

# Complete Inhibition of Rhabdomyosarcoma Xenograft Growth and Neovascularization Requires Blockade of Both Tumor and Host Vascular Endothelial Growth Factor

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## Abstract

Growth of the human rhabdomyosarcoma A673 cell line in nude mice is substantially reduced but not completely suppressed after systemic administration of the antihuman vascular endothelial growth factor (VEGF) monoclonal antibody (Mab) A.4.6.1. Potentially, such escape might be attributable to incomplete local penetration of the antibody because of a diffusion barrier associated with tumor growth. Alternatively, it might reflect a compensatory up-regulation of murine VEGF, produced by the stroma of the host, or of other angiogenic factor genes. To test these potential mechanisms, systemic administration of Mab A.4.6.1 was performed in conjunction with intratumoral administration of an irrelevant antibody, an antihuman VEGF Fab or mFlt(1-3)-IgG that neutralizes both human and murine VEGF. Tumor growth in the systemic-plus-intratumoral anti-VEGF group was not different from that in the systemic anti-VEGF-plus-intratumoral-control antibody group, arguing against the possibility that bioavailability is the factor that limits the antitumor efficacy of Mab A.4.6.1. However, intratumoral mFlt(1-3)-IgG administration dramatically enhanced the activity of systemic anti-VEGF Mab and resulted in complete suppression of tumor growth, which indicated that host VEGF significantly contributes to tumor growth. Systemic administration of mFlt(1-3)-IgG alone replicated these findings. Histological analysis of residual tumor tissues revealed an almost complete absence of host-derived vasculature and massive tumor-cell necrosis in the mFlt(1-3)-IgG groups. Such extensive necrotic areas were not present in the other groups. Real-time reverse transcription-PCR analysis of total RNA derived from tumor tissues indicated strong up-regulation of both human and murine *VEGF* as well as other genes regulated by hypoxia. Our findings emphasize the need to completely block VEGF for maximal inhibition of tumor growth.

## Introduction

There is extensive evidence that the development of a neovascular supply is required for a variety of proliferative processes (1). VEGF<sup>2</sup> is a key regulator of physiological and pathological angiogenesis, and even partial inactivation of the *VEGF* gene results in early embryonic lethality (2, 3). Inhibition of VEGF activity by neutralizing antibodies or other inhibitors results in significant tumor suppression in a broad variety of tumor cell lines (4, 5). Also, administration of a chimeric murine soluble VEGF receptor protein, mFlt(1-3)-IgG, induces growth arrest and lethality in neonatal mice (6). However, safety evaluation studies in fully developed rodents or primates have failed to detect any significant toxicity after VEGF blockade, except inhibition of corpus luteum angiogenesis (6–8). This favorable safety

profile, combined with considerable antitumor efficacy in preclinical models, has made VEGF inhibitors attractive candidates for the treatment of solid tumors. A humanized anti-VEGF Mab has completed Phase I and Phase II trials in cancer patients, and, currently, Phase III studies are under way. Phase II studies have shown initial evidence of clinical efficacy in non-small cell lung and colorectal carcinoma patients (9, 10). Small molecules inhibiting VEGF receptor signal transduction are undergoing clinical testing as well (for review, see Ref. 11).

Systemic administration of the murine Mab A.4.6.1, directed against human VEGF, causes considerable inhibition of human rhabdomyosarcoma xenografts growth in immunodeficient mice (4). When the treatment is initiated at the same time or shortly after tumor-cell inoculation, the growth inhibition is dramatic, exceeding 90–95%. However, if tumors are allowed to reach a significant size prior to the beginning of the treatment, the level of inhibition is less complete, and tumors escape from the inhibition. This phenomenon might reflect molecular and cellular alterations resulting from genomic instability and/or increased mutation rates in the tumor cells. This might provide the molecular framework for a compensatory up-regulation of angiogenic molecules other than VEGF, or alternatively, for the down-regulation of antiangiogenic genes under growth-selective conditions. Such escape could also be potentially mediated by increased production of VEGF by the stroma of the host, because Mab A.4.6.1 does not neutralize murine VEGF (4, 12). Another factor that may potentially account for tumor escape after systemic administration of Mab A.4.6.1 is the blood/tumor barrier, attributable in part to interstitial hypertension, which may prevent systemically delivered therapeutic agents from achieving optimal concentrations in the extravascular space (13). The availability of several inhibitors enabled us to study the relative contribution of all these potential mechanisms involved in angiostatic escape. Our findings indicate that neither insufficient bioavailability nor compensatory regulation of antiangiogenic or angiogenic factor, but, rather, the up-regulation of host-derived VEGF is primarily responsible for the angiostatic escape observed with the A673 rhabdomyosarcoma.

## Materials and Methods

**In Vivo Experiments.** Human A673 rhabdomyosarcoma cells (HTB 1598) were cultured as described previously (4). Five × 10<sup>6</sup> cells in 0.1 ml of Matrigel were injected s.c. in the dorsal flank region of beige nude mice (Harlan Sprague Dawley). Five days after tumor cell inoculation, when the xenografts were clearly established and had reached a volume of 50–100 mm<sup>3</sup>, i.p. administration of Mab A.4.6.1 (4) was initiated, at a dose of 10 mg/kg. The antibody was then given i.p. at the same dose twice weekly. In addition, animals received direct intratumoral injections of an affinity metered recombinant humanized Fab fragment (14) originally derived from Mab A.4.6.1, murine Flt(1-3)-IgG (8) or a control murine Mab directed against HSV glycoprotein D (Clone 3L8) of the same isotype as Mab A.4.6.1., each at the dose of 25 mg/kg. Injections were made directly into the tumor mass, from the side and underneath, using a 28-gauge needle and a 0.5-ml tuberculin syringe.

