 chip (BIAcore AB) was prepared with EGFR ligands for 24 hours.

After serum starvation, the cells were stimulated with dilutions of antibodies were incubated for 30 minutes at a tissue culture incubator with the EC50 concentration of the respective EGFR ligand determined in the assay. After incubation, the antibody ligand mix was put on serum-starved cells and stimulated for 24 hours. After stimulation, the cells were pulsed with BrDU for 4 hours and then analyzed with the colorimetric BrDU ELISA kit according to the manufacturer’s instructions. There were three replicates per concentration on a plate, and the assays were independently run in two to four experiments on different plates. The absorbance values (450–690 nM) were generated on a SpectraMax 190 plate reader (Molecular Devices, Sunnyvale, CA), and the data were imported into software programs Microsoft Excel 2007 (Microsoft, Redmond, WA) and SigmaPlot v.9.01 (Systat Software, San Jose, CA) for analysis and generation of EC50 or IC50 values by use of the four-parameter Hill equation for regression analysis.

Cell-Based ELISA Assay. HT-29 cells (ATCC, Manassas, VA) were incubated overnight in complete DMEM/F-12 medium with or without the TNF-α-converting enzyme (TACE) inhibitor TNF-α protease inhibitor 2 (TAI-2) at 50 µM (Calbiochem/EMD Millipore, Billerica, MA). Cells were then fixed with 2% paraformaldehyde for 20 minutes, washed several times in PBS, subjected to a cell-based ELISA with either a biotinylated isotype control IgG or biotinylated mAb41 (2 µg/ml), and incubated at room temperature for 2 hours. Detection was with a streptavidin HRP and substrate (Jackson ImmunoResearch Laboratories, West Grove, PA). To demonstrate specificity, mAb41 was incubated with a 50-fold molar excess of recombinant human TGFα (R&D Systems) for 30 minutes at 37°C before addition to cells, which resulted in no signal.

Conjugation of Alexa Fluor 488 to Antibodies and Internalization Assay. Alexa Fluor 488 (Invitrogen) was conjugated to antibodies according to the manufacturer’s protocol. In brief, protein was diluted to 2 mg/ml in PBS. To 0.5 ml of this 2 mg/ml solution, 50 µl of 1 M sodium bicarbonate, pH 9.0, was added. The protein solution was then transferred to a vial of dye and stirred at room temperature for 1 hour. The labeled protein was purified using the Bio-Rad BioGel P-30 resin included with the labeling kit. Two experiments were performed to determine whether the antibodies promoted the internalization of target in HT-29 cells (ATCC).

An antibody dose response was performed in the first experiment with 10,000 cells seeded per well of a 96-well plate in DMEM/F-12 (1:1) containing 1-glutamine, 10% heat-inactivated FBS, 1× antibiotic, and 2.438 g/l sodium bicarbonate. The next day, the cells were washed with PBS containing 0.1% BSA (PBSB) and then incubated with Alexa Fluor 488–conjugated antibodies at concentrations ranging from 0 to 88 µg/ml for 2 hours at 37°C in a tissue culture incubator. There were three replicate wells per concentration. After the incubation period, the cells were washed in PBSB, fixed with 4% formaldehyde in PBS for 30 minutes at room temperature, washed with PBS, and then stained with Hoechst dye 33342 at 2 µg/ml in PBS for 30 minutes before analysis.

The second experiment was set up the same as the first experiment with a few changes to examine the time course of antibody binding and internalization. Cells were incubated with Alexa Fluor 488–conjugated antibodies in PBS with 0.1% BSA at a concentration of 2 µg/ml for time periods of 5, 15, 30, 60, or 120 minutes at 37°C in a tissue culture incubator. After incubation with the mAb41, the cells were washed, fixed, stained, and analyzed as described for the first experiment. There were three replicate wells per time point.

The quantitation of internalization was performed as follows: more than 500 cells/well were collected with a Cellomics Arrayscan VTI (Thermo Fisher Scientific) with a 20× objective lens. Image analysis was performed with the “compartmental analysis” biospplications of the system. Cell nuclei were identified by a Hoechst stain (blue masking). Two regions of interest were set to collect fluorescent signals from intracellular spots (red masking) and total green fluorescence (both red and blue) obtained from the masked image. The number, area, and fluorescent intensity from each spot in cells were calculated. The
mean spot total intensity of intracellular spots (red) per cell was chosen for measuring antibody-induced internalization.

