The concept that tumors can be controlled by directly targeting their vascular supply has finally come of age, because clinical trials using a humanized monoclonal antibody that blocks VEGF have demonstrated exciting efficacy in cancer patients, as well as in vascular eye diseases that can lead to blindness. However, data suggest that these current regimens may not provide complete VEGF inhibition and, thus, that the maximum therapeutic potential of VEGF blockade has not yet been achieved. We describe the status of a very potent and high-affinity VEGF blocker, termed the VEGF Trap, that may provide the opportunity to maximize the potential of VEGF blockade in cancer as well as in vascular eye diseases. We also describe use of the VEGF Trap as a research tool, when coupled to high-throughput mouse genetics approaches such as *VelociGene*® that can be exploited in strategies to discover and validate the next generation of angiogenesis targets.

The concept that tumors can be controlled by directly targeting their vascular supply has finally come of age. The first antiangiogenesis approach to be validated in human cancer patients involves blocking vascular endothelial growth factor (VEGF-A). In this regard, the most advanced clinical data have been generated with a humanized monoclonal antibody termed bevacizumab (Avastin) that directly binds and blocks all isoforms of VEGF-A (Ferrara et al. 2004). Despite the promising data achieved to date, dose-response studies suggest that higher doses of bevacizumab may provide even greater benefit (Yang et al. 2003; Yang 2004), implying that current bevacizumab regimens may not provide optimal VEGF inhibition and thus may not have yet demonstrated the maximum potential of VEGF blockade in cancer. In addition to the promise of anti-VEGF approaches in cancer, blocking VEGF-A has also been impressive in maintaining and improving vision in wet age-related macular degeneration (AMD), a disease marked by leaky and proliferating vessels which distort the retina, and these data suggest that VEGF blockade may provide benefit in other eye diseases involving vascular leak and proliferation (Bergsland 2004). Efficacy in wet AMD has most notably been achieved using a modified fragment of the bevacizumab antibody, termed ranibizumab (Lucentis), delivered via monthly intraocular injections (Brown et al. 2006; Heier et al. 2006).

In this paper, we focus on the development and status of a novel VEGF-blocking agent, termed the VEGF Trap, that retains many of the advantages of a blocking antibody but may offer further potential (Holash et al. 2002). The VEGF Trap consists of portions of VEGF receptors that have been fused to the constant region of an antibody, resulting in a fully human biologic with exceedingly high affinity that blocks not only all isoforms of VEGF-A, but also related VEGF family members such as placental growth factor (PlGF). The VEGF Trap also display tended pharmacological half-life, allowing long-te well as very high affinity blockade. The VEGF Tra performed impressively in extensive animal studi cancer and eye diseases, and initial clinical trials a promising. The VEGF Trap may provide the opport to explore the potential of more complete VEGF b ade in cancer, as well as the opportunity for more plete blockade and even longer-interval dosing regi in eye diseases. To conclude this paper, we describe the VEGF Trap can be used as a research tool in effo discover and validate the next generation of targets field of angiogenesis.

DISCOVERY OF VEGF AND ITS REQUISIT ROLES DURING NORMAL DEVELOPMEN AND IN DISEASE SETTINGS

Initial studies by Dvorak and his colleagues (Sen al. 1986; Dvorak et al. 1999) identified a protein in t ascites fluid that was capable of inducing vascular lea permeability, which they termed vascular permeabilit tor (VPF). Independent efforts by Ferrara and his leagues to identify secreted factors that could pro tumor angiogenesis led to the discovery of a prote bovine pituitary follicular cell conditioned medium mitogenic properties for endothelial cells which termed vascular endothelial cell growth factor (V (Ferrara and Henzel 1989; Leung et al. 1989). Upo quencing and further studies, this VEGF protein was pectedly found to correspond to the VPF previously tified by the Dvorak lab. These findings set the stage concerted effort to define the role of VEGF/VPF (h VEGF) in cancer angiogenesis as well as other settin vascular disease, which have led to the realization tha of its initially realized actions—i.e., promoting vas