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<sup>2</sup> The abbreviations used are: VEGF, vascular endothelial growth factor; Mab, monoclonal antibody; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Volume was 0.1 ml and injected slowly. Injections were done every other day throughout the experimental period. The control group received both systemic and intratumoral administration of the control Mab. Tumor size measurements were performed every second day and tumor volume was calculated using the ellipsoid volume formulas ( $\pi/6 \times L \times W \times H$ , where  $L$  = length,  $W$  = width, and  $H$  = height; Ref. 15). In an additional set of experiments, we compared systemic delivery (i.p.) alone of Mab A.4.6.1 (10 mg/kg twice weekly) versus mFlt(1-3)-IgG (1, 10, 25, or 50 mg/kg daily). Statistical analysis of tumor volumes and gene expression data were performed using ANOVA software (Abacus Concepts, Inc., Berkeley, CA).

**Histology and Immunohistochemistry.** Tumors were fixed in 10% neutral buffered formalin for 12–16 h prior to paraffin embedding. H&E staining and immunohistochemistry for Flk-1 and CD31 expression were performed as described previously (6). Briefly, tumor tissue sections either were pretreated with 0.1% trypsin for 10–15 min at 37°C and then incubated with rat anti-mouse CD31 (mAb MEC13.3, PharMingen, San Diego, CA) at 1:500 dilution overnight at 4°C or were pretreated with Trilogy antigen retrieval solution (Cell Marque, Austin, TX) at 99°C for 1 h and then incubated with rat anti-mouse Flk-1 (mAb MALK-1; Ref. 6) at 3.9 mg/ml overnight at 4°C. Immunoreactivities were visualized by the avidin-biotin complex technique using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) with diaminobenzidine as chromogen. Hematoxylin was used as counterstain.

**Real-Time Quantitative RT-PCR Analysis.** RNA was isolated from frozen tumor tissue harvested at necropsy from five specimens of each treatment group using the STAT 60 method (TEL-TEST "B," Friendswood, TX), and purified on RNeasy Quick spin columns (Qiagen, Valencia, CA). One hundred ng of total RNA per reaction were analyzed using the RT-PCR kit from Perkin-Elmer, following the manufacturer's instructions (PE Applied Biosystems, Foster City, CA). Reactions were run in 96-well plates in a Model 7700 Sequence Detector (PE Applied Biosystems), and results were analyzed using Sequence Detection Software (PE Applied Biosystems). RT-PCR conditions were 30 min at 48°C, 10 min at 95°C, and 40 cycles of 30 s at 95°C and 90 s at 60°C. Relative RNA equivalents for each sample were obtained by standardizing to GAPDH levels. Each of the five samples per group was run in duplicates to determine sample reproducibility, and the average relative RNA equivalents per sample pair was used for further analysis. Statistical analysis was performed using ANOVA software (Abacus Concepts, Inc., Berkeley, CA). Species specificity of the probe/primer sets was verified by testing total RNA derived from human epithelial cells or mouse kidney RNA (data not shown). Expression levels have been standardized to the probe/primer sets specific for human or murine GAPDH, respectively.

## Results and Discussion

Systemic administration of the murine antihuman VEGF Mab A.4.6.1, initiated when the tumors are already established and vascularized, causes a significant growth delay but not complete suppression of A673 rhabdomyosarcoma xenografts (4, 16). Because tumor growth is accompanied by a blood/tumor barrier which may limit the ability of therapeutics to diffuse into the tumor (13), it is possible that incomplete inhibition of tumor growth might result from insufficient penetration of the circulating antibody in the extravascular space. We, therefore, examined whether the activity of systemic A.4.6.1 can be enhanced by intratumoral administration of an affinity-matured humanized anti-VEGF Fab (14) that neutralizes human (but not murine) VEGF with 50–100-fold higher potency than the parental Fab (17) and is also substantially more potent in inhibiting VEGF-induced mitogenesis than full-length Mab A.4.6.1. (data not shown). A Fab has a 3-fold lower size than a full-length IgG and is more diffusible in several biological systems (18). However, intratumoral Fab did not cause any additional inhibition of tumor growth in Mab A.4.6.1-treated mice. As a second reagent, we tested mFlt(1-3)-IgG, which is able to inhibit both human and murine VEGF. Intratumoral injection of mFlt(1-3)-IgG, in conjunction with systemic administration of Mab A.4.6.1, resulted in essentially complete inhibition of A673 tumor growth (Fig. 1A) even over a prolonged treatment period lasting 4 weeks (Fig. 1B). These findings indicate that, in this experimental

