Hypoxia

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Induction of erythropoiesis by hypoxia-inducible factor prolyl hydroxylase inhibitors without promotion of tumor initiation, progression, or metastasis in a VEGF-sensitive model of spontaneous breast cancer

This article was published in the following Dove Press journal:
Hypoxia
10 March 2017
Number of times this article has been viewed

Todd W Seeley
Mark D Sternlicht
Stephen J Klaus
Thomas B Neff
David Y Liu

Therapeutics R&D, FibroGen, Inc., San Francisco, CA, USA

Abstract: The effects of pharmacological hypoxia-inducible factor (HIF) stabilization were investigated in the MMTV-Neu^{ndl}-YD5 (NeuYD) mouse model of breast cancer. This study first confirmed the sensitivity of this model to increased vascular endothelial growth factor (VEGF), using bigenic NeuYD;MMTV-VEGF-25 mice. Tumor initiation was dramatically accelerated in bigenic animals. Bigenic tumors were also more aggressive, with shortened doubling times and increased lung metastasis as compared to NeuYD controls. In separate studies, NeuYD mice were treated three times weekly from 7 weeks of age until study end with two different HIF prolyl hydroxylase inhibitors (HIF-PHIs), FG-4497 or roxadustat (FG-4592). In NeuYD mice, HIF-PHI treatments elevated erythropoiesis markers, but no differences were detected in tumor onset or the phenotypes of established tumors.

Keywords: cancer progression, erythropoiesis, hypoxia-inducible factor, hypoxia-inducible factor prolyl hydroxylase inhibitors, vascular endothelial growth factor, MMTV-Neu breast cancer model

Introduction

Important safety concerns are associated with the use of recombinant erythropoiesis-stimulating agents (ESAs, i.e., recombinant erythropoietins [rEPOs] and analogs).[1,2] Novel approaches to managing anemia are therefore of clinical interest. Anemia correction has been reported in phase 2 clinical trials in chronic kidney disease anemia using small molecule inhibitors of hypoxia-inducible factor (HIF) prolyl hydroxylase (HIF-PH).[3–5] Inhibition of these enzymes causes increased levels of HIF, a transcription factor activated in response to low oxygen. These HIF-PH inhibitors (HIF-PHIs) enhance EPO expression, red blood cell (RBC) mass, and hemoglobin (HGB) levels through coordinated gene expression of EPO and genes mediating absorption, transport, storage, and metabolism of iron.[6,7]

Mutations in HIF pathway genes underlie specific familial polycythemias,[8] including mutation of the von Hippel–Lindau protein (pVHL) in Chuvash polycythemia and erythrocytoses associated with mutations in HIF-2α (e.g., *EPAS1* [HIF2A] G537W, G537R, I533V)[9,10] and PHD2 (*EGLN1* [PHD2] P317R, R371H).[11,12] These provide genetic evidence that modulation of HIF pathway genes can be used to increase RBC

Correspondence: Todd W Seeley
Therapeutics R&D, FibroGen, Inc.,
409 Illinois Street, San Francisco,
CA 94158, USA
Tel +1 415 978 1494
Email tseeley@fibrogen.com

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/HP.S130526

mass. Studies of pVHL-mutated Chuvash polycythemia patients have not shown increased tumor predisposition.[13] By contrast, other mutations in pVHL predispose VHL syndrome patients to highly vascularized clear-cell type renal cell carcinoma (RCC) tumors.[14]

The molecular mechanisms underlying the seemingly discrepant phenotypes of Chuvash polycythemia and VHL syndrome remain a matter of considerable scientific interest. Although HIF dysregulation appears common to both disorders, familial VHL-associated erythrocytosis and RCC-associated VHL syndrome involve unique alleles and distinguishing patterns of inheritance. VHL erythrocytoses are associated with autosomal recessive germ-line variants (homozygous *VHL* R200W, Chuvash polycythemia; or compound R200W heterozygosity with other *VHL* alleles in other sporadic polycythemias),[15] such that all cells carry mutations that confer sensitivity to HIF activation. In contrast, VHL syndrome (and RCC risk) is associated with distinct heterozygous germ-line *VHL* mutations and in diseased tissues, somatic mutation of the unaffected *VHL* allele is commonly observed.

Hypoxia is also a common feature of aggressive tumors, with HIF being elevated in many tumor types. Broad roles of HIF and tumor hypoxia in tumor promotion have been proposed.[6] Hypoxias associated with exercise, altitude, respiratory insufficiency, hemorrhage, or local tissue ischemias each exhibit unique features, however, and are not widely regarded as tumor promoting.[16]

Vascular endothelial growth factor (VEGF) is a well-studied hypoxia-responsive gene. VEGF-associated tumor promotion has been cited as a theoretical obstacle to HIF-PHI therapeutics.[17] Here, the effects of pharmacologic HIF activation are characterized in tumor-prone MMTV-Neu^{ndl}-YD5 (NeuYD) mice, known to be sensitive to increased VEGF.[18] NeuYD mice develop relatively normally until about 16 weeks of age, when females spontaneously develop mammary tumors with 100% penetrance. Although MMTV-VEGF-25 mice are phenotypically normal and exhibit normal mammary gland development, in bigenic NeuYD;MMTV-VEGF-25 (NeuYD;VEGF) female mice, tumor initiation, progression, and metastasis are dramatically accelerated versus control NeuYD mice, indicating that the NeuYD model is highly sensitive to increased VEGF. Published results showing that this model is sensitive to increased VEGF were confirmed, and HIF-PHI effects in this model were further characterized by treating NeuYD mice with two reversible, orally bioavailable HIF-PHIs, FG-4497 and roxadustat (also known as FG-4592). FG-4497 induces erythropoiesis in rhesus macaques[19] and exhibits beneficial effects in experimental models of kidney and bone marrow injury and other indications.[20,21] Roxadustat, a structurally related but chemically distinct HIF-PHI, was shown to correct anemia in phase 2 clinical trials in anemic chronic kidney disease patients[3,5,22,23] and is currently in phase 3 clinical development. In the current study, HIF-PHI treatment elicited markers of erythropoiesis without promoting initiation, progression, or metastasis of VEGF-sensitive NeuYD tumors.

