

## Angiopoietin-2 Functions as a Tie2 Agonist in Tumor Models, Where It Limits the Effects of VEGF Inhibition

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### Abstract

The angiopoietins Ang1 (ANGPT1) and Ang2 (ANGPT2) are secreted factors that bind to the endothelial cell-specific receptor tyrosine kinase Tie2 (TEK) and regulate angiogenesis. Ang1 activates Tie2 to promote blood vessel maturation and stabilization. In contrast, Ang2, which is highly expressed by tumor endothelial cells, is thought to inhibit Tie2 activity and destabilize blood vessels, thereby facilitating VEGF-dependent vessel growth. Here, we show that the inhibition of tumor xenograft growth caused by an Ang2-specific antibody (REGN910) is reversed by systemic administration of the Tie2 agonist Ang1. These results indicate that Ang2 blockade inhibits tumor growth by decreasing Tie2 activity, showing that Ang2 is a Tie2 activator. REGN910 treatment of tumors resulted in increased expression of genes that are repressed by Tie2 activation, providing further evidence that REGN910 inhibits Tie2 signaling. Combination treatment with REGN910 plus the VEGF blocker aflibercept reduced tumor vascularity and tumor perfusion more dramatically than either single agent, resulting in more extensive tumor cell death and more potent inhibition of tumor growth. Challenging the prevailing model of Ang2 as a destabilizing factor, our findings indicate that Ang2 plays a protective role in tumor endothelial cells by activating Tie2, thereby limiting the antivasular effects of VEGF inhibition. Thus, blockade of Ang2 might enhance the clinical benefits currently provided by anti-VEGF agents. *Cancer Res*; 73(1); 108–18. ©2012 AACR.

### Introduction

The Angiopoietin/Tie2 signaling system is essential for vascular development and function (1). Tie2 is an endothelial cell-specific tyrosine kinase receptor for the angiopoietin ligands, the best studied of which are angiopoietin-1 (ANGPT1; Ang1) and angiopoietin-2 (ANGPT2; Ang2). Ang1 is a strong Tie2 agonist that is produced primarily by perivascular cells, and Ang1/Tie2 signaling is believed to promote blood vessel maturation and stabilization (1–6). In contrast, Ang2 is produced primarily by the endothelial cells in remodeling blood vessels (1, 7, 8) and is believed to function largely as a Tie2 antagonist to promote tumor angiogenesis and inflammation (1, 7–13). Ang2 expression is upregulated in a wide range of human cancers (14–20), and recent preclinical studies using Ang2 inhibitors and Ang2 knockout mice have established that Ang2 is important for tumor angiogenesis and tumor growth (21–31).

The dominant model for the role of Ang2 in tumors proposes that Ang2 competes with Ang1 for Tie2 binding, thereby

inhibiting Tie2 activity (1, 7, 13). The loss of Tie2 signaling is thought to lead to vessel destabilization, which facilitates VEGF-dependent angiogenesis (1, 7, 13). Consistent with this model, a number of papers have shown that overexpression of Ang2 can disrupt the tumor vasculature (32–34). However, several studies indicate that in certain settings Ang2 can activate Tie2 (35–39). Furthermore, recent data indicate that the effects of an Ang2-specific blocker on tumor growth are not reversed by cotreatment with an Ang1-specific blocker, suggesting that Ang2 does not function to inhibit Ang1 action (22, 28). Thus, the mechanism of Ang2 signaling in tumors remains controversial and data that directly link Ang2 blockade to changes in Tie2 activity are lacking.

While Ang2 inhibition reduces the growth of a broad range of tumors in preclinical models, the effects are relatively modest, with only partial growth inhibition usually observed (21, 24, 27, 30). Interestingly, treatment with a combination of Ang2 and VEGF blockers provides better inhibition of tumor growth than either single agent in a number of tumor models (21–25, 30). However, several of the VEGF blockers used in the published combination experiments are of uncertain specificity (21, 24), or only block human, but not mouse, VEGF (22, 24, 30). Furthermore, in most cases, it was not shown that the VEGF and Ang2 blockers were used at doses that provide maximal inhibition of each individual pathway (21–24, 30). Thus, it remains to be convincingly shown that blockade of Ang2 provides additional antitumor benefit under conditions where VEGF activity is completely and specifically blocked.

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To investigate the molecular mechanism of Ang2 signaling in tumors, we have generated a fully human antibody (REGN910) that is completely specific for Ang2. Here, we provide strong evidence that Ang2 is a Tie2 activator in tumors and show that combination treatment with REGN910 plus aflibercept (ziv-aflibercept in the U.S.; commonly known in the literature as VEGF Trap), a highly specific blocker of both human and mouse VEGF, inhibits tumor angiogenesis and tumor growth more potently than saturating doses of either single agent. Our findings, in contrast to the current model, indicate that Ang2 plays a protective rather than a destabilizing role in tumor endothelial cells. Furthermore, our data indicate that Ang2 signaling limits the antivasular effects of VEGF inhibition, suggesting that blockade of Ang2 might enhance the clinical benefits provided by anti-VEGF therapies.

## Materials and Methods

### Generation of human antibodies against Ang2

VelocImmune mice (with genes encoding human immunoglobulin heavy and kappa light chain variable regions), in which the *Angpt2* gene was replaced with the mouse *Angpt1* cDNA, were immunized with recombinant human Ang2 fibrinogen-like domain. Splensens were harvested for generation of hybridomas or for direct isolation of antigen-positive splenocytes. The cloned human immunoglobulin variable region genes from antibodies exhibiting the desired characteristics were joined to human IgG1 constant region genes for production in chinese hamster ovary (CHO) cells. REGN910 was selected as a lead antibody from more than 1,000 antigen-positive clones, based on *in vitro* biochemical properties as well as the ability to inhibit tumor xenograft growth. The methods used to characterize the biochemical properties of REGN910 are described in detail in the Supplementary Methods.

### Tumor xenograft growth studies

Tumor cells obtained from American Type Culture Collection (ATCC; the identities of our human tumor cell lines were authenticated by short tandem repeat profiling at ATCC) were implanted subcutaneously into the hind flank of 6- to 8-week-old C.B.-17 SCID (for Colo205 and A431 tumors) or NCr nude (for PC3 tumors) mice. Once tumors were established (100–400 mm<sup>3</sup> in volume), mice were randomized into treatment groups ( $n = 5-8$  mice per group) and injected subcutaneously twice per week with human Fc control protein, REGN910, aflibercept (40), or Ang1 (a recombinant version of human Ang1 known as AngF1-Fc-F1 that has been described previously; ref. 41) at the doses indicated in the figure legends. Average tumor growth for each treatment group was compared using one-way ANOVA and Tukey multiple comparison test. All procedures were conducted according to the guidelines of the Regeneron Institutional Animal Care and Use Committee.