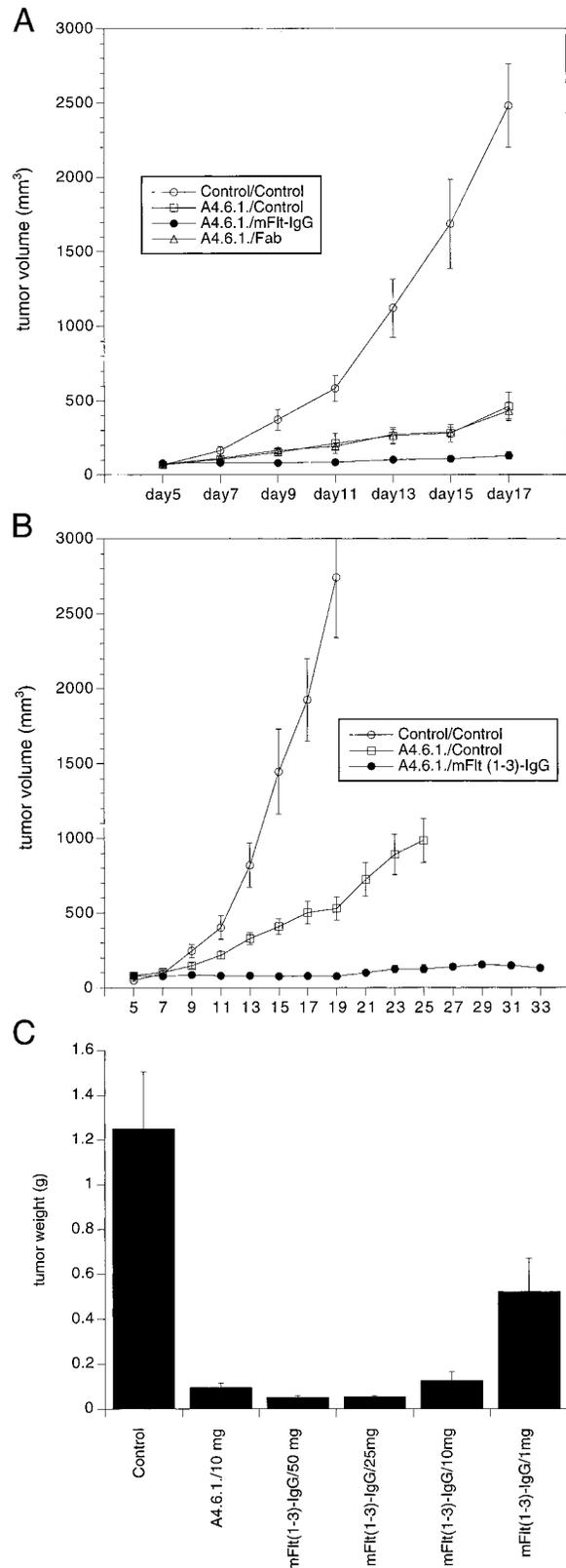


Fig. 1. Xenograft experiments in nude mice. Animals ( $n = 5$  per group) were treated with different anti-VEGF compounds or a control antibody. In A,  $5 \times 10^6$  human A673 rhabdomyosarcoma cells were injected s.c. in the dorsal area. Treatment was started after day 5 by i.p. administration of Mab A.4.6.1 or control Mab at 10 mg/kg, twice weekly. Intratumoral injection of mFlt(1-3)-IgG, Fab, or control Mab was at 25 mg/kg, every other day. In B, mice were treated as indicated. Mice receiving Mab A.4.6.1 plus mFlt(1-3)-IgG were treated until day 33. C, systemic administration of VEGF inhibitors. A673 cells were injected as described above. Animals were injected i.p. with Mab A.4.6.1 at 10 mg/kg twice weekly or with mFlt(1-3)-IgG daily at the doses indicated. Control Mab was administered at the dose of 25 mg/kg. After 2 weeks, the animals were killed, and the tumor weight was determined.