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stages of blood vessel development. Still more remarkably, disruption of even a single VEGF allele in developing mice, which decreases VEGF levels by half, also results in embryonic lethality due to severe vascular abnormalities (Carmeliet et al. 1996; Ferrara et al. 1996), demonstrating the need for exquisite regulation of VEGF levels to form normal vessels. Reciprocally, modest increases in VEGF levels during development also lead to vascular disaster and lethality (Miquerol et al. 2000). VEGF continues to be critical during early postnatal growth and development, as evidenced by the lethality and major growth disturbances caused by conditional disruption of the VEGF gene or by administration of VEGF blockers (Ravindranath et al. 1992; Carmeliet et al. 1996; Ferrara et al. 1996, 1998; Gerber et al. 1999a; Ryan et al. 1999; Fraser et al. 2000; Zimmermann et al. 2001; Hazzard et al. 2002; Eremina et al. 2003). However, VEGF blockade in older animals is much less traumatic, affecting only those structures that continue to depend on ongoing vascular remodeling, such as occurs in bone growth plates or during remodeling of the female reproductive organs (Ferrara et al. 1998; Gerber et al. 1999a,b). As discussed in greater detail below, vascular remodeling is absolutely required in a variety of pathological settings, such as during tumor growth, providing major therapeutic opportunities for VEGF blockade in the adult setting in which such blockade can be tolerated.

VEGF ISOFORMS, VEGF FAMILY MEMBERS, AND VEGF RECEPTORS

Further study of the gene encoding human VEGF revealed eight exons separated by seven introns, which results in the generation of four isoforms of increasing size—$VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (subscripts refer to number of amino acids comprising the isoform, with the VEGF isoforms varying in length at their carboxyl termini). The main purpose of these isoforms appears to relate to their bioavailability such that the 121 isoform is diffusible, whereas the higher-molecular-weight isoforms remain bound to the extracellular matrix, requiring cleavage to be released (Houck et al. 1992; Park et al. 1993; Keyt et al. 1996).

Because of the discovery of additional members of the VEGF family, VEGF is now often referred to as VEGF-A. Other members of the VEGF family were

with VEGF blockade (Persico et al. 1999; Ca
2000). Little is known about VEGF-B, and mice
VEGF-B are overtly healthy and fertile. VEGF-
seem to play more critical roles in the lymphati
lature than in the blood vasculature, showing sp
for a VEGF receptor (see below) expressed on t
culature; administration of both of these factors
lymphatic vessel hyperplasia (Joukov et al. 1996
dini et al. 1996; Olofsson et al. 1999).

Following rapidly on the heels of the disco
VEGF came the identification of two closely
high-affinity receptors for VEGF—FLT1 (FMS
rosine kinase) now termed VEGFR1 (de Vrie
1992), and KDR or Flk1, now termed VEGFR2 (
et al. 1990; Terman et al. 1992; Millauer et al
These high-affinity receptors share features o
other growth factor receptors, in that they contai
tracellular domain which binds and is dimerized
and, and a cytoplasmic tyrosine kinase domain tha
regulated upon binding of ligand to the extracell
main. VEGFR2 seems to be the receptor which r
the major growth and permeability actions of
whereas VEGFR1 may have a negative role, e
acting as a decoy receptor or by suppressing s
through VEGFR2. Thus, mice engineered
VEGFR2 fail to develop a vasculature and have v
endothelial cells (Shalaby et al. 1995), pheno
mice lacking VEGF, whereas mice lacking V
seem to have excess formation of endothelial c
abnormally coalesce into disorganized tubules (
al. 1995). Mice engineered to express only a t
form of VEGFR1, lacking its kinase domain,
rather normal, consistent with the notion that the
role of VEGFR1 may be that of a decoy recepto
suka et al. 1998), and supporting only a minor rol
cytoplasmic kinase domain. The third member o
ceptor family, initially called Flt-4 and now
VEGFR3, does not bind to VEGF-A nor PlGF,
stead binds to VEGF-C and VEGF-D and seems
ate the actions of these latter two factors on the ly
vasculature (Taipale et al. 1999).