### Analysis of tumor vascularity, perfusion, and gene expression

Tumor sections were stained with a rat anti-murine CD31 (PECAM1) antibody (BD Pharmingen) to visualize blood vessels using standard methodology. Tumor cell death was

assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) stain with the In Situ Cell Death Detection Kit (Roche). Tumor perfusion was assessed by contrast-enhanced microultrasound imaging, using Micro-Marker contrast agent and a Vevo 2100 Micro-Ultrasound Imaging System (VisualSonics Inc). Tumor gene expression was measured by real-time PCR under standard TaqMan conditions on the ABI 7900HT instrument using the automatic setting for determining the threshold cycle. Additional detail on these methods is present in the Supplementary Methods.

## Results

### A fully human monoclonal antibody, REGN910, binds Ang2 with high affinity and specificity and inhibits Ang2/Tie2 signaling

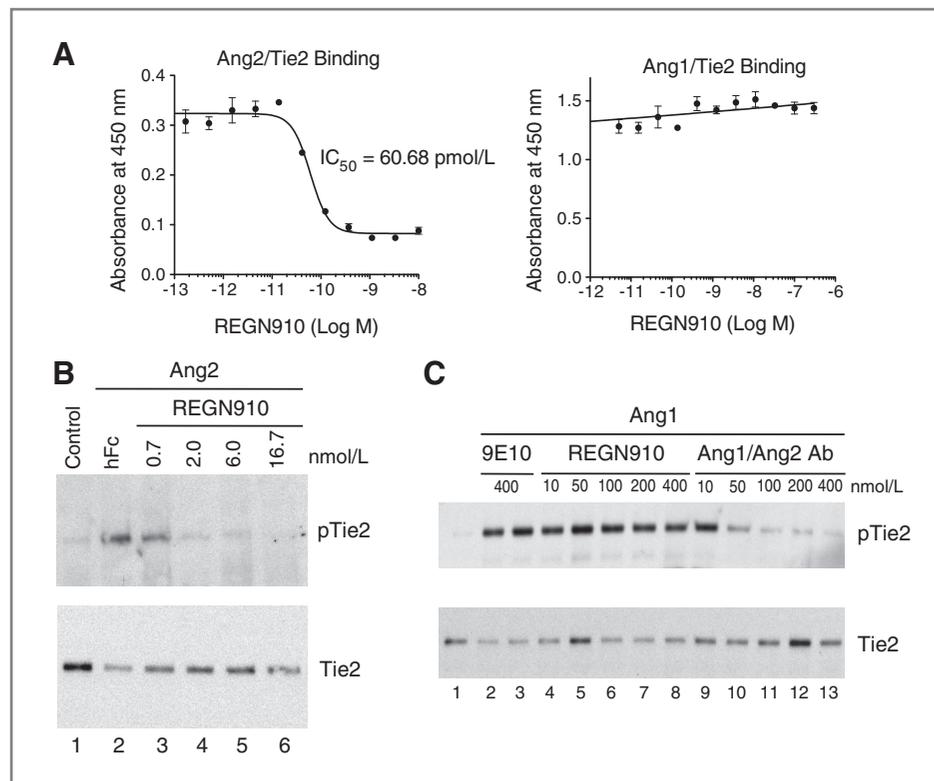
Human antibodies against Ang2 were generated as described in Materials and Methods. The lead antibody REGN910 (selected from more than 1,000 antigen-positive clones) exhibits high-affinity binding to human, monkey, and mouse Ang2 ( $K_D \sim 18$  pmol/L for human Ang2), but does not bind to Ang1 at all, as measured in SPR Biacore experiments (Supplementary Table S1). Furthermore, REGN910 blocked the binding of Ang2 to Tie2 with an  $IC_{50}$  of approximately 60 pmol/L, but had no effect on Ang1/Tie2 binding, even at a high antibody concentration (Fig. 1A). Thus, REGN910 is a potent and specific inhibitor of the Ang2/Tie2 interaction.

To confirm that REGN910 inhibits Ang2 signaling, we tested its ability to inhibit Ang2-dependent Tie2 phosphorylation in human umbilical vein endothelial cells (HUVEC) in which Ang2 expression is induced by the transcription factor FOXO1 (35). HUVECs expressing elevated levels of Ang2 exhibited a marked increase in Tie2 phosphorylation (Fig. 1B, lane 2), which was completely inhibited by the addition of REGN910 at concentrations of 2 nmol/L or above (Fig. 1B, lanes 4–6), confirming that REGN910 inhibits autocrine Ang2-mediated Tie2 activation. Consistent with its inability to bind Ang1, REGN910 had no effect on Ang1-dependent Tie2 phosphorylation (Fig. 1C).

### REGN910 inhibits tumor xenograft growth and decreases tumor vascularity

To assess the effect of REGN910 on tumor growth, Colo205 (colorectal), PC3 (prostate), and A431 (epidermoid) tumor xenografts were grown in immunocompromised mice. As shown in Supplementary Table S1, REGN910 binds to mouse Ang2 with high affinity and is therefore competent to bind to host endothelial cell-derived Ang2 in these xenograft models. Once tumors were established (100–200 mm<sup>3</sup>), mice were treated twice per week with human Fc control protein or with REGN910 at various doses. REGN910 significantly inhibited the growth of all 3 tumors, with maximal efficacy achieved with the 2.5 mg/kg dose (50%, 70%, and 54% inhibition of Colo205, PC3 and A431 tumor growth, respectively; Fig. 2A–C). Thus, REGN910 as a monotherapy significantly delays the growth of multiple tumor xenografts.