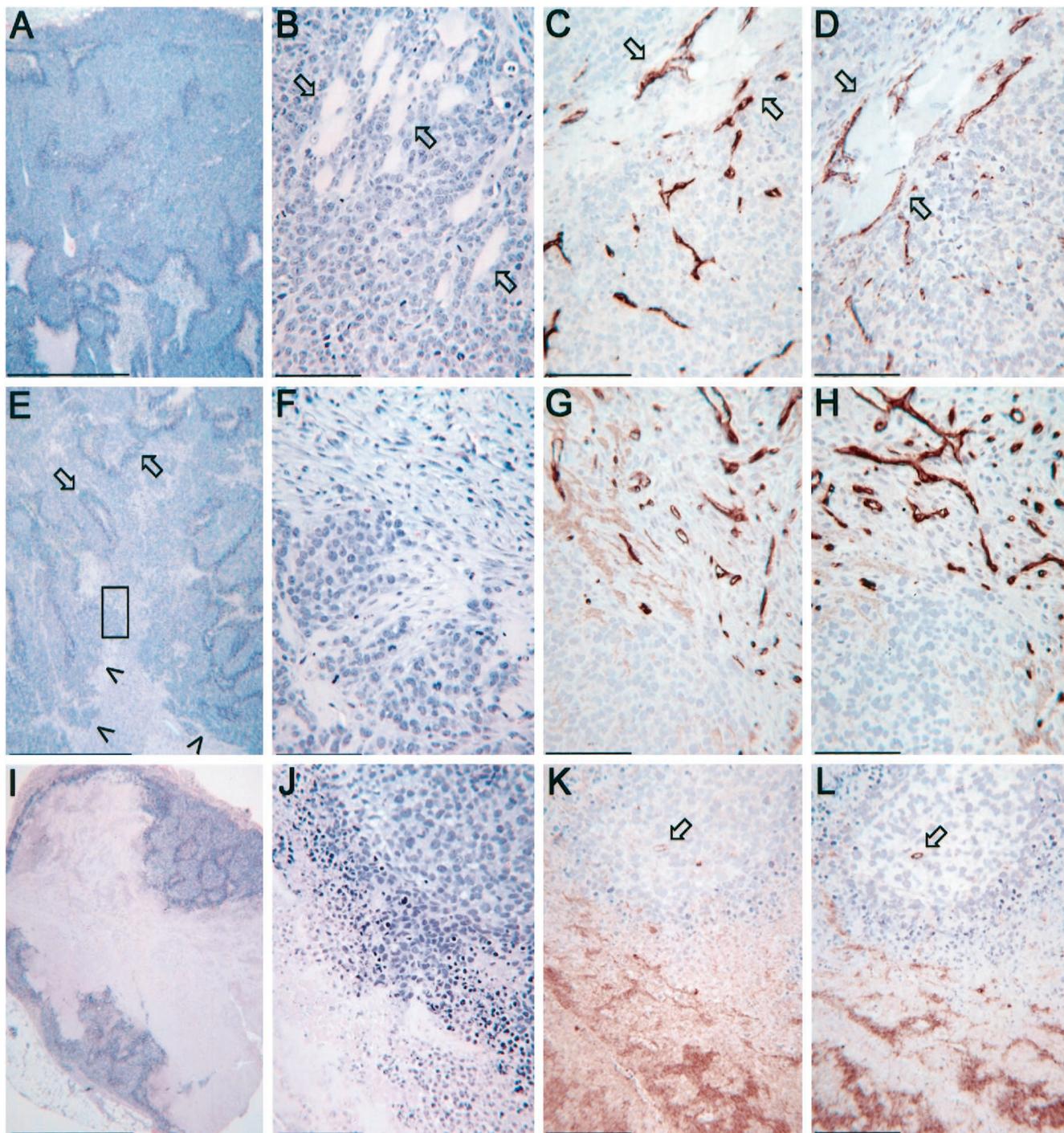


Fig. 2. Effects of anti-VEGF treatments on the histopathology of A673 xenografts. Serial sections of tumors harvested from mice treated with control antibody (A–D), systemic Mab 4.6.1 and intratumoral Fab (E–H), or systemic Mab 4.6.1 and intratumoral mFlt(1-3)-IgG (I–L) were stained with H&E (A, B, E, F, I, J) or by immunoperoxidase for the endothelial cell markers Flk-1 (C, G, K) and CD31 (D, H, L). In A, control tumors showed regions of geographic necrosis that were often most pronounced in deeper areas (bottom). In B, entrapped host-derived elements such as skeletal muscle fibers (arrows) were frequently seen in the invasive tumors. In C and D, tumor microvessels were often present in increased numbers near host elements such as entrapped muscle fibers (arrows). In E, Mab A.4.6.1-treated tumors also showed geographic necrosis. In some areas, the tumor-necrosis interface stained more deeply than the viable tumor (arrows); this is attributable to nuclear pyknosis, an early necrotic change (see J also). In other areas, the tumor-necrosis interface lacked pyknotic changes (arrowheads and box). The interface region within the box (E) is shown at higher magnification in F, G, and H. In F, areas of previous necrosis began to resolve with ingrowth of granulation tissue stroma (upper right). The adjacent tumor (lower left) appeared to be viable and proliferative, without nuclear pyknosis and karyorrhexis in the border zone. In G and H, immunostains for endothelial cell markers demonstrated the high vascularity of the stromal ingrowth. In I, mFlt(1-3)IgG/Mab A4.6.1-treated tumors were largely necrotic. A thin peripheral zone of viable tumor remains adjacent to the border of the tumor mass and surrounding host tissues. In J, the tumor-necrosis interface in mFlt-1 (1-3)IgG-treated tumors showed progression from viable tumor through pyknosis and karyorrhexis, indicating continuing cell death. In K and L, immunostains for endothelial cell markers revealed a complete lack of neovascularization at the tumor-necrosis interface. Nonspecific staining of the necrotic regions as shown here was also seen in nonspecific isotype-matched primary antibody controls. A single vessel is present within the viable tumor (arrow). It is notable that in control tumors, immunoreactivity for Flk-1 (C) was more intense than for CD31 (D), whereas in the anti-VEGF-treated tumors, this relationship was reversed (G and K versus H and L). Scale bars: 1 mm (A, E, and I); 100  $\mu$ m (B–D, F–H, and J–L).

system, lack of bioavailability is not the factor mediating resistance to Mab A.4.6.1. A likely explanation is that an inhibitor of angiogenesis such as anti-VEGF Mab has a more limited need to diffuse in the extravascular space relative to conventional anticancer agents. Instead, complete suppression of tumor growth required neutralization of host-derived as well as tumor-derived VEGF. To further verify these findings, we compared systemic delivery alone of Mab A.4.6.1 *versus* several doses of mFlt(1-3)-IgG. As illustrated in Fig. 1C, mFlt(1-3)-IgG administration resulted in a dose-dependent suppression of tumor growth. At the higher doses, the inhibition as assessed by tumor weight was approximately 97%. The inhibition afforded by Mab A.4.6.1. was approximately 90%.