Methods

Ethical statement

Animal studies were performed at Mispro Biotechnology Services Inc. (Montréal, Québec, Canada). Mispro Biotechnology Services Inc. is accredited with the Canadian Council on Animal Care and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and strictly complies with the norms and requirements of these bodies. Accordingly, Mispro's Institutional Animal Care and Use Committee approved this study.

Mice

Drs WJ Muller (McGill University, Montréal, Québec, Canada) and RG Oshima (Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA) kindly provided FVB background NeuYD and MMTV-VEGF-25 mice. FVB mice were obtained from Charles River. Transgene presence was verified by polymerase chain reaction (PCR) genotyping of tail clips (Dr Michel L Tremblay, McGill University). Controlled matings were performed to obtain sufficient number of female pups of the desired genotypes. Because of the large number of animals required, animals from multiple litters were pooled over a narrow range of ±2 days. Thus, "days age" reflects a group average. At the time of treatment initiation, mice were assigned by weight and age to groups. Animal studies were conducted in strict compliance with AAALAC guidelines for animal care.

FG-4497 pharmacokinetics study

FVB females 7–14 weeks of age were treated with a single oral dose of carboxymethyl cellulose (CMC) vehicle or FG-4497 (20 and 40 mg/kg). Plasma and tissue were taken at t=2, 4, 8, 12, and 24 h and compared to the CMC vehicle at t=24 h, n=3/time-point. Plasma drug was determined by liquid chromatography–mass spectrometry. VEGF and EPO mRNA levels were determined from total kidney mRNA by

reverse transcription-PCR and normalized to β-actin mRNA (ABI; Mm00433126_m1, EPO; Mm00437304_m1, VEGF; and 4352341E, β-actin). The VEGF mRNA assay detects all alternatively spliced isoforms. Plasma EPO and VEGF protein were determined by enzyme-linked immunosorbent assay (ELISA; R&D systems kits MEP00, EPO; and MMV00, VEGF). The VEGF ELISA recognizes both the 164 and 120 amino acid residue forms of mouse VEGF.

Tumor studies

NeuYD mouse studies were conducted by Mispro Biotech Services in collaboration with Insymbiosis Discovery, Inc. (Montréal, Québec, Canada). NeuYD;VEGF, FG-4497, and roxadustat effects were each examined in separate studies. NeuYD;VEGF study animals were dosed by oral gavage three times per week (TIW; Monday–Wednesday–Friday) with 10 mL/kg CMC vehicle control, starting at 47 days average age through study end. In HIF-PHI studies, mice were dosed TIW by oral gavage with 10 mL/kg CMC vehicle or HIF-PHI starting at 52 days average age. Dose was adjusted weekly based on average cohort weights. Animals were weighed once weekly, and the clinical status was monitored on treatment days. Treatments were well tolerated, and no unscheduled deaths were reported.

Tumor volumes were determined TIW by caliper, using the formula $L \times W^2/2$. In place of Oshima's linear aggregated tumor growth model,[18] the doubling time of the single largest tumor per animal was estimated by linear regression of $\log_2$ of the initial 3–6 measurements >25 mm^3 tumor size. Estimated doubling times were then compared by analysis of variance (ANOVA).

EDTA-preserved blood samples were analyzed by complete blood count analysis. Individual determinations were excluded when sample clotting was suspected.

Histology

Whole lungs were harvested and fixed. Representative 5 µm sections (using a Leica EG 1160 microtome, Wetzlar, Germany) were stained with hematoxylin and eosin (Leica Autostainer XL), and the number of tumor emboli per section was determined.

Data analysis

Parametric *t*-tests (unpaired, two-tailed), one-way ANOVA, regression analyses, χ^2 tests, and log-rank (Mantel–Cox) tests to assess differences in tumor-free survival were performed using GraphPad PRISM (GraphPad Software, Inc., San Diego, CA, USA). Data are presented as mean ± standard error with $p<0.05$ considered statistically significant unless indicated otherwise.

Results

Pharmacologic response to single dose administration of HIF-PHI FG-4497

Following a single oral dose, FG-4497 was detected in FVB mouse plasmas at the earliest time-point in this study (2 h), and levels declined rapidly thereafter (Figure 1). Treatment was also associated with dose-dependent increases in EPO kidney mRNA and EPO plasma protein, which resolved over time. In contrast, VEGF kidney mRNA levels appeared relatively unchanged (Figure 1). A transient and mild (<2-fold) change in plasma VEGF protein at 2 h did not seem to be linked to changes in kidney VEGF mRNA. Thus, FG-4497 treatment of FVB mice resulted in drug exposure, followed by transient elevations of EPO mRNA and protein, with minimal effects on VEGF.