The inhibition of tumor growth by REGN910 was associated with a significant decrease in tumor vascularity (Fig. 2D), consistent with previous reports that Ang2 blockade inhibits



**Figure 1.** REGN910 is a potent and specific inhibitor of Ang2. **A**, REGN910 at the indicated concentrations was incubated with 0.6 nmol/L His-tagged human Ang2 or Ang1 for 1 hour at room temperature. The mixtures were then added to an ELISA plate coated with human Tie2. Bound Ang2 or Ang1 was detected with an HRP-conjugated anti-His tag antibody. **B**, HUVECs induced to express Ang2 (lanes 2–6) were treated with 50 nmol/L human Fc (lane 2) or REGN910 at concentrations ranging from 0.7 to 16.7 nmol/L (lanes 3–6). Tie2 was immunoprecipitated and Western blots were conducted to assess the levels of phosphorylated and total Tie2. **C**, EA.hy926 human endothelial cells were untreated (lane 1) or stimulated for 10 minutes with 0.5  $\mu\text{g/mL}$  of human Ang1 in the presence of 9E10 control antibody (lanes 2 and 3), REGN910 (lanes 4–8), or a dual Ang1/Ang2 blocking antibody (lanes 9–13) at concentrations ranging from 10 to 400 nmol/L. Tie2 was immunoprecipitated and Western blots were conducted to assess the levels of phosphorylated and total Tie2.

tumor angiogenesis (21–24, 28–31). To assess whether the decrease in tumor vascularity reflects increased endothelial cell apoptosis and/or decreased endothelial cell proliferation, we conducted immunohistochemistry to quantitate dual-positive CD31/TUNEL and CD31/BrdUrd pixels following REGN910 treatment. As shown in Supplementary Fig. S1, REGN910 treatment modestly increased endothelial cell apoptosis and decreased endothelial cell proliferation. While the changes observed at a single time point (10-day treatment) were not statistically significant (due to high intertumor variability), our data suggest that the decrease in tumor vascularity observed following Ang2 blockade might be attributable to modulation of both endothelial cell apoptosis and proliferation, consistent with earlier reports (22, 29, 31). Furthermore, consistent with previous studies (23, 26, 28), REGN910 treatment significantly increased the percentage of blood vessels that were closely associated with pericytes (Supplementary Fig. S2).

#### Ang2 functions as a Tie2 agonist in tumors

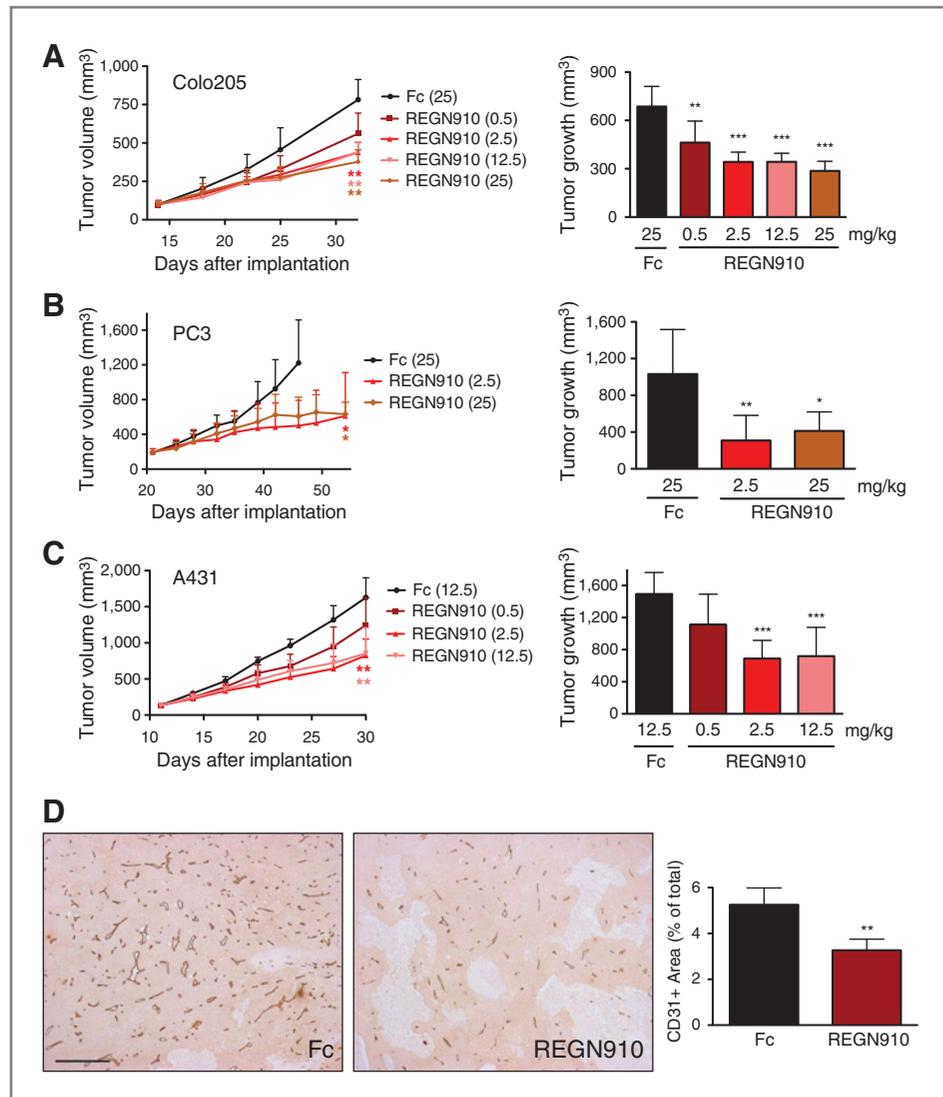
While a role for Ang2 in promoting tumor growth is well established, it remains unclear whether Ang2 functions as a Tie2 antagonist or agonist in tumors. If Ang2 is a Tie2 agonist, then REGN910 would decrease Tie2 activity, and the addition

of Ang1 should reverse the effect of REGN910 on tumor growth by restoring Tie2 signaling. On the other hand, if Ang2 is a Tie2 antagonist, then REGN910 would increase Tie2 activity and Ang1 would, if anything, enhance the effect of REGN910 on tumor growth.

To assess the effect of Ang1 on the ability of REGN910 to inhibit tumor growth, mice bearing Colo205 or A431 xenografts were treated systemically with REGN910, Ang1, or the combination of REGN910 plus Ang1. Systemic administration of Ang1 promotes rapid phosphorylation of Tie2 in mouse tissues (35) and modulates the expression of Tie2 target genes in tumors (see below). Ang1 completely reversed the inhibitory effect of REGN910 on tumor growth in both models (Fig. 3A, B), indicating that Ang2 functions as a Tie2 agonist in these settings. Ang1 did not by itself increase tumor growth versus Fc control treatment (Fig. 3A and B), presumably because the level of Tie2 phosphorylation in these tumors is already sufficiently high to support maximal tumor growth. Ang1 also blunted the decrease in tumor vascularity caused by REGN910 (Fig. 3C), indicating that Ang1-mediated rescue of tumor growth reflects an effect on the tumor vasculature.