Quantitative Real-Time Polymerase Chain Reaction Analysis. HT-29 cells were seeded in 6-well plates (100,000 cells/well) in McCoy's 5A (Gibco) supplemented 10% fetal calf serum. After a 2- to 3-day recovery from passage, the cells were serum starved (McCoy's 5A with no serum) for 48 hours. On the day of the experiment, the cells were treated with mAb41 antibody or control IgG at 30 μg per well. Thirty minutes after the antibody addition, the cells were stimulated with either TNF-α (3 ng/ml) or thrombin (10 U/well). Thirty minutes after the addition of growth factors, the cellular RNA was isolated with TRIzol reagent (Life Technologies) following the manufacturer's directions. The RNA was reverse transcribed into cDNA using Superscript III kit (Invitrogen). The probes used include: ribosomal 18S (A309829), Tgfa (HS00608187), HB-EGF (HS00181813), and Ereg (HS00154995). All probes were purchased from Applied Biosystems and were used at 1:20 dilution with TaqMan Universal PCR Master Mix (Applied Biosystems). Detection and quantification was performed on the ABI 7900HT Sequence Detection System (Applied Biosystems), and data were collected and analyzed with the SDS 2.2.2 software from Applied Biosystems. Data were analyzed by calculating the Ct value, ΔCt in reference to 18S, ΔΔCt, and ultimately fold change calculated from the ΔΔCt value. N = 3 for each group in which the quantitative real-time polymerase chain reaction (RT-PCR) for each was run in triplicate.

Animal Studies. In all studies involving animals, animal care and use were performed in accordance with federal and local laws, polices, regulations, and standards in effect at the time of their conduct (e.g., Animal Welfare Act of 1990 and Regulations, PL 89-544; USDA 1985). Laboratories conducting these studies were accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all study protocols were approved by each laboratory's institutional animal care and use committee. Male and female Tgfa knockout (KO) (B6.129-Tgfatm1Arldj) and control C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Pregnant female and male 129S6 mice as well as male unilateral ureteral obstructed (unilateral ureteral obstruction) C57BL/6J mice were obtained from Taconic Farms (Germantown, NY). Tgfa KO or wild-type C57BL/6 mice were immunized with recombinant human Tgfa protein. The resulting antigen-specific spleen cells were enriched by standard sorting methods using biotinylated antibody. mAb were obtained by cloning variable regions of antibody genes resulting from RT-PCR of RNA prepared from spleen cell lysates by standard recombinant DNA cloning methods. mAb were expressed by standard transient transfection methods into 293 cells, and supernatants containing mAb were confirmed to bind to rat and human Tgfa in plate binding assays.

The effect of mAb41 on mouse development was assessed by dosing either mAb41 or an isotype control antibody (mouse IgG1) as a single injection subcutaneous at 30 mg/kg to pregnant 129S6 mice at postvaginal-plug day 14 with analysis of pups at birth. In another experiment, mAb41 or an isotype control IgG was dosed once weekly at 20 mg/kg s.c. to male and female 129S6 postnatal day 2 pups out to postnatal day 28. Body weight and length measurements were obtained weekly for the pups as well as observations of hair and whisker growth. At the end of the neonate pup experiment, tissues were taken, fixed, processed, decalcified (femur and tibia), and stained for histologic analysis and then analyzed by an institutional pathologist. Femur and tibia sections were examined histologically for scoring of the growth plate cartilage (total, proliferative zone, and hypertrophic zone). Whole-slide digital images were scanned at 20× magnification using an Aperio Scanscope (Leica Biosystems; Wetzlar, Germany), and the components of the physical growth plate were measured as described by Usmani et al. (2012).

Pharmacokinetic (PK) analyses for mAb41 were conducted in male 129S6 mice (n = 5/group) C57BL/6J mice and in Tgfa KO mice after a single intravenous dose of 1 or 10 mg/kg. PK analysis of LY3016859 was conducted in healthy male cynomolgus monkeys after a single intravenous dose of 1, 10, or 30 mg/kg or a single subcutaneous administration of 1 or 10 mg/kg. In the normal mouse PK studies, blood samples were collected from two animals per treatment group per time point at 0.083 (intravenous only), 1 (intravenous only), 6, 12, 24, 48, 72, 120, 168, 240, and 336 hours after administration. In the PK studies of Tgfa KO mice, blood samples were collected from two animals per treatment group per time point at 0.083, 48, 96, 168, and 288 hours after administration. In both mouse studies, all blood was collected by submandibular puncture and immediately transferred into tubes containing no anticoagulant. In male cynomolgus monkeys, blood samples were collected from the femoral vein at 0.083, 1, and 6 hours postdose after intravenous administration and at 12, 24, 48, 72, 96, 120, 144, 168, 192, 216, 240, 288, 336, 384, 456, 528, 600, and 672 hours postdose after intravenous and subcutaneous administration from two animals per time point per treatment group. Each monkey blood sample was maintained under ambient conditions after collection. In both mouse studies and the single dose monkey PK study, blood was processed to serum. All serum samples were stored frozen at ~80°C until assayed.

Anti-EGFR antibodies have been shown to induce skin toxicity in cynomolgus monkeys (Skartved et al., 2011). A 6-week study with the neutralizing anti-TGFα/Epiregulin humanized IgG4 antibody LY3016859 was conducted in female cynomolgus monkeys at doses of 0, 10, and 100 mg/kg i.v. dosed weekly with necropsy at approximately 7 days after the last dose to obtain plasma and tissues for analysis. There were four animals per group, with the exception of the control group (N = 2). The animals were monitored for skin changes daily. A standard pathologic analysis of tissues was conducted at the end of the study. For PK analysis, approximately 1 ml of whole blood was collected via a femoral vein/artery at predose and as follows: Dose 1: 0.083 hour; Dose 2, 3, 4, and 5: 5 minutes postdose; and Dose 6: 5 minutes and 1, 6, 24, 48, 72, 120, 144, and 168 hours postdose. All blood was collected into tubes containing K3 EDTA anticoagulant. Samples were centrifuged to obtain plasma and aliquoted into individual polypropylene storage tubes. Individual plasma samples were harvested and split into two aliquots before storage at approximately ~60°C.