Histopathological examination revealed that neovascularization of the xenografts still occurred in mice treated with A.4.6.1 alone and in combination with intratumoral Fab but was nearly completely abolished in mice treated with Mab A.4.6.1 together with mFlt(1-3)-IgG (Fig. 2). Although the Mab A.4.6.1-treated tumors plus Fab or control antibody were markedly smaller than controls, they displayed qualitatively similar histological appearances, with scattered regions of geographic necrosis in various stages of evolution. In later stages, the tumors recruited the ingrowth of highly vascular granulation tissue-like stroma, which appeared to replace necrotic areas and allow tumor regrowth. However, tumors treated with Mab A.4.6.1 together with

mFlt(1-3)-IgG showed large areas of confluent necrosis, with only a thin peripheral rim of viable cells remaining in much of the tumors. Strikingly, there was a complete lack of recruitment of new vessels into the necrotic areas. These findings indicate that in the A673 xenograft model, VEGF is a key regulator of tumor angiogenesis. The inability of Mab A.4.6.1 alone to completely suppress tumor neovascularization and growth seemed to result primarily from the production of murine VEGF by host cells associated with the tumor. The A673 xenografts grew with the invasion of adjacent tissues, which resulted in the frequent entrapment of host skeletal muscle fibers and adipocytes within the tumor mass (Fig. 2). Thus, these cells as well as vessel-associated pericytes and infiltrating macrophages (19) may represent sources that contribute to increased levels of host VEGF in the tumors. The histological picture of tumors from animals treated with systemically delivered mFlt(1-3)-IgG at the doses of 25 or 50 mg/kg was indistinguishable from that of the groups that received intratumoral mFlt(1-3)-IgG, with extensive area of necrosis. However, such extensive necrotic areas were not present in the Mab-alone groups (data not shown).

To identify candidate angiogenic factors that might be compensatorily up-regulated under complete VEGF block conditions, we analyzed a panel of known genes, involved in the regulation of angiogenesis. Peripheral tumor tissues from all of the treatment groups were

Table 1 Sequences of primers and probes used for real-time RT-PCR analysis of tumor RNA