To provide further support for the hypothesis that Ang2 is a Tie2 agonist in tumors, we investigated the effects of REGN910 on the expression of Tie2 target genes. Our previous work has

**Figure 2.** REGN910 inhibits tumor xenograft growth and reduces tumor vascularity. Mice bearing established Colo205 (A), PC3 (B), or A431 (C) tumors were treated twice per week with human Fc control protein or REGN910 at doses ranging from 0.5 to 25 mg/kg as indicated. The line graphs depict the average tumor volumes over the course of treatment. Error bars represent SD. Tumor growth curves were compared with the Fc control group by repeated measures one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). The bar graphs depict average tumor growth from the start of treatment through the end of the experiment. Error bars represent SD. Tumor growth in the treatment groups was compared with the Fc control group by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). D, mice-bearing established Colo205 tumors were treated with 15 mg/kg of human Fc control protein or REGN910 (mice received 2 injections over 7 days). Tumors were excised, sectioned, and stained with an antibody to CD31 (PECAM1) to visualize the blood vessels (left). The scale bar is 500  $\mu\text{m}$ . Whole tumor sections were analyzed with NIH Image to calculate the percentage of CD31-positive area (right). Values were compared by *t* test. Error bars represent SD (\*\*,  $P < 0.01$ ).



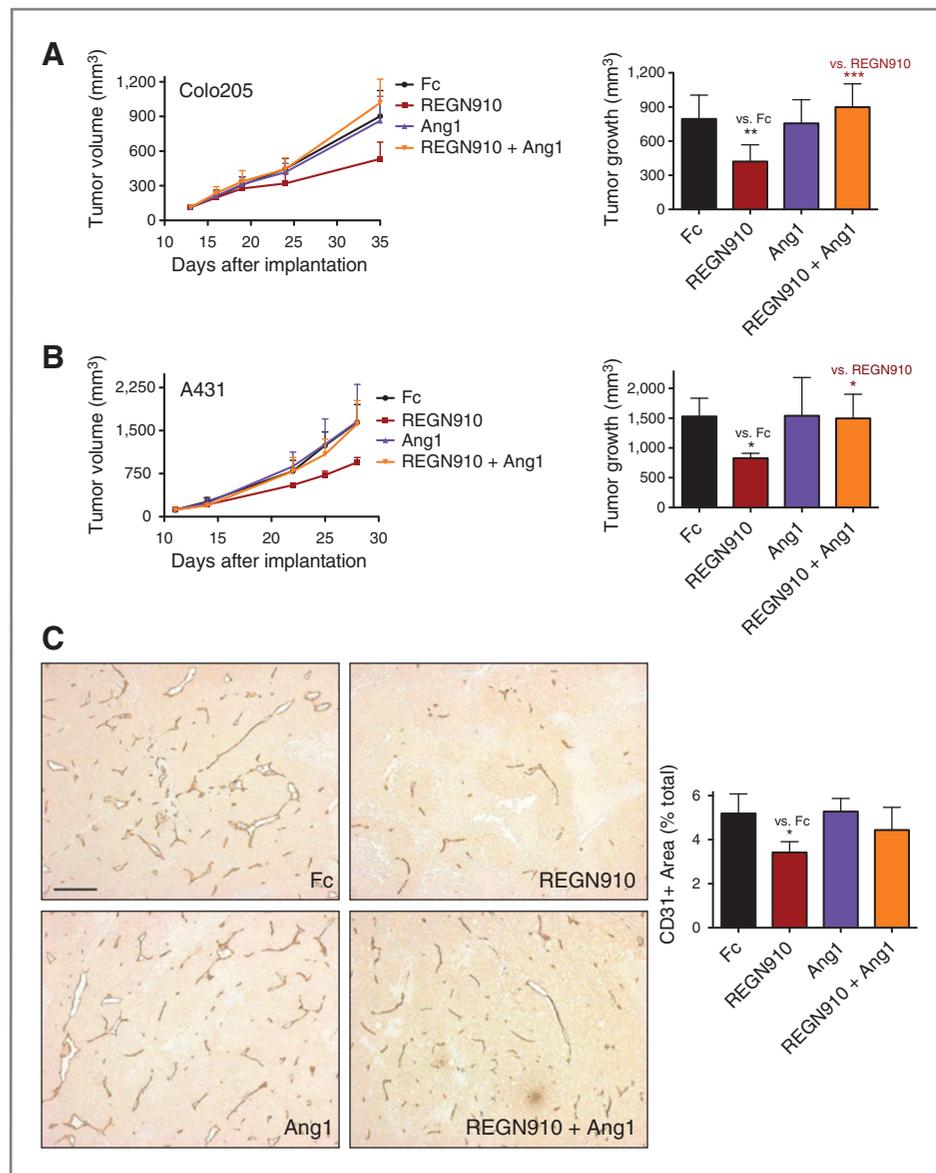
established that Tie2 regulates gene expression by strongly activating the Akt pathway, which results in inhibition of FOXO transcription factors (42). Three genes whose expression is significantly inhibited by Akt and induced by FOXO in endothelial cells are Ang2, endothelial cell-specific molecule 1 (ESM1), and placental growth factor (PlGF; ref. 42). To confirm that these genes are directly regulated by Tie2 activation, we assessed the effect of Ang1 on their expression. Treatment with Ang1 rapidly decreased the expression of these genes in cultured endothelial cells and/or in mouse tissues, confirming that these genes are directly regulated by Tie2 activation (Fig. 4A and B). Furthermore, microarray analysis of cultured endothelial cells revealed that PlGF and Ang2 are among the 10 genes most strongly repressed by Ang1 (data not shown), providing additional support for the contention that changes in the expression of these genes is a reliable surrogate measure for changes in Tie2 activity.

To determine the effects of REGN910, Ang1, or the combination of REGN910 plus Ang1 on the expression of Tie2 target

genes, mice bearing established A431 or PC3 tumors were treated for 72 hours and tumor lysates were subjected to TaqMan real-time PCR analysis. REGN910 treatment increased the expression of the host (mouse) Ang2, ESM1, and PlGF genes in tumors, indicating that REGN910 inhibits Tie2 signaling and by extension that Ang2 activates Tie2 (Fig. 4C and D). While treatment with Ang1 alone did not repress the expression of these genes versus control treatment (presumably because Tie2 is already strongly activated in these tumors), Ang1 completely reversed the increase in gene expression caused by REGN910 (Fig. 4C and D), confirming that the increased expression is due to decreased Tie2 signaling.