Concentrations of mAb41 in murine blood samples or LY3016859 in monkey blood samples were determined using an antigen capture ELISA with TGFα. In brief, microtiter plate (Nunc Polysorb Immobilizer; Thermo Fisher Scientific, Rochester, NY) wells were coated with 0.05 μg per well of human Tgfa. The mAb41 standards and samples were prepared in mouse serum, and LY3016859 standards and samples were prepared in cynomolgus monkey serum or plasma. After preparation, the samples were incubated for 1 hour at room temperature on Tgfa-coated plates. After washing, the mAb41- and LY3016859-bound antibodies were detected with an anti-mouse HRP conjugate (Bethyl Laboratories, Montgomery, TX) or anti-human kappa HRP conjugate (Southern Biotech, Birmingham, AL), respectively. The standard curve ranged from 3 to 400 ng/ml, with 13.2 and 12.5 ng/ml being the lower limit of quantitation in mouse and cynomolgus monkey serum or plasma, respectively. The PK parameters were calculated using the WinNonlin Professional (version 3.2) software package (Pharsight Corporation, Mountain View, CA). Serum or plasma concentration-time data were calculated using a model-independent approach based on the standard moment theory. The parameters calculated included the area under the curve (AU0–∞), clearance (CL), volume of distribution (Vss), and elimination half-life (t1/2).

Statistical Methods. All data were analyzed with SAS or JMP version 8.0 software (SAS Institute, Cary, NC). Data were evaluated by ANOVA with log transformed data (if the data were skewed) and a Student’s unpaired t test. P < 0.05 was considered statistically significant.
Results

Identification of a High-Affinity Tgfa-Neutralizing Monoclonal Antibody. Attempts at obtaining Tgfa antibodies in wild-type mice were unsuccessful, but Tgfa KO mice immunized with human Tgfa yielded several distinct antibodies that bound to both human and mouse Tgfa. Antibody clone 1F9 bound both human and mouse TGFα with moderate affinity by BLAcore AB (KD of 18.47 ± 0.22 and 38.16 ± 2.01 nM, respectively) and inhibited the mitotic activity of 0.5 nM soluble mouse Tgfa in a cell proliferation assay (IC50 value of 65 nM). Clone 1F9 was then optimized for improved binding to human and mouse TGFα and converted into the high-affinity IgG1 monoclonal antibody mAb41 (KD of 36.5 ± 8 and 38.0 ± 13.6 pM to human and mouse TGFα, respectively, by Biacore analysis, Table 1). Antibody mAb41 inhibited the cell-proliferative activity of both human and mouse TGFα at 0.5 nM with IC50 values of 0.52 ± 0.04 nM and 0.13 ± 0.01 nM, respectively (Fig. 1; Table 2).

Approximately 30% sequence similarity on average exists between the seven EGFR ligands in the EGF domain; thus, the selectivity of the mAb41 antibody to all seven ligands was examined in the cell proliferation assay. In addition to TGFα, mAb41 was found to neutralize soluble Ereg. Although antibody mAb41 was found to neutralize soluble Ereg, yet showed no neutralization of mouse Epgn in the cell-proliferation assay (Table 2). Because there was weak binding affinity to mouse Epgn with a KD of 346.6 ± 36.5 pM, respectively (Table 1), antibody mAb41 also exhibited weak binding affinity to mouse Epgn with a KD value of 365 nM (Table 1), yet showed no neutralization of mouse Epgn in the cell proliferation assay (Table 2). Because there was binding of mAb41 to these three EGF ligands, the amino acid sequences of all three EGF domains were compared. This analysis identified a stretch of nine common amino acids in the C-terminal region of the EGF domain that is known to be involved in EGFR binding (Garrett et al., 2002). Considerable sequence similarity exists between the three ligands in this region, consistent with it contributing to the mAb41 epitope (Fig. 2). To rule out binding to other potential proteins, this stretch of sequence was compared with all other known protein sequences by a Blast analysis and identified as unique.

The mouse mAb41 antibody was humanized by cloning the complementary domain regions (CDRs) into human germline frameworks without additional mutations, and this humanized monoclonal IgG4 molecule was designated LY3016859. The binding and neutralization properties of LY3016859 are not different from mAb41 (Fig. 1, Tables 1 and 2). LY3016859 detects increasing quantities of recombinant mature human TGFα protein (12.5–200 ng) by Western blot analysis only under nonreducing conditions, suggesting that secondary structure is important for binding (Fig. 3A). To explore the mechanism of ligand neutralization by mAb41 and LY3016859, experiments were conducted in colon adenocarcinoma HT-29 cells to determine the ability of the antibodies to bind and internalize cell surface ligand. The human HT-29 cell line expresses both TGFα and Ereg (Pai et al., 2002; Wu et al., 2009). TAPI-2 is an ADAM17 inhibitor known to decrease EGFR ligand shedding and thus increase cell surface ligand (Le Gall et al., 2003). Incubation of HT-29 cells with mAb41 exhibited an increased signal in the presence of TAPI-2 consistent with increased membrane-associated ligand binding (Fig. 3B). Fluorescent labeling of LY3016859 with Alexa Fluor 488 revealed that this TGFα Ereg monoclonal antibody dose-dependently binds to the HT-29 cell surface and becomes internalized within 2 hours (Fig. 3, C–E).