probe name	probe sequence	forward primer	reverse primer
HSPLGF 1168.RP	CCCAGCAGAATCCGCATCCCTACTTTG	CCGGCTCGTGTATTTATTACCG	GGCAACCCTGTTCTCCAGAGC
HSUPAR 469.FP	TGGGTACACACATCCAGGCCTGTTCT	GGCTGTACCTATTTCCCGAAGC	GCATGGTGGTCTCAGGGCAGTA
HUVEGF 350.RP	CTGCATTCACACTGGCTGTGTTCTCCA	GATCCGGTACCCGAGCAGTCAG	ATCCGGGACCCAGTGGGTTGA
HUMPAI 1782.FP	CCTTTCATCTCAGAGTCCACTGTGGCA	GGATGGAACACTACGGGGCTTACA	GGGCACGCATCTGACATTTCTT
HSANG1-1932T	TGTGAAGTCACCAAGGTTCTTGACC	CCTTCCAGCAATAAGTGGTAGTT	CAACCGGCTCCAGATTCA
HSANG2-2116T	TTTACAGACGCTGCTGTGCAACCA	GAGGCTGAGAATCAGACTGACA	TTACTGATAAATTTGCACATAACATCT
HSANG5-1383T	TGGATGGATCAACATTTTGGTTGATTTATAGAGTA	TCTTGGAAAGTCTCAAAATGGAA	TCATTCAAGGCTTTCTGAATCTG
HSHG 2145T	CAACATAAAATGAGAATGGTTCTTGGTGTCA	GGTGGCCACTTGTGTTGT	CATCCAGCACCAGGAACA
HSPDFB 1937.T	AAGAAGGACTGAACTCCATCGCCAT	CCCAGCAGCTCAAGAAGAA	TCCCAAGTCTTGGAGTTAAGG
HSVHL 4662.T	TTTTTCCCATGAAAACAGAACCCAAAA	AAACATTCACATCGAAACAAAA	GCTGGTGAATACTAACCACCTACC
HSVEGF-C.1077.FP	CTGCCCCCAATACATGTGGAATAATCA	CAGTGTGAGGCGAGCAAA	CTTCTGAGCCAGGCATCTG
HSc-Met.269.T	CCGAAATGCGCCCATCTTT	CTAAAATGCTGGCACCCCTAAA	ATCCGGGACCCAGTGGGTTGA
HSIL-8.4972.T	CAGTAACTGAGGAGTAAACTGATAATGTGAGGCA	TTTAGCATAGCTGGACATTAAGAA	GCAAAATGCTTAGGCTTTAACC
HSTGF-b.2695.T	TATTTCTAAATTTAATCTGAGCCTCCTTCTACAGGG	ATGCTGTTTATGAAACGGAAATC	GCCTTCCAGTCACATGAT
HSbFGF.3530.T	TGGAGACAAAAGCCTTATTTGCCAT	TGAATCACTAACTGACTGAAAATTT	GAAGGGTCTCCCGCACTACT
HSLeptin.637.T	ATGGACACCCCTTATCCAGGACTCTG	TCCAGGATTGAAGAGCATTG	CTTAGAGGATCAGGGAAATTGA
HSGLUT-1.1668.T	CTGCTCCAGCAGCCCTAAGGA	GTGTGAGTCCGCCAGAT	AGTGAAGTCACTGGGATCCAGTGA
HSLDH-A.1467.T	AGTGAGTCACATCTGGGATCCAGTGTA	GCACAAGACATGATATTGGATTT	GAACATGCCTAGTCCAACATTT
HUMeph-B2.1652T	AGAAATTTGTTTGTACTCAAGGTCGCC	TCCATGGTAAATCCGTTCA	ACAGCCTAGCATCTGGCTAAA
HSBH-EGF.300.FP	CTGGCTGCAAGTCTCTCGGCACTG	TGAACAGTGAAGTATGCTGAACT	CTCCAGGCTCTCGCCAGTC
HSANG4-1377T	AGGCCACAGGCGTCAAACCA	GTAGTAGACGCGTGGAGTT	TGTGCCAGGTGATGTCT
HSVEGF.294.FP	TGTGCCCACTGAGGAGTCCAAACATCA	AATGACGAGGGCCTGGAGT	TTGATCCGCATAATCTGCATG
HSJagged.	TGCCAGAACCGTCCCAAGTCTACAAAC	TGGGGTCACTGTCAGAAATGAA	TGTTCCCGTGAAGCCTTTGTA
MMVEGF-A 150.FP	CCAGCGAAGCTACTGCCGTCCAATT	TGTACCTCCACCATGCCAAGT	TGGAAGATGCCACCAGGGT
MMFh-1.6018T	CAGCGGATGAGACAGAGGCTACCA	GTCGGCTCAGTGTGTAAGT	TGCTGTTCTCATCCGTTCT
MMFik-1.5161.T	CTGGTGTCTTCTCTATCTCCACTCCTGCT	TGTCAAGTGGCGGTAAGG	CACAAAGCTAAAATCTGAGGACTTG
MMANG3-1452T	CATCCGCTGGCACTACTTCCGA	TGTGCCGTGCAAGTGA	TGTGCCGTGCAAGTGA
MMANG5-1356T	ATCCAAAATGATGCTCCAGCCCA	CAGTTCAGTTGAAGCTTCTTAGGT	GCAGAAAGCTCTATGCTATCAAAAT
MMGLUT1.2311.T	CTGTGCACTGAGGGCCACACAA	GGGCTGCCAGGTTCTAGTC	GCTCCGAGGCTCCTTCTCA
MMLDH-A-1441.T	CCTGCTGCATTGCTGCCCTG	GTCGGCTAGGCTGTTG	GTCGGCTAGGCTGTTG
ss.MMPECAM-1-2325T	CCGAGAAGAGCAGCCGATTCT	CTGGGAGTCTGCTCATG	CACAGGACTCTCGCAATCC
MMANG2.2141.FP	AAGTGGTGGGCACAGGCTATCATCTGG	GCAGCCAGTATCCAGGGACTT	GCACCCAAAGAGGAAAACAATG
MMANG1.351.FP	TCACATAGGTTGACGCAACCCAGCG	TGCATTTCTGCTGCCATT	TCTCCCTCCGTTTTCTGGATT
MMTie2.599.FP	TCAGCCTTCCACAGGCTGATTGTTCC	CGCCAGGTACATAGGAGGAA	CCCCACTTCTGAGCTTCCAC
MMFh-4.3764T	AACCAGGGTCTCAGCCCTC	AGCCTGGATCTGATTTGCTGT	CAGCCATGTGGCTCTGCT
MMHGf.2113.T	ACACAAAATGAGAATGTTCTTGGTGTCA	GGCCCACTATTTGTGAAC	CATCCACAGCCAGGAAC
MMPiGF.1242.T	ACACACAACCCAGACTTGTATCGGTCA	GCAGTAGCCGCTGGACTT	CGTCCAGGCTTCCACCAACTG
MMFGf-b.2297.T	CGGTCCAGGTTCTCCACCAACTG	CCTCTCAGAGACCTACGTTCAA	GGAGGTCAAGGCCACAAT
MMVEGFb.367.T	CAGTGGGCAACACCAAGTCCG	GCAGATGGCCTGGAATGT	GGTACTGGATCATGAGGATCTG
MMVEGFc.1674.T	CCCACTAAAATATTGTTCTGCACTTATTTTATAGCA	CCACTAAAATATTGTTCTGCACT	TGATCAGAGTGAAGCTTTACCAAT
MMTNFb.1075.T	CACGAAGCTCTAAAAGCCAGCCACTG	GACCAAGGGTCTTAGAAGAAA	GGGTCCCTGTGGATGTCTA
MMTNFa.1535.T	ACCAGGCTGTGCTACATCACTGAAC	TTGTCTTAATAACGCTGATTTGGT	CAATTGACTGTAGGGCAATTAACA
MMp75TNFR.2285.T	CCCAGCAGGGCTCAGGAACAC	CTTGATCAGGGCTCGAATAAC	GATTTGCGCAAGGATGAAC
MMp55TNFR.2015.T	ATTGATACAAAGATTGCTTATCAAAAGTGA AAAAGGC	GCCTCCTCAGTGGGTTT	CTTACCTTACACAGTTCATCCATTAGT
MMENDOG.2690.T	CATCTGGCCCCAGAAGCCTCC	GCAGGCAAGAAGCTCAGACAT	AGCTCCCTCAGTCTCTGTTT
MMETN1.1346.T	CTCCCTGAAATGTGCCAGGCG	AAAGTCTTATGGGAGTGTTCGT	TTTGCATGGATTTGGAGTT
MMID1.677.T	TTGATCAACAGAGCCTCACCCCTCCTC	GATCGTCCAGTGGGTAGA	GTCTCTGAGGCTGAAAGGT
MMHB-EGF.2145T	GTCAATCCGGGGCTAAAC	GTTGGTACCAGGCTGAGAGT	TGGGTAGCAGCTGGTTTG
MMTIE1 2398.FP	AGCTGCCTACATCGGAGACGCCAC	GCCCTTTTAGCCTTGGTGTG	CTTACCCGATCCGACTGG
MMNPILIN 1963.FP	CGTTCGAAGGCCAACCAACTATGACACAC	GACAGCAAGCCAAAGGCTA	AGGTGAAAACGTCGGAAGCTC
MMGAPDH	AAGCCATCACATCTCCAGGAGCGAGA	ATGTTCCAGTATGACTCCACTCAC	GAAGACACCAGTAGACTCCACGACA