VEGF overexpression accelerates tumor phenotypes of NeuYD mammary cancers

The tumor-prone transgenic NeuYD mouse line developed and characterized in the laboratory of Dr William Muller[24,25] was used in subsequent studies. Females of this FVB background line develop mammary tumors with a median time to tumor appearance of ~110–120 days, whereas males exhibit normal health and unaltered life expectancy. Tumor initiation, progression, and metastasis in the NeuYD model are dramatically accelerated by the presence of a VEGF transgene, as reported by Oshima et al[18] and confirmed here (Figure 2). Compared with NeuYD control animals, time of initial tumor appearance in NeuYD;VEGF mice was dramatically accelerated, with a median tumor-free survival of 61 days in NeuYD;VEGF mice versus 106 days in NeuYD controls (Mantel–Cox hazard ratio =25, $p<0.0001$). In NeuYD;VEGF mice, tumors were more aggressive, with shortened doubling times as compared with NeuYD controls ($p<0.01$, *t*-test). Lung tumor emboli were rare in NeuYD mice, but were frequent in NeuYD;VEGF mice, with 95% of double transgenic mice bearing 1–20 tumor emboli per lung section versus a solitary embolus detected in one NeuYD control animal (χ^2, $p<0.0001$).

Plasma VEGF protein levels were ~9-fold elevated at termination in bigenic NeuYD;VEGF mice compared with NeuYD controls. Bigenic animals exhibited decreased RBC, HGB, and hematocrit (HCT) ($p<0.0001$ by *t*-test) versus

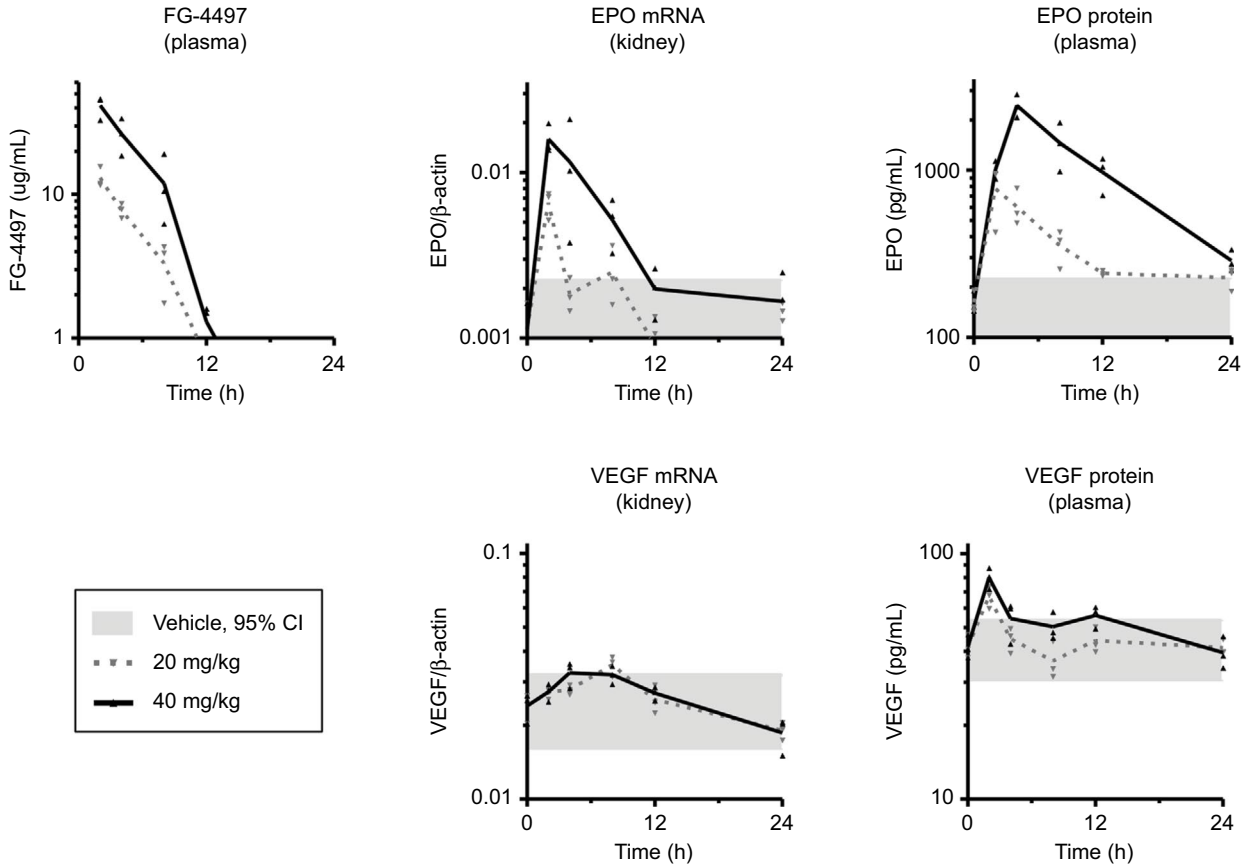

Figure 1 Oral administration of HIF-PHI FG-4497 to normal FVB mice induces transient increases in kidney EPO mRNA and circulating EPO protein.
Notes: Mean time-course plots and individual data-points for each time-point (n=3) are shown as well as the 95% CI (gray area) for 24 h vehicle controls.
Abbreviations: CI, confidence interval; EPO, erythropoietin; HIF-PHI, hypoxia-inducible factor prolyl hydroxylase inhibitor; mRNA, messenger RNA; VEGF, vascular endothelial growth factor.

NeuYD controls suggesting a more profound disease state in these animals at the time of sacrifice.

HIF-PHI stimulates erythropoiesis without affecting NeuYD mammary cancer progression

In a second NeuYD study, females were treated with the HIF-PHI FG-4497 (20 or 40 mg/kg), starting at 52 days average age and continued to study end. At the 40 mg/kg dose, animal weights initially increased more slowly than in the vehicle and low dose groups, recovering thereafter (Figure 3). Median times to first appearance of tumors between groups were not significant (log-rank $p>0.05$) (Figure 3). The age at which half or more of the animals were scored as bearing tumors in the vehicle cohort was 96 days, and 100 days in the 20 and 40 mg/kg cohorts. Tumor doubling times in FG-4497 treatment groups were similar to controls (not significant, ANOVA, Dunnett's test). At sacrifice (117 days for vehicle-treated mice and 118 days average age for 20 and 40 mg/kg FG-4497-treated groups), the frequency of lung tumor emboli in both instances was very low (Figure 3).