Both thrombin and TNF-α have been shown to induce EGFR phosphorylation and ERK1/2 activation through EGFR transactivation (Darmoul et al., 2004; Ueno et al., 2005). EGFR transactivation was originally described as a mechanism by which G protein–coupled receptors could activate the MAPK pathway and transduce mitogenic signals through an intermediate step involving phosphorylation and activation of the EGFR (Daub et al., 1996). This EGFR transactivation appears to result from activation of membrane-bound metalloproteases, resulting in the release of EGFR ligands (Prenzel et al., 1999). Ligand-stimulated EGFR activation may also induce cell autonomous expression of other EGFR ligands, a phenomenon known as cross-induction (Barnard et al., 1994). The effect of mAb41 on ligand-induced EGFR phosphorylation,

<table>
<thead>
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<th>Ligand</th>
<th>Antibody</th>
<th>Mean ± S.D. (n = 3)</th>
<th>On Rate (k_on)</th>
<th>Off Rate (k_off)</th>
<th>Affinity (K_D)</th>
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<tbody>
<tr>
<td>Mouse Tgfa</td>
<td>mAb41</td>
<td>5.41 ± 0.50 x 10^5</td>
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<td>mAb41</td>
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<td>TGFα</td>
<td>LY3016859</td>
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<td>4.28 ± 2.63 x 10^-5</td>
<td>101.5 ± 43.1 pM</td>
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<tr>
<td>Mouse Ereg</td>
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<td>6.55 ± 0.38 x 10^4</td>
<td>1.41 ± 0.09 x 10^-2</td>
<td>215 ± 15 nM</td>
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<td>Human Ereg</td>
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<td>5.78 ± 0.18 x 10^6</td>
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<tr>
<td>Cyan Ereg</td>
<td>LY3016859</td>
<td>6.73 ± 0.71 x 10^6</td>
<td>7.05 ± 0.23 x 10^-4</td>
<td>1.05 ± 0.09 nM</td>
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<td>Mouse Epgn</td>
<td>mAb41</td>
<td>3.92 ± 0.26 x 10^4</td>
<td>1.43 ± 0.10 x 10^-2</td>
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<tr>
<td>Human/Cyno</td>
<td>LY3016859</td>
<td>ND</td>
<td>ND</td>
<td>&gt;2 μM*</td>
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</tr>
</tbody>
</table>

ND, not determined as binding too weak for appropriate kinetic analysis.

*Calculated as K_D = k_off/k_on.

*Identical epitope, not measured separately.

*Half saturation not achieved at 2 μM ligand concentration.
EGFR transactivation, and ligand cross-induction was tested in HT-29 cells by quantitative Western blot and RT-PCR analysis. Western blot data were quantified by normalizing to loading controls (GAPDH) and displayed as arbitrary units (AU) reflecting increases in phosphorylated EGFR or ERK1/2 relative to total EGFR or ERK1/2, respectively. Treatment with vehicle was set as the baseline with a value of 1.0

$0.06$ AU for $p$EGFR and $1.0

$0.04$ AU for $p$ERK1/2.

Incubation of HT-29 cells with 3 ng/ml TGF-$\alpha$ or 25 ng/ml Ereg in the presence of a control IgG stimulated the phosphorylation of the EGFR to $30.1

$3.4$ and $8.4

$0.6$ AU, respectively (Fig. 4A). Addition of mAb41 to the ligands significantly reduced EGFR phosphorylation by TGF-$\alpha$ to $5.3

$1.1$ AU and by Ereg to $1.8

$0.5$ AU (Fig. 4A). TGFA and Ereg also stimulated the phosphorylation of ERK1/2 (7.9

$1.1$ and $4.9

$0.6$ AU, respectively), whereas the inclusion of mAb41 was able to significantly reduce this effect to increases of $2.0

$0.3$ AU for TGFA and $2.1

$0.2$ AU for Ereg (Fig. 4B).

The addition of 3 ng/ml TNF-$\alpha$ or 10 U/ml thrombin to HT-29 cells in the presence of control IgG also resulted in significant phosphorylation of the EGFR (2.1

$0.2$ and $3.1

$0.2$ AU, respectively), and this effect was significantly attenuated by inclusion of mAb41 with a return of phosphorylated EGFR levels to near baseline of $1.3

$0.1$ AU for TNF-$\alpha$ and $1.9

$0.1$ AU for thrombin (Fig. 4C). TNF-$\alpha$ and thrombin also increased phosphorylation of ERK1/2 (2.1

$0.3$ and $2.0

$0.1$ AU, respectively), and this increase was also significantly reduced by inclusion of mAb41 to $1.5

$0.2$ AU for TNF-$\alpha$ and $1.0

$0.3$ AU for thrombin (Fig. 4D).