In addition to these primary receptors, a numbe
tential accessory receptors for the VEGFs ha
identified, although the requisite roles of these r
in mediating VEGF responses have not been clea
cidated. These potential accessory receptors inc
neuropilins (Soker et al. 1998).

1997; Ellis et al. 1998; Tomisawa et al. 1999). However, out of these studies came the interesting finding that one tumor type, renal cell carcinoma, had particularly high VEGF expression which correlated with inactivation of the von Hippel Lindau locus, resulting in loss of control of the tumor's oxygen sensor, hypoxia-inducible factor (HIF) (Iliopoulos et al. 1996; Lonser et al. 2003). The upregulation of VEGF in an attempt to reoxygenate the tumor through revascularization led to the belief that this tumor may either be highly sensitive to anti-VEGF therapy or highly refractory. Fortunately, the former seems to be the case (Yang et al. 2003).

Concomitant with the analysis of human tumors for VEGF expression came the development of animal models of cancer where the hypothesis that VEGF was required for tumor vasculature, and thus tumor growth, could be tested. In 1993, 4 years after their discovery of VEGF, Ferrara and colleagues demonstrated that a mouse monoclonal antibody to human VEGF (A.4.6.1) could inhibit the growth of several human tumor types in nude mice with inhibition ranging from 70% to more than 90% (Kim et al. 1993). Subsequent to this observation, a number of laboratories using different strategies to inhibit VEGF signaling have shown to a greater or lesser extent that inhibition of VEGF can have a major impact on tumor growth in mice. In addition to numerous studies using the VEGF-blocking antibody, other strategies to block VEGF in tumor models included blocking antibodies targeting VEGFR2 (Prewett et al. 1999), soluble VEGF receptors acting as circulating decoys to capture VEGF and preventing it from binding cell-surface receptors (Ferrara et al. 1998; Gerber et al. 1999a,b; Liang et al. 2006), dominant-negative VEGF receptors expressed at high levels on tumor surfaces, small-molecule inhibitors of VEGF receptor kinases and other kinases (Smith et al. 2004), antisense oligonucleotides targeting VEGF, and VEGF siRNA (Grunweller and Hartmann 2005; Lu et al. 2005).

As the number of studies increased comparing the different modes of inhibiting VEGF, it became apparent that blocking tumor-derived VEGF without blocking stromal VEGF was not as efficacious, implicating stromal VEGF as a crucial player in tumor growth and angiogenesis. Thus, antibodies such as A.4.6.1 which only block human VEGF did not fare as well in blocking human tumor growth in immunocompromised mice as reagents blocking both tumor and host stroma-derived VEGF (Gerber et al. 2000; Liang et al. 2006).

with 5–20 picomolar binding affinity for VEGF
tumor experiments this VEGFR1-Fc reagent was
cious at approximately 500-fold lower concen
than a similar VEGFR2-Fc construct (Kuo et al.
Despite its high affinity, the VEGFR1-Fc was no
sible clinical candidate because of its poor pha
kinetic profile; in rodent studies, this protein ha
administered frequently and at very high d
achieve efficacious levels. In addition, this ag
peared to have nonspecific toxicity effects that
seem to be accounted for by its blocking of VEG
et al. 2001). We decided to exploit our Trap tech
platform (Economides et al. 2003), which in
defining and fusing minimal binding units from
ent receptor components to generate chimeric
proteins that act as high-affinity soluble blocker
attempt to create a potent and well-behaved T
VEGF. The result was a chimeric fusion prote
taining a modified domain 2 of VEGFR1 and th
Ig domain of VEGFR2 fused to the Fc region of
IgG1, resulting in a fully human protein that w
VEGF Trap (Holash et al. 2002). This reagent
advantage of being fully human and thus pot
non-immunogenic, as well as being substa
smaller than previous fusion proteins and anti
raising the possibility that it might allow impro
sue and tumor penetration. In addition, this VEC
had greatly improved pharmacological bioavai
as compared to the initial VEGFR1-Fc reagent, e
ing about a 300-fold increase in the maximum c
tration achieved in the circulation (i.e., C_{max}), as
about a 1000-fold increase in total circulation ex
(i.e., AUC) (Holash et al. 2002). Importantly, th
ity of VEGF Trap binding to both mouse and
VEGF isoforms (0.58 pM, 0.46 pM) was superior
of the parental VEGFR1-Fc (~ 5–20 pM) (Holas
2002). In addition, the VEGF Trap also boun
with high affinity (1.8 pM).