#### REGN910 potentiates the antitumor effects of aflibercept

While REGN910 provided significant inhibition of tumor growth in multiple xenograft models, the effects were relatively modest. Thus, we assessed the effect of combining REGN910 with aflibercept, a potent VEGF blocker (40). Aflibercept

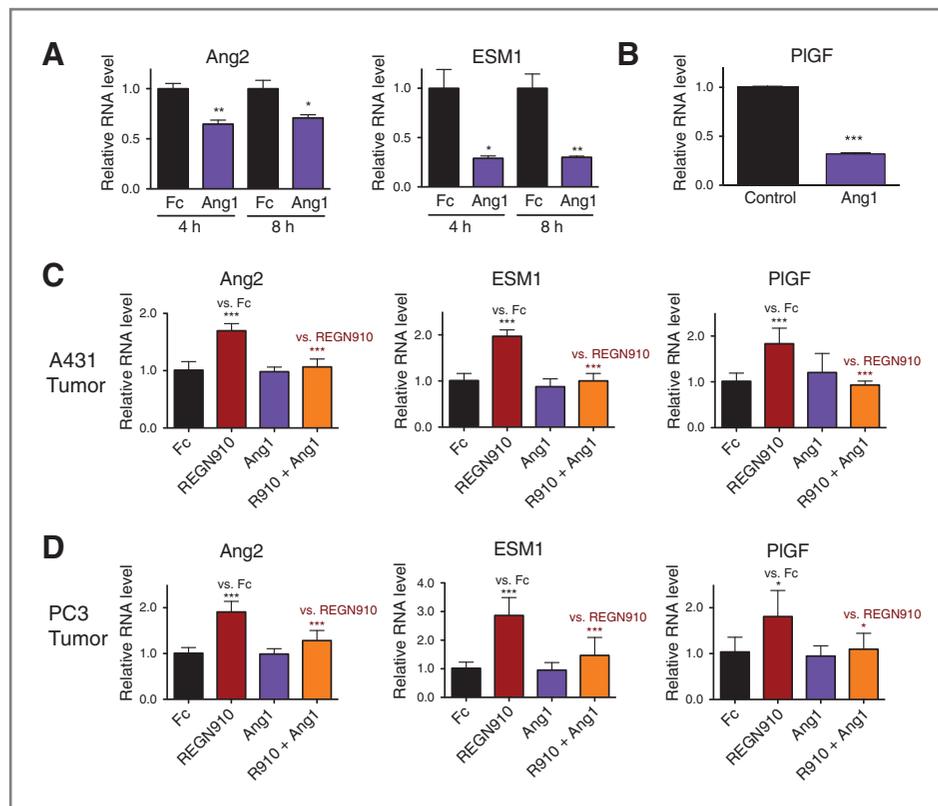


**Figure 3.** Ang1 reverses the effects of REGN910 on tumor growth and vascularity. Mice bearing established Colo205 (A) or A431 (B) tumors were treated twice per week with human Fc control protein (25 mg/kg), REGN910 (12.5 mg/kg), recombinant Ang1 (12.5 mg/kg), or the combination of REGN910 plus Ang1 (12.5 mg/kg each). The line graphs depict the average tumor volumes over the course of treatment. Error bars represent SD. The bar graphs depict average tumor growth from the start of treatment through the end of the experiment. Error bars represent SD. Tumor growth in the treatment groups was compared by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). C, Colo205 tumors treated as described above were excised, sectioned, and stained with an antibody to CD31 to visualize the blood vessels (left). The scale bar is 200  $\mu$ m. Whole tumor sections were analyzed with NIH Image to calculate the percentage of CD31-positive area (right). Values were compared by one-way ANOVA with Tukey multiple comparison test. Error bars represent SD (\*,  $P < 0.05$ ).

exhibits high affinity binding to all isoforms of VEGF-A as well as VEGF-B and PlGF (40, 43). In addition, aflibercept binds to both human and mouse VEGF, thereby providing complete blockade of VEGF in mouse tumor models (40). Colo205, PC3, and MMT (mammary) tumors were grown to approximately 400 mm<sup>3</sup> and then treated with human Fc, REGN910, aflibercept, or the combination of REGN910 plus aflibercept at doses that provide maximal efficacy. REGN910 as a single agent significantly reduced the growth of Colo205 and PC3 tumors, but had little effect on MMT growth (Fig. 5A–C). While aflibercept inhibited the growth of all 3 tumors, the combination of REGN910 plus aflibercept was significantly more efficacious than either single agent in all 3 models (Fig. 5A–C). For example, Colo205 tumors shrank by 35% following treatment with aflibercept alone and by 65% following combination treatment (Fig. 5A). Importantly, the

combination treatment was superior to 25 mg/kg of either single agent in the MMT model, doses that we show here to be saturating (Fig. 5C). Thus, blockade of Ang2 provides additional antitumor benefit under conditions where VEGF is maximally inhibited.