**TABLE 2**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC$_{50}$ Range$^a$</th>
<th>Mean ± S.E.M. ($n$ = 3–4 Experiments)</th>
<th>mAb41 IC$_{50}$</th>
<th>LY3016859 IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pM$</td>
<td>$nM$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Tgfa (0.5 nM)$^b$</td>
<td>13–14</td>
<td>0.13 ± 0.01</td>
<td>0.19 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Human/Cyno TGF$\alpha$ (0.5 nM)$^b$</td>
<td>11–12</td>
<td>0.52 ± 0.04</td>
<td>0.46 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Mouse Ereg (0.5 nM)$^b$</td>
<td>163–320</td>
<td>214 ± 49</td>
<td>334 ± 41</td>
<td></td>
</tr>
<tr>
<td>Human Ereg (0.5 nM)$^b$</td>
<td>78–282</td>
<td>1.12 ± 0.36</td>
<td>3.15 ± 1.04</td>
<td></td>
</tr>
<tr>
<td>Cyno Ereg (0.5 nM)$^b$</td>
<td>104–139</td>
<td>ND</td>
<td>3.06 ± 0.99</td>
<td></td>
</tr>
<tr>
<td>Mouse Epgn (50 nM)$^b$</td>
<td>6860–8600</td>
<td>$&gt;2000^a$</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Human/Cyno Epgn (100 nM)$^b$</td>
<td>3797–18987</td>
<td>ND</td>
<td>480–1357$^a$</td>
<td></td>
</tr>
</tbody>
</table>

ND, not determined.

$^a$Data shown as a range of the means derived from two independent experiments.

$^b$Concentration used in assay with antibody.

$^c$Identical epitope, not measured separately.
Pharmacokinetic Properties of mAb41 and LY3016859.

To explore the in vivo effects of mAb41, a PK analysis of the antibody was conducted in healthy wild-type 129S6 mice. Because mAb41 binds mouse Tgfa with much greater affinity than mouse Ereg (>5000-fold difference), it was assumed that in vivo clearance of the antibody in mice would be driven by this ligand. A wide range of antibody dose levels from 1–30 mg/kg were chosen because it is not known how much Tgfa membrane-bound protein is present in mouse tissues, and circulating Tgfa is assumed to be undetectable because it is a membrane-bound ligand that must be proteolytically released. After a single intravenous administration of 1, 10, or 30 mg/kg, mAb41 displayed nonlinear PK with clearance (CL) decreasing and elimination half-life increasing with increasing dose (Fig. 5A; Table 3). After intravenous administration, the CL decreased from 0.33 to 0.13 ml/h/kg over the dose range examined in normal mice. The PK data were consistent, with a saturable target-mediated mechanism of CL for mAb41 especially apparent at circulating concentrations of ≤10 μg/ml antibody (Fig. 5A). Comparison of the AUCs after a single subcutaneous or intravenous administration of mAb41 at 10 mg/kg show the bioavailability of mAb41 to be roughly 87% in healthy wild-type 129S6 mice (Fig. 5A; Table 3). To further delineate that the saturable mAb41 CL in normal mice was related to Tgfa binding in vivo, a PK analysis was conducted in Tgfa KO mice after a single intravenous administration of 1 or 10 mg/kg. Unlike the observations in wild-type mice, in Tgfa KO mice there were reasonably dose-proportional increases in AUC, indicating that the nonlinear PK observed in wild-type mice was due to Tgfa-mediated CL (Fig. 5B; Table 3). Taken together, the PK observations in mice suggested once weekly dosing at a dose level ≥10 mg/kg would be sufficient to provide adequate exposure in wild-type mice for the in vivo evaluation of mAb41.

A similar PK analysis of LY3016859 was conducted in male cynomolgus monkeys. As LY3016859 binds both cynomolgus monkey TGFα and Ereg, CL would presumably be driven by both ligands, although the concentrations of these ligands in monkey tissues are not known. Comparable to the PK properties of mAb41 in mice, LY3016859 displayed nonlinear PK with the CL decreasing and elimination half-life increasing with increasing dose (Fig. 5C; Table 3). The CL decreased from ∼0.47 to 0.23 ml/h per kg, and the half-life (t1/2) ranged from ∼20 to ∼178 hours with increasing dose. The PK data were consistent, with a saturable target-mediated mechanism of CL for LY3016859 especially apparent at circulating concentrations of ≤5 μg/ml antibody (Fig. 5C). Based on the kinetic profiles, there was no evidence for the formation of anti-LY3016859 antibodies that would lead to the wide range in reported half-life. The subcutaneous bioavailability of LY3016859 in monkeys was ∼83%–100% (Table 3).

In Vivo Activity of mAb41 in Mice. Tgfa KO mice have curly whiskers, wavy hair, and stunted growth (Luetteke et al., 1993; Mann et al., 1993) and, more recently, have been reported to have a growth plate cartilage phenotype similar to animals treated with EGFR inhibitors, namely ephypseal growth plate thickening and expansion of the cartilage hypertrophic zone (Usmani et al., 2012). To determine whether mAb41 neutralization of Tgfa in vivo phenocopies the binding of mAb41. 