FG-4497 treatment induced erythropoiesis as demonstrated by increases in hematologic parameters (40 mg/kg HGB $p<0.01$, HCT $p<0.05$ by one-way ANOVA, Dunnett's test) (Figure 3). EPO and VEGF levels measured 24 h after the final dose were not altered in any clear pattern (Figure 3), consistent with resolution of immediate FG-4497 effects by this time-point.

A third MMTV-NeuYD study examined roxadustat treatment (40 and 80 mg/kg). Again, onset of tumor appearance did not differ significantly between HIF-PHI treated groups and the vehicle-treated group (log-rank and Gehan–Breslow–Wilcoxon tests). The age at which half or more of the animals were scored as bearing tumors was comparable in the vehicle, 40 mg/kg roxadustat, and 80 mg/kg roxadustat groups at 104, 99.5, and 102 days, respectively (Figure 4). Tumor growth rates were also comparable between groups ($p>0.05$). At sacrifice (111 days, all animals), plasmas were harvested 6 h after final dose. At this time-point, roxadustat

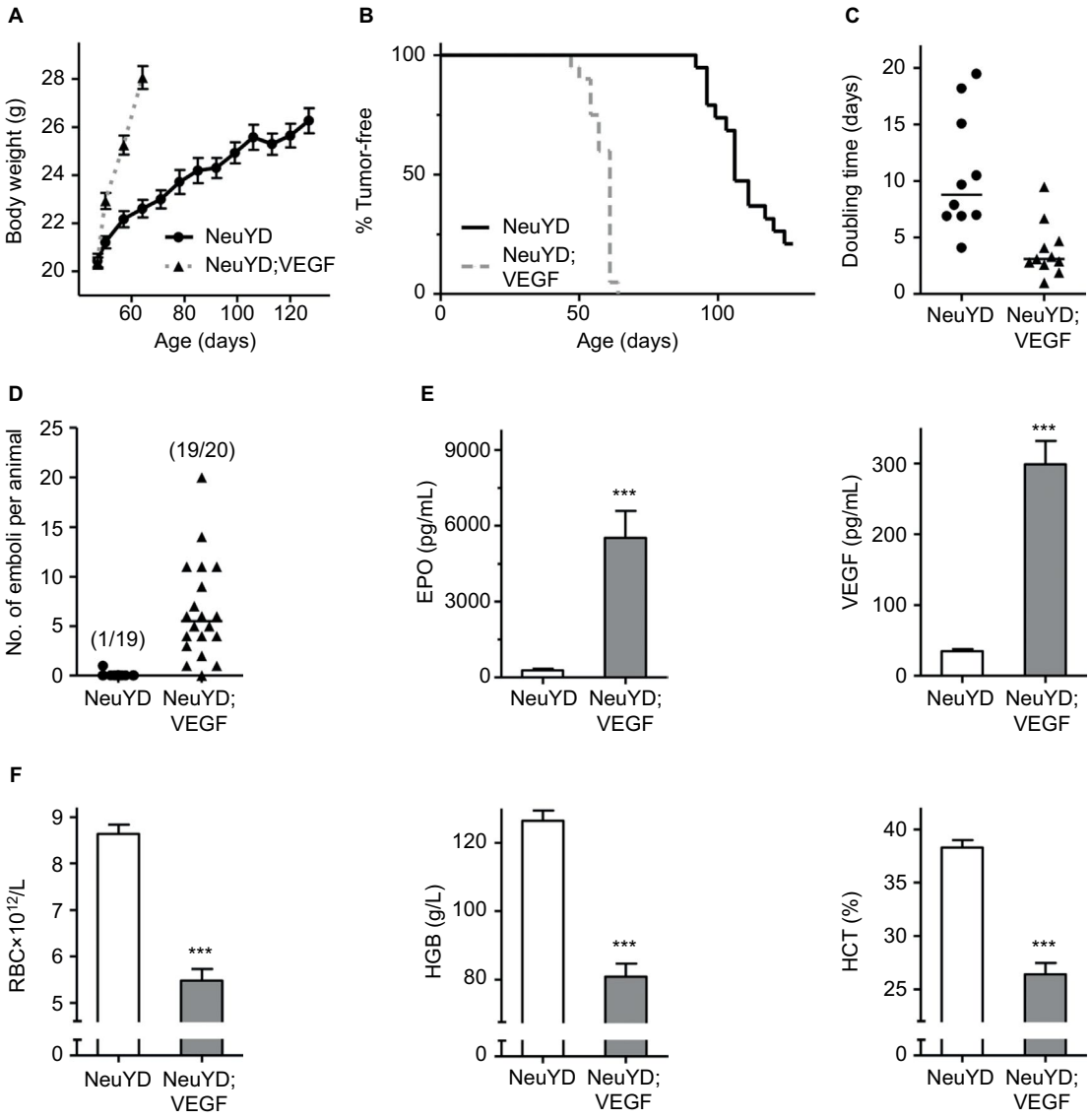

Figure 2 VEGF accelerates NeuYD tumor onset, growth, and metastasis.

Notes: Changes in body weight (**A**; n=19–20/group), tumor onset (**B**; n=19–20), tumor doubling times (**C**; n=10–11), number of pulmonary tumor emboli (**D**), plasma cytokines (**E**; n=17–19), and hematologic indicators of erythropoiesis (**F**; n=15–18) are shown for NeuYD (●, solid lines, open bars) and NeuYD;VEGF mice (▲, dashed lines, solid bars). Data indicate mean ± SE (**A, E, F**) or median plus data for individual biological replicates (**C, D**). ***$p<0.001$.