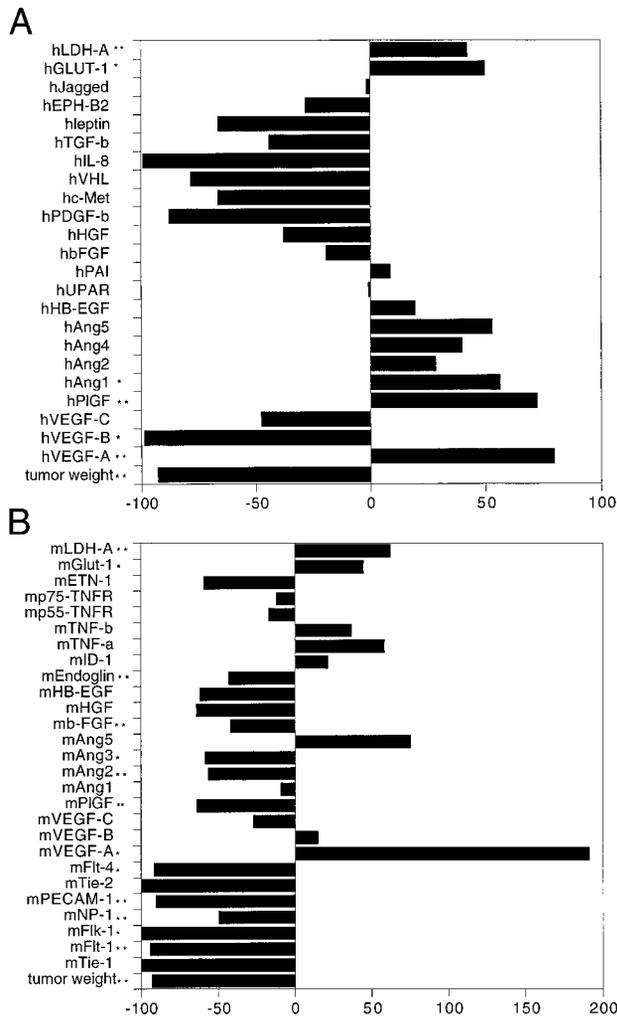


Fig. 3. RT-PCR gene expression analysis of control tumors and tumors treated with Mab A4.6.1/Flt-1gG. *A*, comparison of relative RNA levels of human genes between total RNA isolated from tumors harvested from mice treated with Mab A.4.6.1/Flt(1-3)-IgG or control/control during a 12-day treatment period. Black bars, relative changes in percentage to control-treated tumors. Statistically significant changes: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  ( $n = 5$ ). Expression levels have been standardized to the probe/primer sets specific for human or murine GAPDH, respectively. *B*, comparison of RNA levels of mouse genes with total RNA isolated from tumors as defined in *A*. \*,  $P < 0.05$ ; \*\*,  $P < 0.005$  ( $n = 5$ ).

harvested at necropsy, and total RNA was isolated and analyzed for gene expression by real-time RT-PCR. To determine whether such genes are expressed by the tumor or the host cells, we used primer sets (Table 1) to differentially amplify human and murine counterparts. Because all three of the anti-VEGF treatment groups displayed similar changes in their gene expression profile relative to controls, data are shown for the Mab A.4.6.1/mFlt(1-3)-IgG versus irrelevant control Mab tumors. From a panel of 21 human genes analyzed, VEGF itself (1.8-fold) and angiopoietin 1 (1.6-fold) were found to be significantly up-regulated. There was a moderate induction of other members of the human angiopoietin family such as Ang2 (1.3-fold), Ang4 (1.4-fold), which, however, did not reach statistical significance (Fig. 3A). Among the VEGF-related genes, *VEGF-B* and *VEGF-C* were down-regulated. In contrast, the *PIGF* gene was significantly induced. Interestingly, the Flt-1 receptor (VEGFR-1) is known to bind PIGF and VEGF-B in addition to VEGF (for review, see Ref. 20). Therefore, the possibility exists that neutralization of at least PIGF contributes to the effects of mFlt(1-3)-IgG reported here. However, available data indicate that both VEGF-B and PIGF have little or no direct mitogenic effect on endothelial cells, and their action depends largely