Fig. 2. Alignment of EGF domains showing proposed binding region of mAb41. An alignment of Tgfa (A), Ereg (B), and Epigen (C) amino acid sequences of the EGF domain from human, cynomolgus monkey, and mouse. The underlined stretch of amino acids in the C-terminal region of the EGF domain identifies a proposed epitope region for the binding of mAb41.
the KO mice, we administered mAb41 or a control IgG at dose level of 20 mg/kg s.c. once weekly for 26 days to male and female neonate mice beginning at postnatal day 2. We chose this high dose to provide enough exposure to target a complete knockout of the Tgfa protein based on the PK data in mice. The mAb41 treatment significantly reduced body weight and length in both males and females over the 4-week period (Fig. 6, A and B). In addition, mAb41 treatment in neonates was associated with a widening of the hypertrophic zone of growth plate cartilage in the femur and tibia as reported in Tgfa KO mice (Fig. 6, C and D). Notably, postnatal administration of mAb41 to neonate pups did not result in curly whiskers or wavy hair or any other macroscopic or microscopic skin abnormality. To determine whether the curly whiskers phenotype of the Tgfa KO mice could be reproduced, a single dose of mAb41 or a control isotype antibody was administered at 30 mg/kg to pregnant female mice at day 14 postvaginal plug, again using a high enough dose of antibody to target a complete knockout of Tgfa protein. In utero exposure of mice to mAb41, but not a control IgG, resulted in newborn pups with misaligned wavy whiskers (data not shown).

Toxicology Assessment of LY3016859 in Monkeys.

Nonclinical and clinical data indicate that skin is a target organ for toxicity when EGFR is inhibited (via a monoclonal antibody or small molecule inhibitor approaches). Based on this, a 6-week non–Good Laboratory Practice toxicology study was conducted in female cynomolgus monkeys to evaluate whether inhibition of Tgfa and Ereg would lead to similar skin toxicity. Monkeys were dosed intravenously with 10 or 100 mg/kg LY3016859 antibody on a weekly basis for 6 weeks. The dose levels were chosen to provide enough exposure to neutralize the ligands with the antibody based on the single dose pharmacokinetic data from both mouse and monkey as well as the in vivo efficacy data in mice. Exposure of the animals to the antibody was confirmed throughout the study by a PK analysis with data shown in Table 3. Based on the kinetic profiles, there was possible evidence for the formation of anti-LY3016859 antibodies in one of the high-dose animals after the sixth (final) dose. Because of the significant reduction of LY3016859 plasma concentration and the possibility of anti-LY3016859 antibodies, mean PK and plasma concentrations are shown with and without data from the one animal with this anti-LY3016859 response (Table 3).
Fig. 4. Antibody mAb41 blocks ligand-stimulated EGFR phosphorylation, transactivation, and ligand cross-induction in HT-29 cells. (A and B) Stimulation with 3 ng/ml Tgfa or 25 ng/ml Ereg in the presence of a control IgG increased the amount of phosphorylated EGFR (A) and ERK1/2 (B) compared with vehicle-treated controls and was significantly attenuated when ligands were preincubated with 30 μg/well mAb41. (C and D) Stimulation of HT-29 cells with 3 ng/ml TNF-α or 10 U/well thrombin in the presence of a control IgG significantly increased phosphorylated EGFR (C) and ERK1/2 (D) compared with vehicle-treated controls. Increases in phosphorylated EGFR or ERK1/2 were significantly reduced in cells preincubated with 30 μg/well mAb41 before stimulation. Data for A–D were quantitated by normalizing to loading controls (GAPDH) and displayed as arbitrary units (AU), reflecting increases in pEGFR or pERK1/2 relative to total EGFR or ERK1/2, respectively. Vehicle treatment was set as baseline with an AU of 1.0 (N = 3 samples for each group; *P < 0.05 compared with vehicle-treated cells; †P < 0.05 as compared with respective IgG + growth factor control). (E–G) Cross-induction of EGF ligand expression is shown after stimulation of EGFR transactivation with 3 ng/ml TNF-α or 10 U/well thrombin in the presence of a control IgG. Data shown represent increases in TGFα (E), HB-EGF (F), and Ereg (G) mRNA levels compared with vehicle-treated controls, which were significantly abolished by pretreatment of cells with 30 μg/well mAb41. Data are shown as arbitrary units (AU) reflecting the fold increase over vehicle control as measurement of ΔΔCt after normalizing ΔCt to ribosomal 18S (n = 3, samples each were run in triplicate in quantitative RT-PCR; *P < 0.05 compared with vehicle-treated cells; †P < 0.05 compared with respective IgG + growth factor control).
maximal plasma concentrations ($C_{\text{max}}$) increased in an approximately dose proportional fashion for both doses (Table 3). No significant accumulation was seen over the 6 weeks of dosing.