Abbreviations: EPO, erythropoietin; HCT, hematocrit; HGB, hemoglobin; No., number; RBC, red blood cell; SE, standard error; VEGF, vascular endothelial growth factor.

induced a substantial increase in circulating EPO protein (33×), whereas VEGF levels appeared unaffected by treatment. Histopathologic examination of lung tissues collected from this study did not reveal tumor emboli in any animal.

Discussion

Although FG-4497 and roxadustat treatments were not associated with prominent increases in VEGF, small changes in local VEGF in the tumor microenvironment could influence tumor progression. The NeuYD-dependent mouse model is exquisitely sensitive to increases in VEGF, as reported by Oshima et al[18] and confirmed here. In bigenic NeuYD;VEGF females, increased circulating VEGF was associated with a dramatically shorter time to appearance of initial tumors. These tumors also exhibited increased aggressiveness as measured by growth rates and rates of metastasis to lung, as previously reported.[18]

Using two distinct HIF-PHIs, no effects of extended HIF-PHI treatment were observed on tumor initiation, progression, or metastasis, under conditions where elevations of erythropoiesis markers were scored. Time to median tumor onset in HIF-PHI treatment groups varied in a manner similar to that observed in the NeuYD control groups across the three studies (98, 104, and 106 days). It is concluded that the HIF-PHI treatments used here do not elicit sufficient VEGF to alter the phenotypes of tumors in this model.

Since no HIF-PHI-dependent increases were observed in tumor initiation, progression, or metastasis, it can be

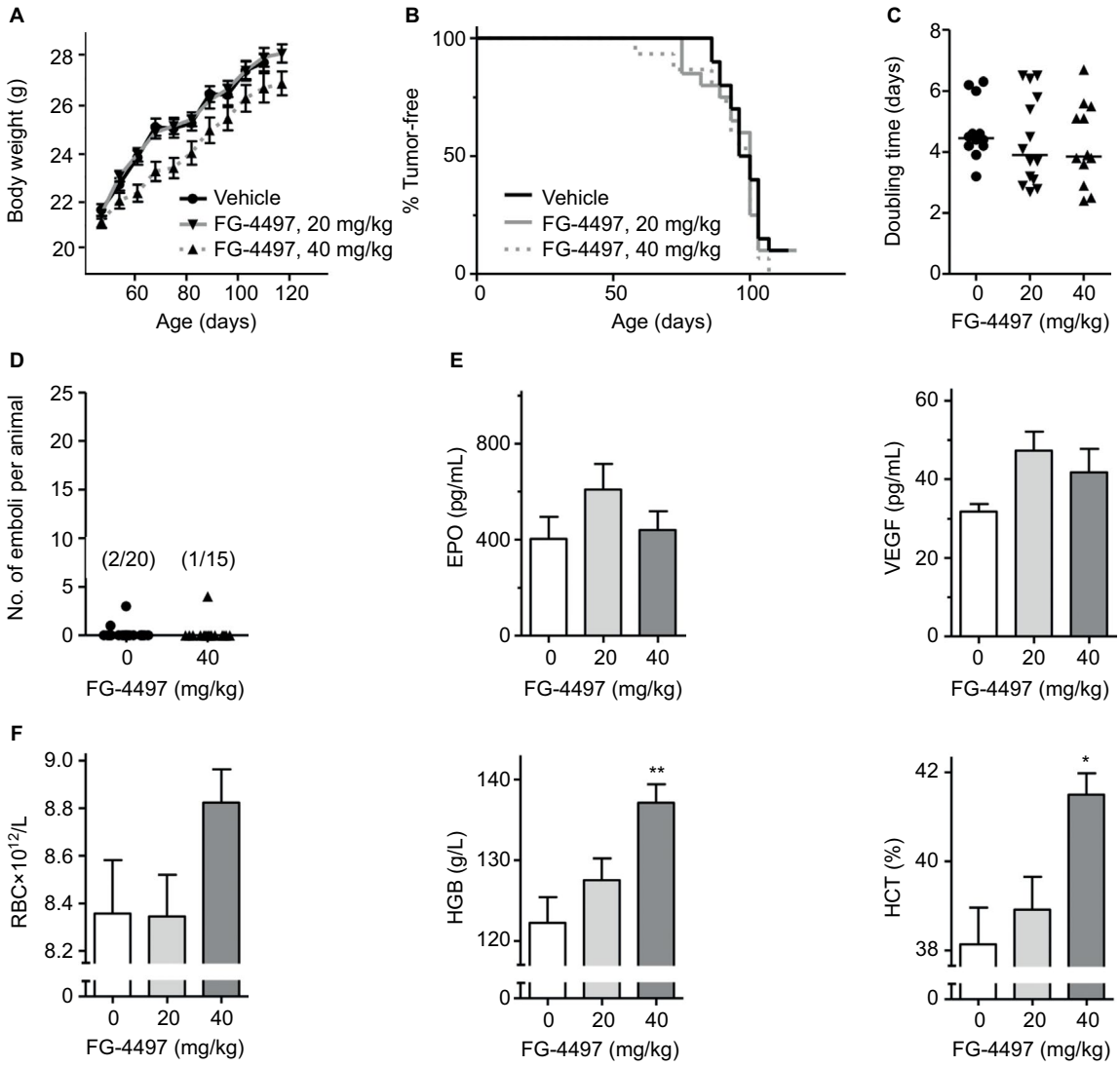

Figure 3 FG-4497 stimulates erythropoiesis without affecting NeuYD tumor onset, growth, or metastasis.