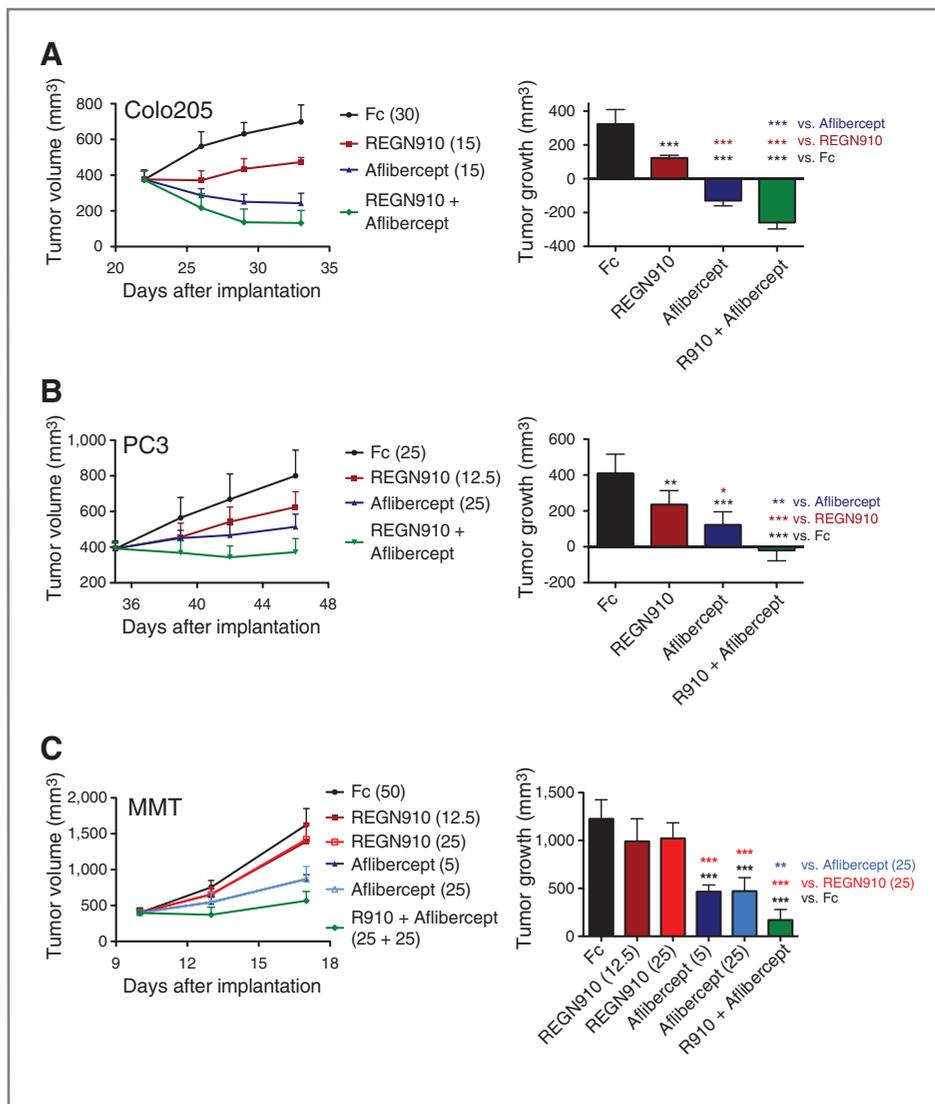
Consistent with its more potent effect on tumor growth, combination treatment with REGN910 plus aflibercept decreased Colo205 tumor vascularity to a greater extent than either single agent (Fig. 6A, left). At 72 hours following a single administration of REGN910, aflibercept or the combination, CD31-positive area was reduced by 23%, 29% and 50%, respectively, versus the Fc control (Fig. 6A, right). These findings suggest that the more pronounced inhibition of tumor growth provided by the combination treatment versus the single agents is attributable to a more dramatic decrease in tumor vascularity.



**Figure 4.** REGN910 regulates the expression of Tie2 target genes in tumors. **A**, mice were injected via the tail vein with 8 mg/kg of human Fc control protein or Ang1. Hearts were excised at 4 or 8 hours after treatment, RNA was isolated, and the levels of Ang2 and ESM1 RNA (normalized to GAPDH) were determined by TaqMan real-time PCR. The bar graphs depict the relative Ang2 or ESM1 RNA levels versus the Fc control group (assigned a value of 1.0). Error bars represent SD. The differences in Ang2 and ESM1 RNA levels between the Fc- and Ang1-treated groups were statistically significant at both 4 and 8 hours by *t* test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). **B**, cultured rat heart microvessel endothelial cells were either untreated or treated with Ang1 for 2 hours. RNA was isolated and the level of PIGF RNA (normalized to GAPDH) was determined by TaqMan real-time PCR. The bar graph depicts the relative PIGF RNA level versus the control group (assigned a value of 1.0). Error bars represent SD. The difference in PIGF RNA level between the untreated and Ang1-treated cells was statistically significant by *t* test (\*\*\*,  $P < 0.001$ ). **C** and **D**, mice-bearing established A431 or PC3 tumors were injected with human Fc control protein (25 mg/kg), REGN910 (15 mg/kg), Ang1 (25 mg/kg), or REGN910 plus Ang1. At 72 hours after treatment, tumors were harvested and RNA was prepared. The levels of mouse Ang2, ESM1, and PIGF RNAs (normalized to mouse Robo4 RNA level) were determined by TaqMan real-time PCR. The bar graphs depict the relative Ang2, ESM1, or PIGF RNA levels versus the Fc control group (assigned a value of 1.0). Error bars represent SD. Treatment groups were compared by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

To test the effects of the REGN910 plus aflibercept combination on tumor perfusion, we used contrast-enhanced microultrasound imaging. Mice-bearing Colo205 tumors were given a single injection of Fc control protein, REGN910, aflibercept, or the combination of REGN910 plus aflibercept, and perfusion was assessed 24 hours later. While REGN910 and aflibercept as single agents caused only modest decreases in tumor perfusion at this early time point, the combination of REGN910 plus aflibercept had a substantially greater effect, causing a 70% reduction in perfusion (Fig. 6B, left). Importantly, none of the treatments had an effect on blood vessel perfusion in adjacent normal skin (Fig. 6B, right), indicating that the actively remodeling blood vessels in tumors are preferentially dependent on Ang2 and VEGF. The more potent effect of the REGN910 plus aflibercept combination on tumor perfusion is consistent with the more pronounced decrease in tumor vascularity that is observed following combination treatment as compared with the single-agent treatments.

Finally, to assess the effect of decreased vascularity and perfusion on tumor cells, we used the TUNEL assay to assess tumor cell death 72 hours after treatment. While both REGN910 and aflibercept as single agents caused an increase in tumor cell death versus control, the combination treatment had a much more dramatic effect, promoting extensive tumor cell death (Fig. 6C, left). On average, approximately 75% of the tumor area in the combination treatment group was TUNEL positive (Fig. 6C, right; see Supplementary Fig. S3 for images of whole tumor sections that highlight the effect of the combination treatment on tumor cell death). Thus, the more potent effects of the REGN910 plus aflibercept combination on tumor vascularity and tumor perfusion translate into a greater induction of tumor cell death, consistent with the rapid tumor regression observed in the Colo205 model in response to the combination treatment (Fig. 5A). Interestingly, at an earlier time point (24 hours), we were able to detect an increase in endothelial cell death in tumors treated with the combination (Supplementary Fig. S4). While the overall percentage of dead



**Figure 5.** REGN910 plus aflibercept combination treatment inhibits tumor growth more potently than either single agent. Mice bearing established Colo205 (A), PC3 (B), or MMT (C) tumors (~ 400 mm<sup>3</sup>) were treated with the indicated doses of human Fc control protein, REGN910, aflibercept, or the combination of REGN910 plus aflibercept. Mice were treated 3 times over 11 days (Colo205 and PC3) or 2 times over 7 days (MMT). The line graphs depict the average tumor volumes over the course of treatment. The bar graphs depict average tumor growth from the start of treatment through the end of the experiment. Error bars represent SD. Tumor growth in the treatment groups was compared by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