Over the dose range examined, LY3016859 displayed linear toxicokinetics, with CL and elimination half-life remaining constant regardless of dose and AUC$_{\text{last}}$ increasing dose proportionally (Table 3), excluding the one animal in the high-dose group. Unlike the single-dose PK study at lower doses, the toxicokinetics data did not show a saturable target-mediated clearance component for LY3016859 in cynomolgus monkeys within the tested dose range. No observed adverse effects were seen at doses up to 100 mg/kg. Importantly, no skin changes were observed in treated animals, and there were no other important pathologic changes noted from tissues taken at the end of the study.

### Discussion

The EGFR pathway plays an important role in the pathophysiology of cancer (Normanno et al., 2006), cardiovascular disease (Nakata et al., 1996; Reape et al., 1997; Asakura et al., 2002; Kagiyama et al., 2002; Flamant et al., 2003), psoriasis (Sergi et al., 2000; Overbeck and Griesinger, 2012), arthritis (Hallbeck et al., 2005; Swanson et al., 2012), and experimental chronic kidney disease (Terzi et al., 2000; Lautrette et al., 2005; Liu et al., 2012). Therapeutic drugs targeting this pathway have potential to treat diverse human diseases in addition to cancer. However, the occurrence of an accompanying skin rash associated with the use of EGFR inhibitors has discouraged their use in these other diseases. Neutralization of relevant EGFR ligands offers an alternative strategy that may provide efficacy and circumvent the associated skin toxicity. Neutralization of selected ligands may allow for the preservation of key functions provided by the other EGFR ligands. There are relatively few reports describing the characterization of neutralizing monoclonal antibodies to either TGF-$\alpha$ or Ereg, and to our knowledge, this is the first report of a monoclonal antibody that binds and neutralizes both EGFR ligands.

Immunization of wild-type mice with recombinant Tgfa or several different Tgfa fusion proteins failed to yield anti-Tgfa antibodies. Through subsequent immunization of Tgfa-deficient mice with human TGF-$\alpha$, we successfully identified and affinity optimized a monoclonal antibody that bound both human and mouse TGF-$\alpha$. The mAb41 antibody blocked the mitogenic activity of both human TGF-$\alpha$ and Ereg. Neutralization of mouse Ereg was substantially less. Ereg is expressed in human renal mesangial cells (Mishra et al., 2002) and has been suggested to play a pathophysiologic role in arterial remodeling and cardiovascular disease (Taylor et al., 1999; Koo and Kim, 2003; White et al., 2010). Thus, Ereg neutralization might also provide benefit in patients with chronic kidney disease who are at increased risk for cardiovascular events (McClellan et al., 2004; Karras et al., 2012).

Ereg and TGF-$\alpha$ share near identical C-terminal sequences comprising their EGF domains. We speculate that this linear region may represent an important part of the mAb41 epitope, although the epitope is most likely more complex and is dependent on proper secondary structure as the antibody failed to recognize the TGF-$\alpha$ protein on Western blots under reducing conditions. There were also differences in this stretch between mouse and human Ereg, which could explain the difference in binding and neutralization activities between these species of Ereg. A comparison of this conserved stretch of amino acids in all seven ligands suggested that Epgn might also bind to mAb41, which was confirmed by Biacore analysis, although binding to Epgn was significantly weaker. The humanized monoclonal antibody LY3016859 is
expected to effectively neutralize the activities of both TGFα and Ereg when tested clinically.

As mAb41 and LY3016859 bind to and internalize the membrane-associated ligands, we speculated that target-mediated clearance of the antibody could play a prominent role in the PK properties of these antibodies. Indeed, both antibodies demonstrated nonlinear clearance in the PK studies, with clearance decreasing and elimination half-life increasing with increasing dose. The PK data indicated a saturable target-mediated clearance component for these antibodies in each species within the tested dose range. Taken together with the known low concentrations of circulating soluble TGFα, the nonlinear PK observations are consistent with binding and subsequent systemic clearance of the antibody by membrane-bound target. Furthermore, in Tgfa KO mice, the disappearance of nonlinearity in the clearance of the antibody also demonstrates that tissue-associated target-mediated clearance of the antibody could play a prominent role in the PK properties of these antibodies. Indeed, both antibodies demonstrated nonlinear clearance in the PK studies, with clearance decreasing and elimination half-life increasing with increasing dose. The PK data indicated a saturable target-mediated clearance component for these antibodies in each species within the tested dose range. Taken together with the known low concentrations of circulating soluble TGFα, the nonlinear PK observations are consistent with binding and subsequent systemic clearance of the antibody by membrane-bound target. Furthermore, in Tgfa KO mice, the disappearance of nonlinearity in the clearance of the antibody also demonstrates that tissue-associated target-mediated clearance of the antibody is the predominant factor driving the systemic clearance of the antibodies.