Notes: Changes in body weight (**A**; n=15–20), tumor onset (**B**; n=15–20), tumor doubling times (**C**), number of pulmonary tumor emboli (**D**; n=12–14), plasma cytokines (**E**; 24 h post-final dose, n=15–20), and hematologic indicators of erythropoiesis (**F**; n=10–15) are shown for NeuYD mice treated with vehicle (●, solid black lines, open bars), 20 mg/kg FG-4497 (▼, solid gray lines, light gray bars), and 40 mg/kg FG-4497 (▲, dashed lines, dark gray bars). Data indicate mean ± SE (**A, E, F**) or median plus data for individual biologic replicates (**C, D**). *p<0.05; **p<0.01.

Abbreviations: EPO, erythropoietin; HCT, hematocrit; HGB, hemoglobin; No., number; RBC, red blood cell; SE, standard error; VEGF, vascular endothelial growth factor.

concluded that non-VEGF pro-tumorigenic pathways were not recruited in this study either, including EPO-responsive pathways. Systematic surveys of xenografted tumor models suggest EPO-responsiveness is uncommon,[26] and current literature provides limited guidance as to which solid tumor types, if any, may be of special concern with respect to EPO elevation. In the NeuYD model here, circulating EPO elevations associated with HIF-PHI treatment were not associated with tumor promoting effects.

Although the NeuYD model used here was selected on the basis of findings that VEGF promoted tumor development,[18] the model can be considered broadly representative of human HER2/neu-associated breast cancer tumor promotion. Intratumoral hypoxia is a common feature of breast tumors, and HIF-1α overexpression and adverse outcomes are correlated in many studies of breast cancer patients. Both cell proliferation and HIF stabilization are inhibited in the NeuYD model upon treatment with rapamycin,[27] consistent with a model where proliferative signaling is a prerequisite for HIF-responsiveness. Due to associations between proliferation, hypoxia, and HIF stabilization, HIF target genes have been broadly proposed to be involved in promoting primary tumor growth, vascularization, invasion, and metastasis.[28] Hypotheses suggesting increased

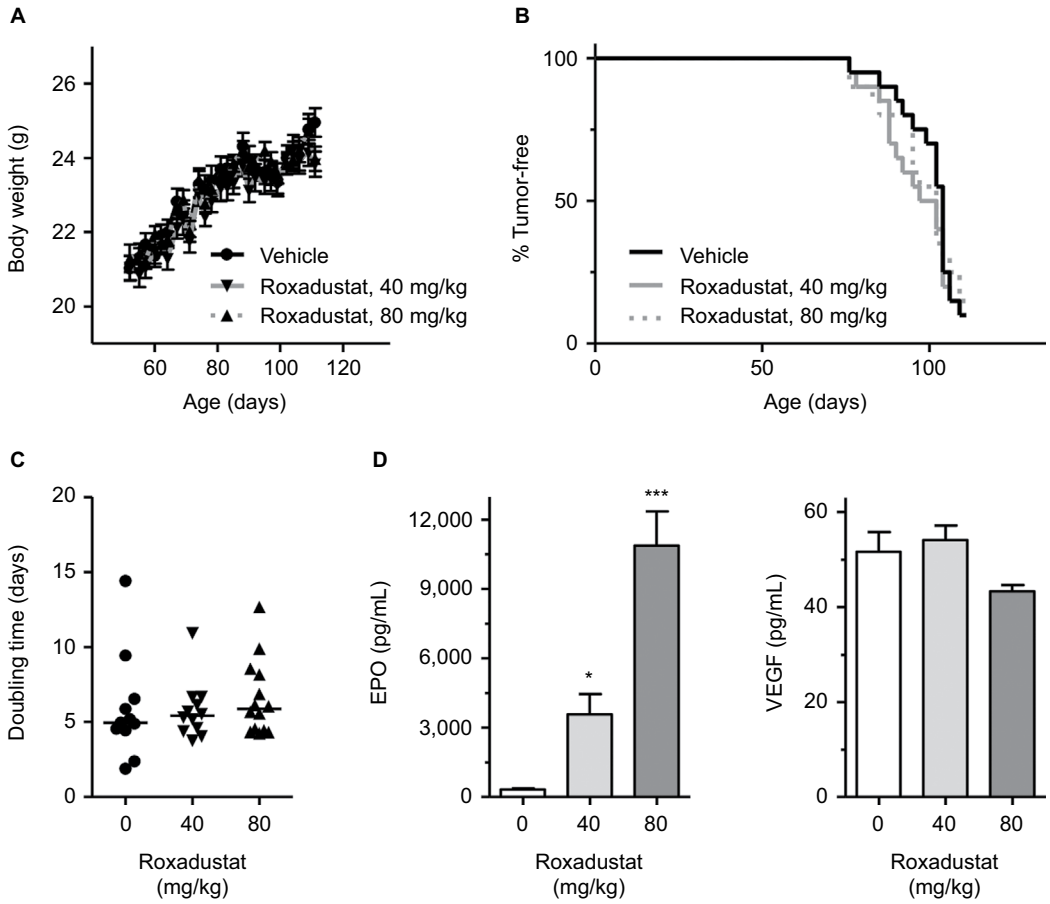

Figure 4 Roxadustat stimulates circulating EPO production without affecting plasma VEGF or the growth or onset of NeuYD tumors.
Notes: Changes in body weight (**A**; n=20), tumor onset (**B**; n=20), tumor doubling times (**C**; n=11–14), and plasma cytokines (**D**; 6 h post-final dose, n=20) are shown for NeuYD mice treated with vehicle (●, solid black lines, open bars), 40 mg/kg roxadustat (▼, solid gray lines, light gray bars), and 80 mg/kg roxadustat (▲, dashed lines, dark gray bars). Final hematology was not available for this study. Data indicate mean ± SE (**A, D**) or median plus data for individual biologic replicates (**C**). *p<0.05; ***p<0.001.
Abbreviations: EPO, erythropoietin; SE, standard error; VEGF, vascular endothelial growth factor.