To determine whether the improved pharmaco
bioavailabity and high-affinity binding of VEG
translated into superior performance in vivo, v
used a short-term and quantitative in vivo m
VEGF activity in which a single dose of VEGF
a stereotypic reduction in blood pressure. In thi
assay model, we found that equivalent doses of
Trap were indeed far superior to that of the p
VEGFR1-Fc (Holash et al. 2002).

many different investigators, with impressive results in almost every case. In addition to its activity in multiple subcutaneous models of melanoma, glioma, and rhabdomyosarcoma tumors (Holash et al. 2002), the VEGF Trap has been shown to work in multiple pancreatic cancer models (Fukasawa and Korc 2004), Wilms' tumor (Huang et al. 2003), Ewing's sarcoma (Dalal et al. 2005), glioblastoma (Wachsberger et al. 2005), and models of ovarian cancer as well as associated malignant ascites (Hu et al. 2005).

In addition to the above published studies, recent unpublished temporal studies indicate that vascular regression can be seen in most tumors within hours of VEGF Trap treatment, resulting in marked and widespread hypoxia within the tumors. In addition, transcription profiling studies during these temporal studies have revealed a set of endothelial-specific genes that are rapidly and profoundly regulated in response to VEGF Trap treatment. Further studies on some of these genes have led to their identification as potential targets for new antiangiogenesis therapies (see below).

In summary, animal tumor studies have indicated that treatment with VEGF Trap effectively inhibited tumor growth of a wide variety of murine, rat, and human tumor cell lines implanted either subcutaneously or orthotopically in mice. VEGF Trap treatment inhibited the growth of tumors representing a variety of tumor types, including melanoma, glioma, rhabdomyosarcoma, ovarian, pancreatic, renal, and mammary tumor tissue, with a broad therapeutic index. Growth of small established tumors was also inhibited. Histological analysis indicated that treatment with VEGF Trap resulted in the formation of largely avascular and necrotic tumors, demonstrating that tumor-induced angiogenesis was blocked. VEGF Trap was also active in blocking tumor growth in similar animal tumor models in combination with paclitaxel, docetaxel, or radiation, and was synergistic with 5-fluorouracil. VEGF Trap as a single agent and in combination with paclitaxel also prevented the formation of ascites in mouse tumor models (Byrne et al. 2003; Hu et al. 2005).

VEGF TRAP IN CLINICAL TRIALS FOR CANCER

The above results in animal tumor models supported the exploration of the VEGF Trap in human studies. Initial clinical studies are promising (Dupont et al. 2005;

VEGF TRAP EXHIBITS IMPRESSIV EFFICACY IN PRECLINICAL MODELS VASCULAR EYE DISEASES

In addition to the role for VEGF in tumor angio a variety of studies have indicated that VEGF ma key pathological role in vascular eye diseases, in lar in diabetic edema and retinopathy settings, and related macular degeneration (AMD), which are causes of vision loss and blindness. In these disea cess VEGF is thought to result in vascular leak t tributes to abnormal swelling of the retina and r vision impairment, as well as in the abnormal gr choroidal and retinal vessels that can destroy norr nal architecture. Consistent with these possibili VEGF Trap has demonstrated impressive effica assortment of animal models of these eye disease

Preclinical studies in rodents have shown tha Trap can inhibit choroidal (Saishin et al. 20 corneal (Wiegand et al. 2003) neovascularization as suppress vascular leak into the retina (Qau 2001), and that the VEGF Trap can also promote vival of corneal transplants by inhibiting associa vascularization (Cursiefen et al. 2004). In additi primate model of AMD, in which choroidal neo lesions and vascular leak are induced by using a create small lesions in the retinas of adult cyno macaques, both systemically and intravitreally d VEGF Trap not only prevented development of leak and neovascular membranes when admi prior to laser lesion, but also induced regression w ministered after lesions had developed (Wiegar 2005). These preclinical results support a role fo blockade, and in particular for local delivery of th Trap, in multiple vascular eye diseases rangi AMD and diabetic eye diseases to corneal inj transplantation.