To demonstrate that mAb41 neutralizes Tgfa activity in vivo, the effects of the antibody were assessed in pregnant and newborn mice to see whether the reported phenotype associated with Tgfa KO mice is healthy and fertile, yet have a distinctive feature in that there is a pronounced waviness to the coat and curly whiskers (Luetteke et al., 1993; Mann et al., 1993). In fact, curly whiskers are easily identified in newborn Tgfa-deficient mice (Mann et al., 1993). Tgfa deficiency also results in stunted growth with an approximate 10% decrease in body weight relative to wild-type pups and some eye abnormalities (Luetteke et al., 1993). The only other reported phenotype associated with Tgfa deficiency is a widening of the hypertrophic zone in growth plate cartilage (Usmani et al., 2012). Although we did not observe an effect of mAb41 treatment on whiskers or hair growth when initiating dosing at postnatal day 2, we did observe an effect on postnatal growth with about a 10% decrease in body weight and length. We also noted a widening of the hypertrophic zone in the growth plate cartilage with dosing of mAb41 from postnatal day 2 to day 28. Thus, both of these phenotypes indicate that mAb41 at the dose level administered is completely neutralizing the Tgfa target in vivo. It is possible that we did not see an effect on whisker or hair growth as we did not initiate dosing of mAb41 until postnatal day 2 and in mice the follicles that produce coat hairs are established in the skin by that time (Claxton, 1966). However, when dosing was initiated at day 14 after plug in pregnant females, pups were born with misaligned wavy whiskers, supporting the idea that the timing of Tgfa neutralization is critical for observing this phenotype.

A major drawback to using EGFR inhibitors clinically in the treatment of diseases outside of cancer is the effect of this inhibition on skin toxicity. An off-target effect of EGFR inhibition in the skin results in the development of a papulopustular follicular eruption that is most pronounced on the face, chest, and upper back (Rhee et al., 2005; Li and Perez-Soler, 2009). This cutaneous rash may be accompanied by dry skin, itching, and hair alterations that can have a negative impact on patient quality of life and in extreme cases may lead to a discontinuation of EGFR inhibitor therapy. As Tgfa-deficient mice show hair follicle abnormalities, there was a concern that Tgfa neutralization with a monoclonal antibody might result in skin toxicities similar to that observed with EGFR inhibitors. However, no skin toxicities were observed while dosing of cynomolgus monkeys with LY3016859 for 6 weeks at doses as high as 100 mg/kg weekly. Skin is known to express many of the EGFR ligands (Stoll et al., 2010) and while blocking the EGFR prevents signaling from all the...
ligands, neutralization of only TGF-α and Ereg appears to still allow for sufficient signaling through the remaining ligands to prevent the development of skin toxicity. Additionally, no other target organs for toxicity were observed with LY3016859 after 6 weeks of treatment.

Accumulating evidence implicates TGF-α as a promising target for several diseases, including Ménétrier’s disease (Dempsey et al., 1992), psoriasis (Vassar and Fuchs, 1991), COPD (Cockayne et al., 2012) and CKD (Lautrette et al., 2005; Laouari et al., 2011). For instance, TGF-α expression appears mislocalized and overexpressed in the gastric mucosa of patients with Ménétrier’s disease (Dempsey et al., 1992) and transgenic mice that overexpress Tgfa in the gastric mucosa show a number of features in common with Ménétrier’s disease (Goldenring et al., 1996). In addition, not only are circulating levels of Tgfa significantly elevated in smokers versus nonsmokers as well as in patients with COPD and show a specific association with COPD disease severity (Cockayne et al., 2012), but pulmonary fibrosis from bleomycin-induced lung injury is also significantly reduced in Tgfa-deficient mice (Madtes et al., 1999). Tgfa has also been shown to be induced by angiotensin II and renal lesions from angiotensin II infusion are substantially reduced in Tgfa-deficient mice (Lautrette et al., 2005). Moreover, Tgfa has been shown to be critically involved in the genetic predisposition to the progression of experimental CKD (Laouari et al., 2011, 2012). These studies suggest that an anti–TGFα-neutralizing antibody may show promise as a potential therapeutic in the treatment of these various diseases.

In conclusion, we have demonstrated development of a humanized monoclonal antibody that binds and neutralizes human Tgfa and Ereg. Furthermore, the antibody binds to the membrane bound version of these ligands and promotes their internalization, thus clearing them from the cell surface and making them unavailable for signaling through the EGFR. In addition, we have shown that the antibody neutralizes the target in vivo as mAb41 can reproduce the phenotype of Tgfa-deficient mice. Despite this, the antibody is devoid of the skin toxicity in cynomolgus monkeys that is seen with EGFR inhibitors. Thus, we have shown that LY3016859 has a safe profile and allows for continued development in human patients as a potential therapy for many diseases.

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![Fig. 6](https://example.com/figure6.png)
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Authorship Contributions

**Participated in research design:** Beidler,Connor,Petrovan,Boyles,Witcher,Datta-Mannan,Staub,-Chu,Harlan,Heuer. **Performed the experiments:** Beidler,Connor,Petrovan,Boyles,Elis,-Chu,Yang,Harlan,Heuer. **Contributed new reagents or analytic tools:** Beidler,Connor,Petrovan,Boyles,Witcher,Datta-Mannan,Staub,-Chu,Harlan,Heuer.

References


Mann GB, Fowler KJ, Gabriel A, Nice EC, Williams RL, and Dunn AR (1993) Mice with targeted deletion of the β2 integrin gene have normal hair, bone structure, hair, and curly whiskers and often develop cornelal inflammation. Cell 73:249-261.


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