HIF may contribute to tumor progression have yielded mixed results in genetically engineered cells, however, in no clear pattern with respect to tumor types or within tumor types.[29–36]

In non-RCC tumor types, HIF activation is mediated not by genetic lesions but by tumor ischemia resulting from vascular insufficiency and erratic blood flow.[37] Although many cancer researchers equate tumor hypoxia with ischemia, some studies suggest hypoxia may not be the main cause of heightened VEGF expression by ischemic tissues or tumors.[38] Robust normoxic regulation of VEGF mRNA by nutrient limitation[39–41] is mediated by ATF4 independently of HIFs.[42,43] VEGF was rapidly and robustly induced >10-fold by normoxic amino acid deprivation in human tumor cells of different lineages,[44] comparable to circulating VEGF increases associated with tumor promotion in bigenic NeuYD;VEGF control animals in the current study.

In summary, FG-4497 and roxadustat treatments that stimulated EPO and erythropoiesis were not observed to exacerbate tumor development or progression in the NeuYD model, via VEGF-dependent or other pathways. The ability to control responses by pharmacologically manipulating magnitude, duration, and interval of HIF stabilization is unique, and responses are likely to differ from models employing genetic manipulation of HIF pathway genes. Further studies will be required to determine if the results reported here relate to specific aspects of HIF-PHI pharmacology or to intrinsic differences in how tumors receive and process HIF signals.

Acknowledgments

Adrienne Scott (Insymbiosis Discovery, Inc.) and Daniel Goulet (Mispro Biotech Services) provided expert assistance with animal breeding, tumor studies, sample collection, and data analysis. FibroGen's Pharmacology group is acknowledged

for the PK/PD animal study. Compounds were provided by FibroGen's Medicinal Chemistry and Formulations groups. Analytical support was provided by FibroGen's Cell Biology, Small Molecule Evaluation, and Molecular Biology groups.

Disclosure

TWS, MDS, and TBN are employees of and hold stock and/or options in FibroGen. SJK and DYL are former employees of FibroGen. The authors report no other conflicts of interest in this work.