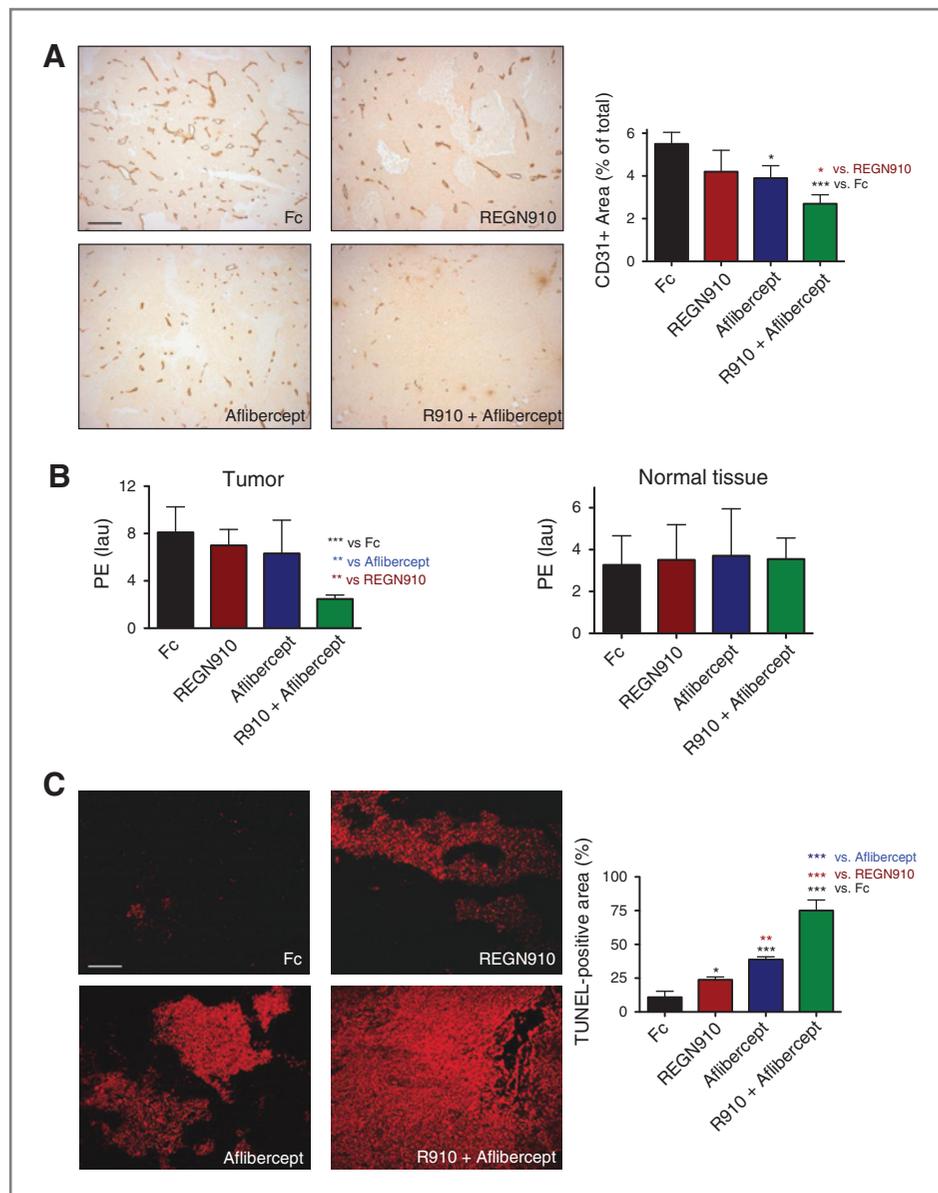
endothelial cells detected at this single time point was quite low (< 1% of the total endothelial cells), this finding suggests the possibility that the more potent effect of the combination treatment on tumor vascularity is at least partly due to increased endothelial cell death. Together, our results indicate that both Ang2 and VEGF significantly contribute to tumor angiogenesis and that combined blockade of both factors results in more potent inhibition of tumor vascular function and of tumor growth than blockade of either factor individually.

## Discussion

In this article, we used a novel, fully human Ang2 antibody, REGN910, to investigate the mechanism of Ang2 signaling in tumors. The dominant model for Ang2 function in tumors proposes that Ang2 inhibits Tie2 signaling and destabilizes tumor blood vessels, thereby facilitating VEGF-dependent angiogenesis (1, 7, 13). However, in this study, we show that systemic Ang1 reverses the effects of REGN910 on tumor

growth and vascularity and that REGN910 increases the expression of genes that are repressed by Tie2 activation. These findings strongly suggest that REGN910 decreases Tie2 signaling in tumors and, therefore, that Ang2 functions as a Tie2 agonist.

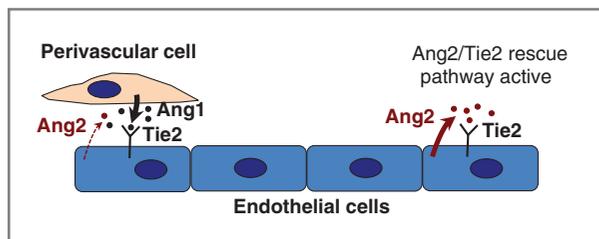
Our previous work established that Ang2 expression is induced in cultured endothelial cells by FOXO transcription factors in response to low Akt activity, and that in this setting, Ang2 activates Tie2 (35, 42). Those findings suggested that Ang2 induction is an adaptive mechanism to restore Tie2/Akt signaling. On the basis of the current study, we propose that in tumors, Ang2 functions to activate Tie2, particularly in endothelial cells that are not exposed to significant levels of pericyte-derived Ang1 (likely the majority of tumor endothelial cells; see Fig. 7 for a model). Thus, in our model, Ang2 expression is induced not to inhibit Ang1/Tie2 signaling, but rather to compensate for weak Ang1 signaling by restoring Tie2 activity and promoting the survival and/or proliferation of tumor endothelial cells. This model is consistent with reports