VEGF TRAP IN CLINICAL TRIALS FO VASCULAR EYE DISEASES

The above results in animal models have suppo exploration of the VEGF Trap in human studies o lar eye diseases. Initial clinical studies in human suffering from both AMD and diabetic ede retinopathy appear quite promising, with evid early trials that the VEGF Trap can rapidly and sively decrease retinal swelling, and that these marked regression and/or very long term stabilization, other tumors can continue to grow even in the face of anti-VEGF treatments. The realization that some tumors can be relatively resistant to anti-VEGF approaches raises the need for additional antiangiogenesis approaches that might be useful in such settings. Toward this end, as noted above, we performed transcriptional profiling screens to identify endothelial-specific targets that are markedly regulated either by VEGF blockade or by excess VEGF activity, reasoning that such targets might prove interesting as new antiangiogenesis targets. Confirming the potential of such a screen, one target that was "rediscovered" via such screens was Angiopoietin-2. We had previously independently identified the Angiopoietins as key new angiogenic regulators that seemed to work in tandem with the VEGFs (Davis et al. 1996; Suri et al. 1996; Maisonpierre et al. 1997; Valenzuela et al. 1999; Yancopoulos et al. 2000; Gale et al. 2002), and moreover, obtained substantial data that Angiopoietin-2 in particular was specifically induced in tumor vasculature and that it was important for tumor angiogenesis (Holash et al. 1999); a recent study employing Angiopoietin-2-blocking antibodies confirmed notable antitumor effects (Oliner et al. 2004). On the basis of the confidence in these transcriptional profiling screens engendered by the reidentification of Angiopoietin-2, we explored additional potential targets identified by the screens. Among these targets we have reported the identification of Delta-like ligand 4 (Dll4) (a ligand for the Notch family of receptors) as a gene that is markedly and specifically induced in tumor vasculature (Gale et al. 2004). Moreover, Dll4 is strikingly up-regulated in VEGF-overexpressing tumors and down-regulated in tumors by VEGF blockade. Using VelociGene® technology, which provides a high-throughput approach to create mouse mutants for genes of interest (Valenzuela et al. 2003), we found that mice lacking Dll4 exhibit profound vascular defects early in development (Gale et al. 2004). Remarkably, and as previously seen only for VEGF (see above), deletion of even just one of the two Dll4 alleles in developing embryos resulted in embryonic lethality due to vascular defects (Gale et al. 2004). All this evidence for a critical role for Dll4 in normal as well as tumor angiogenesis provided a rationale to develop blockers for Dll4. Recent testing in tumor models indicates that Dll4 may indeed prove to be an important new antiangiogenesis target, either alone or in combination with the VEGF Trap, or in settings of relative resistance to anti-VEGF therapies.

types of tumors and can even cause frank tumor
sion in some settings. In other preclinical cancer
we have found that combination of VEGF Trap wi
totoxic agent can result in potency far greater than
either single agent. Furthermore, the VEGF Trap
very effective in animal models of vascular eye d
The impressive efficacy in preclinical models of
and eye diseases provided a rationale for advance
the VEGF Trap into clinical trials, where it is pro
promising initial results in both cancer and eye di

In addition to its potential therapeutic value in
and vascular eye diseases, the VEGF Trap is also
valuable research tool. Transcription profiling scre
ing VEGF Trap have allowed a number of strateg
signed to identify new antiangiogenesis target
hoped that these strategies are helping to identify t
generation of antiangiogenesis targets, which ma
either alone or in combination with the VEGF Tra
settings of relative resistance to anti-VEGF therap

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