References

1. Teehan G, Benz RL. An update on the controversies in anemia management in chronic kidney disease: lessons learned and lost. *Anemia.* 2011;2011:623673.
2. Del Vecchio L, Locatelli F. An overview on safety issues related to erythropoiesis-stimulating agents for the treatment of anaemia in patients with chronic kidney disease. *Expert Opin Drug Saf.* 2016;15(8):1021–1030.
3. Koury MJ, Haase VH. Anaemia in kidney disease: harnessing hypoxia responses for therapy. *Nat Rev Nephrol.* 2015;11:394–410.
4. Besarab A, Chernyavskaya E, Motylev I, et al. Roxadustat (FG-4592): correction of anemia in incident dialysis patients. *J Am Soc Nephrol.* 2016;27:1225–1233.
5. Besarab A, Provenzano R, Hertel J, et al. Randomized placebo-controlled dose-ranging and pharmacodynamics study of roxadustat (FG-4592) to treat anemia in nondialysis-dependent chronic kidney disease (NDD-CKD) patients. *Nephrol Dial Transplant.* 2015;30:1665–1673.
6. Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. *Annu Rev Pathol.* 2014;9:47–71.
7. Haase VH. Hypoxic regulation of erythropoiesis and iron metabolism. *Am J Physiol Renal Physiol.* 2010;299:F1–F13.
8. Bento C, Percy MJ, Gardie B, et al. Genetic basis of congenital erythrocytosis: mutation update and online databases. *Hum Mutat.* 2014;35:15–26.
9. Percy MJ, Furlow PW, Lucas GS, et al. A gain-of-function mutation in the HIF2A gene in familial erythrocytosis. *N Engl J Med.* 2008;358:162–168.
10. Perrotta S, Stiehl DP, Punzo F, et al. Congenital erythrocytosis associated with gain-of-function HIF2A gene mutations and erythropoietin levels in the normal range. *Haematologica.* 2013;98:1624–1632.
11. Percy MJ, Zhao Q, Flores A, et al. A family with erythrocytosis establishes a role for prolyl hydroxylase domain protein 2 in oxygen homeostasis. *Proc Natl Acad Sci U S A.* 2006;103:654–659.
12. Percy MJ, Furlow PW, Beer PA, Lappin TR, McMullin MF, Lee FS. A novel erythrocytosis-associated PHD2 mutation suggests the location of a HIF binding groove. *Blood.* 2007;110:2193–2196.
13. Gordeuk VR, Sergueeva AI, Miasnikova GY, et al. Congenital disorder of oxygen-sensing: association of the homozygous Chuvash polycythemia VHL mutation with thrombosis and vascular abnormalities but not tumors. *Blood.* 2004;103:3924–3932.
14. Baldewijns MM, van Vlodrop IJ, Vermeulen PB, Soetekouw PM, van Engeland M, de Bruine AP. VHL and HIF signalling in renal cell carcinogenesis. *J Pathol.* 2010;221:125–138.
15. Pastore YD, Jelinek J, Ang S, et al. Mutations in the VHL gene in sporadic apparently congenital polycythemia. *Blood.* 2003;101:1591–1595.
16. Winkelmayer WC, Liu J, Brookhart MA. Altitude and all-cause mortality in incident dialysis patients. *JAMA.* 2009;301:508–512.
17. Maxwell PH, Eckardt KU. HIF prolyl hydroxylase inhibitors for the treatment of renal anaemia and beyond. *Nat Rev Nephrol.* 2016;12:157–168.
18. Oshima RG, Lesperance J, Munoz V, et al. Angiogenic acceleration of Neu induced mammary tumor progression and metastasis. *Cancer Res.* 2004;64:169–179.
19. Hsieh MM, Linde NS, Wynter A, et al. HIF prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis, and modest fetal hemoglobin expression in rhesus macaques. *Blood.* 2007;110:2140–2147.
20. Rosenberger C, Rosen S, Shina A, et al. Activation of hypoxia-inducible factors ameliorates hypoxic distal tubular injury in the isolated perfused rat kidney. *Nephrol Dial Transplant*. 2008;23:3472–3478.
21. Forristal CE, Winkler IG, Nowlan B, Barbier V, Walkinshaw G, Levesque JP. Pharmacological stabilization of HIF-1α increases hematopoietic stem cell quiescence in vivo and accelerates blood recovery following severe irradiation. *Blood.* 2013;121:759–769.
22. Besarab A, Chernyavskaya E, Motylev I, et al. Roxadustat (FG-4592): correction of anemia in incident dialysis patients. *J Am Soc Nephrol.* 2016;27(4):1225–1233.
23. Provenzano R, Besarab A, Sun CH, et al. Oral hypoxia-inducible factor prolyl hydroxylase inhibitor Roxadustat (FG-4592) for the treatment of anemia in patients with CKD. *Clin J Am Soc Nephrol.* 2016;11:982–991.
24. Dankort D, Maslikowski B, Warner N, et al. Grb2 and Shc adapter proteins play distinct roles in Neu (ErbB-2)-induced mammary tumorigenesis: implications for human breast cancer. *Mol Cell Biol.* 2001;21:1540–1551.
25. Khoury H, Dankort DL, Sadekova S, Naujokas MA, Muller WJ, Park M. Distinct tyrosine autophosphorylation sites mediate induction of epithelial mesenchymal like transition by an activated ErbB-2/Neu receptor. *Oncogene.* 2001;20:788–799.
26. Elliott S, Sinclair AM. The effect of erythropoietin on normal and neoplastic cells. *Biologics.* 2012;6:163–189.
27. Liu M, Howes A, Lesperance J, et al. Antitumor activity of rapamycin in a transgenic mouse model of ErbB2-dependent human breast cancer. *Cancer Res.* 2005;65:5325–5336.
28. Gilkes DM, Semenza GL. Role of hypoxia-inducible factors in breast cancer metastasis. *Future Oncol.* 2013;9:1623–1636.
29. Acker T, Diez-Juan A, Aragones J, et al. Genetic evidence for a tumor suppressor role of HIF-2alpha. *Cancer Cell.* 2005;8:131–141.
30. Blouw B, Song H, Tihan T, et al. The hypoxic response of tumors is dependent on their microenvironment. *Cancer Cell.* 2003;4:133–146.
31. Carmeliet P, Dor Y, Herbert JM, et al. Role of HIF-1a in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature.* 1998;394:485–490.
32. Mack FA, Rathmell WK, Arsham AM, Gnarra J, Keith B, Simon MC. Loss of pVHL is sufficient to cause HIF dysregulation in primary cells but does not promote tumor growth. *Cancer Cell.* 2003;3:75–88.
33. Mack FA, Patel JH, Biju MP, Haase VH, Simon MC. Decreased growth of Vhl-/- fibrosarcomas is associated with elevated levels of cyclin kinase inhibitors p21 and p27. *Mol Cell Biol.* 2005;25:4565–4578.
34. Maxwell PH, Dachs GU, Gleadle JM, et al. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci U S A.* 1997;94:8104–8109.
35. Tsuzuki Y, Fukumura D, Oosthuyse B, Koike C, Carmeliet P, Jain RK. Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha--> hypoxia response element--> VEGF cascade differentially regulates vascular response and growth rate in tumors. *Cancer Res.* 2000;60:6248–6252.
36. Kuchnio A, Moens S, Bruning U, et al. The cancer cell oxygen sensor PHD2 promotes metastasis via activation of cancer-associated fibroblasts. *Cell Rep.* 2015;12:992–1005.
37. Jain RK. Vascular and interstitial biology of tumors. In: Abeloff MD, Armitage JO, Niederhuber JE, Kastan MB, McKenna WG, editors. *Clinical Oncology*. 3rd ed. New York: Elsevier; 2004:153–172.
38. Jubb AM, Pham TQ, Hanby AM, et al. Expression of vascular endothelial growth factor, hypoxia inducible factor 1alpha, and carbonic anhydrase IX in human tumours. *J Clin Pathol.* 2004;57:504–512.

39. Wang Y, Alam GN, Ning Y, et al. The unfolded protein response induces the angiogenic switch in human tumor cells through the PERK/ATF4 pathway. *Cancer Res.* 2012;72:5396–5406.
40. Marjon PL, Bobrovnikova-Marjon EV, Abcouwer SF. Expression of the pro-angiogenic factors vascular endothelial growth factor and interleukin-8/CXCL8 by human breast carcinomas is responsive to nutrient deprivation and endoplasmic reticulum stress. *Mol Cancer.* 2004;3:4.
41. Singleton DC, Harris AL. Targeting the ATF4 pathway in cancer therapy. *Expert Opin Ther Targets.* 2012;16:1189–1202.
42. Ameri K, Lewis CE, Raida M, Sowter H, Hai T, Harris AL. Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells. *Blood.* 2004;103:1876–1882.
43. Abcouwer SF, Marjon PL, Loper RK, Vander Jagt DL. Response of VEGF expression to amino acid deprivation and inducers of endoplasmic reticulum stress. *Invest Ophthalmol Vis Sci.* 2002;43:2791–2798.
44. Wang Y, Ning Y, Alam GN, et al. Amino acid deprivation promotes tumor angiogenesis through the GCN2/ATF4 pathway. *Neoplasia.* 2013;15:989–997.