**Figure 6.** REGN910 plus aflibercept combination treatment potently decreases tumor vascularity and perfusion and promotes extensive tumor cell death. **A**, mice-bearing established Colo205 tumors (~400 mm<sup>3</sup>) were treated with Fc control protein (30 mg/kg), REGN910 (15 mg/kg), aflibercept (15 mg/kg), or the combination of REGN910 plus aflibercept (15 mg/kg each). At 72 hours after treatment, tumors were excised, sectioned, and stained with an antibody to CD31 to visualize the tumor vasculature (left). The scale bar is 200  $\mu$ m. Whole tumor sections were analyzed with NIH Image to calculate the percentage of CD31-positive area (right). Error bars represent SD. Values were compared by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ). **B**, mice bearing established Colo205 tumors (~100 mm<sup>3</sup>) were given a single injection of human Fc control protein (25 mg/kg), REGN910 (10 mg/kg), aflibercept (25 mg/kg), or the combination of REGN910 plus aflibercept (10 + 25 mg/kg). At 24 hours after treatment, tumor perfusion (and perfusion in adjacent normal skin) was assessed as described in Materials and Methods. The bar graphs depict the relative perfusion. Error bars represent SD. Treatment groups were compared by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). **C**, mice bearing established Colo205 tumors (~400 mm<sup>3</sup>) were treated with Fc control protein (30 mg/kg), REGN910 (15 mg/kg), aflibercept (15 mg/kg), or the combination of REGN910 plus aflibercept (15 mg/kg each). At 72 hours after treatment, tumors were excised, sectioned, and subjected to TUNEL staining to visualize tumor cell death (left). Scale bar, 200  $\mu$ m. To determine the TUNEL-positive area (right), tumor sections ( $n = 4$  per group) were imaged in their entirety. Image J was used to calculate the percentage of TUNEL-positive area. Error bars represent SD. Treatment groups were compared by one-way ANOVA with Tukey multiple comparison test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

indicating that Ang2 blockade results in decreased proliferation of tumor endothelial cells (22), decreased numbers of endothelial sprouts (23, 28), and increased vessel regression (29, 31).

Although our data show that the overall effect of Ang2 blockade in tumors is a decrease in Tie2 activity, some of the morphologic effects of Ang2 inhibition (e.g., increased pericyte association, endothelial junctional maturation; ref. 28) are



**Figure 7.** Model for the role of Ang2 signaling in tumor angiogenesis. The model proposes that Ang2 functions to activate Tie2 signaling, particularly in endothelial cells that are not exposed to significant levels of pericyte-derived Ang1 (likely the majority of tumor endothelial cells). Thus, Ang2 expression is induced to compensate for weak Ang1 signaling rather than to inhibit Ang1 as the original model proposed. In endothelial cells that are associated with pericytes, Ang1 activates Tie2 and may repress Ang2 expression via activation of the Akt pathway and inhibition of FOXO transcription factors. Important roles for Ang2/Tie2 signaling in tumors may be to maintain the caliber of blood vessels and to promote the proliferation and/or survival of endothelial cells. See Discussion for further details.

likely to reflect an increase in Tie2 signaling in a subset of endothelial cells. One potential explanation for this apparent discrepancy is that in the subset of tumor endothelial cells exposed to both Ang2 and to pericyte-derived Ang1, specific blockade of the weaker agonist Ang2 would lead to greater Tie2 activity (Fig. 7). This subset of endothelial cells may represent a relatively small percentage of the total, as published reports (23, 26, 28) and the data in Supplementary Fig. S2 suggest that only approximately 25% of the vessels in tumor xenografts are closely associated with pericytes. Thus, the increased pericyte association observed following Ang2 blockade is not necessarily inconsistent with the fact that the overall effect of Ang2 blockade is a decrease in Tie2 signaling.

Interestingly, a recent report suggests the possibility that Ang2 can promote angiogenesis via binding to integrins on endothelial cells that express low levels of Tie2 (44). However, we believe that in our tumor models, the inhibition of tumor growth/angiogenesis that results from Ang2 blockade is attributable to decreased Tie2 activity, as these effects are reversed by the Tie2 agonist Ang1 and they are accompanied by changes in expression of well-established Tie2 target genes.

The fact that systemic delivery of exogenous Ang1 reverses the tumor growth inhibition caused by REGN910 suggests the possibility that Ang1, if present at high levels, might promote resistance of tumors to Ang2-specific blockers. However, published reports have shown that an Ang1-specific blocker has no effect on tumor growth (22, 28) and that dual Ang1/Ang2 blockade provides, at best, a marginal and inconsistent additional benefit over specific Ang2 blockade in one tumor xenograft model (Colo205; refs. 22, 28). Thus, the currently available data suggest that endogenous tumor Ang1 does not compensate in a significant way for the inhibition of Ang2.

While Ang2/Tie2 signaling has been mostly studied in endothelial cells, recent work has identified a population of monocytes that express Tie2 (45). These cells, known as Tie2-expressing monocytes (TEM), have been implicated in the regulation of tumor angiogenesis, and Ang2 has been suggested to stimulate the proangiogenic activity of TEMs (46).

Antibody-mediated blockade of Ang2, while not inhibiting TEM infiltration into tumors, seems to inhibit the association of TEMs with tumor blood vessels, potentially limiting their ability to stimulate angiogenesis (31). While conditional knock-down of Tie2 expression in TEMs inhibits tumor angiogenesis (but not tumor growth), suggesting that TEMs do contribute to tumor angiogenesis in a Tie2-dependent manner (31), it remains to be established that the effects of Ang2 blockade on tumor growth are attributable to some degree to the modulation of TEM function.

While REGN910 alone has a fairly modest effect on tumor growth, we show that combination treatment with maximally efficacious doses of REGN910 plus aflibercept results in much more dramatic decreases in tumor vascularity and tumor growth than treatment with either single agent. Importantly, as aflibercept specifically and potently inhibits both mouse and human VEGF (40), our data clearly show that blockade of Ang2 provides additional antitumor benefit under conditions of maximal VEGF blockade. While the precise mechanisms underlying the cooperative effects of REGN910 and aflibercept are not known, one possibility is that Ang2/Tie2 signaling increases the survival of endothelial cells, thereby limiting the ability of aflibercept to promote regression of tumor vessels. Our observations are consistent with a recent report showing that dual blockade of Ang2 and VEGF causes a more pronounced regression of tumor vessels than blockade of either pathway alone (23), and with a study showing that activation of Tie2 by systemic Ang1 delivery impairs the ability of aflibercept to promote tumor xenograft regression (47). Intriguingly, a recent report shows that high serum Ang2 levels are associated with a worse response to the anti-VEGF antibody bevacizumab in patients with colorectal cancer (48). While this finding is preliminary, it suggests the possibility that patients with high circulating Ang2 levels might be less responsive to anti-VEGF treatments and might therefore benefit from the combined blockade of Ang2 and VEGF.

REGN910 is now in a phase I trial (NCT01271972) for patients with advanced solid malignancies. The findings presented in this study suggest that specific blockade of Ang2 with REGN910 could enhance the clinical benefits provided by anti-VEGF therapies.

#### Disclosure of Potential Conflicts of Interest

C. Daly and G. Thurston have ownership interest (including patents) in patent application. No potential conflicts of interest were disclosed by the other authors.

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## Angiopoietin-2 Functions as a Tie2 Agonist in Tumor Models, Where It Limits the Effects of VEGF Inhibition

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