on the formation of heterodimers with VEGF (21, 22) or by rendering the vascular endothelium more sensitive to the action of VEGF (23). Both models imply that VEGF is required for much of the biological activity of these molecules. It is noteworthy that not only mFlt(1-3)-IgG but also Mab A.4.6.1 is able to inhibit the mitogenic activity of PIGF/VEGF heterodimers. Mab A.4.6.1, at the concentration of 5  $\mu\text{g/ml}$  was able to completely inhibit the endothelial cell-mitogenic activity of 200 ng/ml PIGF/VEGF heterodimers. Therefore, the greater tumor-suppressing activity of mFlt(1-3)-IgG is not likely to be attributable to inhibition of PIGF signaling. However, it is possible that PIGF/VEGF heterodimers contribute to the overall process of tumor angiogenesis. Additional studies with truly specific inhibitors are required to fully assess the contribution of the other members of the VEGF family. Among the 24 murine genes examined, VEGF displays the strongest up-regulation (2.9-fold). This finding supports the hypothesis that host-derived VEGF is a significant contributor to the overall process of tumor angiogenesis. Interestingly, other murine angiogenic genes, including PIGF, were found to be repressed in the presence of such complete VEGF-blockade (Fig. 3B). There was also a striking correlation between tumor size and expression levels of endothelial cell marker genes such as *PECAM*, *Tie1*, and *Tie2* as well as the VEGF receptors. Also, a set of human and mouse genes belonging to the family of hypoxia-inducible genes such as *Glut-1* LDH-A were found to be induced under VEGF blockade conditions (Fig. 3, A and B), which indicated that anti-VEGF-treated tumors are hypoxic when compared with control tumors, which could contribute to the up-regulation of VEGF.

The hypothesis that stromal-derived VEGF is implicated in tumor angiogenesis has been also tested in a transgenic mouse model, using a VEGF promoter fragment to drive the expression of green fluorescent protein (GFP; Ref. 24). Inoculation of tumor cells strongly induced expression of the VEGF promoter in the stroma of the host (24). However, subsequent studies using a similar transgenic model but with a different VEGF promoter fragment have yielded a lower GFP expression in the stroma and a greater expression in the epithelium (25). Thus, the extent to which stromal-derived VEGF contributes to the process of tumor angiogenesis so far has been unclear.

It is noteworthy that our findings argue against the possibility that a partial blockade might be sufficient for optimal inhibition of VEGF-dependent tumor angiogenesis. This is in apparent contrast to the findings in gene knock out experiments in mice, in which even partial inactivation of the *VEGF* gene results in embryonic lethality (2, 3). In conclusion, we demonstrate that in the A673 xenograft model, systemic Mab A.4.6.1 antibody exerted a maximal efficacy, which could not be improved by local administration of an antihuman VEGF Fab. However, a reagent that blocked host VEGF completely suppressed residual tumor growth. Such complete inhibition was accompanied by a striking morphological change relative to partial inhibition: the presence of extensive necrosis. It remains to be seen whether other tumor cell types can induce an adaptive response to anti-VEGF treatment and, hence, escape in the presence of a total VEGF blockade.

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#### References

1. Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, 1: 27-31, 1995.
2. Ferrara, N., Carver Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell Braxton, L., Hillan, K. J., and Moore, M. W. Heterozygous embryonic lethality

- induced by targeted inactivation of the VEGF gene. *Nature (Lond.)*, *380*: 439–442, 1996.
3. Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W., and Nagy, A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature (Lond.)*, *380*: 435–439, 1996.
  4. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth *in vivo*. *Nature (Lond.)*, *362*: 841–844, 1993.
  5. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., and Ullrich, A. Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. *Nature (Lond.)*, *367*: 576–579, 1994.
  6. Gerber, H. P., Hillan, K. J., Ryan, A. M., Kowalski, J., Keller, G.-A., Rangell, L., Wright, B. D., Radtke, F., Aguet, M., and Ferrara, N. VEGF is required for growth and survival in neonatal mice. *Development (Camb.)*, *126*: 1149–1159, 1999.
  7. Ryan, A. M., Eppler, D. B., Hagler, K. E., Bruner, R. H., Thomford, P. J., Hall, R. L., Shopp, G. M., and O'Neill, C. A. Preclinical safety evaluation of rhuMABVEGF, an antiangiogenic humanized monoclonal antibody. *Toxicol. Pathol.*, *27*: 78–86, 1999.
  8. Ferrara, N., Chen, H., Davis-Smyth, T., Gerber, H.-P., Nguyen, T.-N., Peers, D., Chisholm, V., Hillan, K. J., and Schwall, R. H. Vascular endothelial growth factor is essential for corpus luteum angiogenesis. *Nat. Med.*, *4*: 336–340, 1998.
  9. DeVore, R., Herbst, R. S., Langer, C. J., Kelly, K., Gaudreault, J., Holmgren, E., Novotny, W. F., and Kabbinavar, F. A. Randomized Phase II trial comparing rhumab VEGF (recombinant humanized monoclonal antibody to vascular endothelial cell growth factor) plus carboplatin/paclitaxel (CP) to CP alone in patients with stage IIIB/IV NSCLC. *Proc. Am. Soc. Clin. Oncol.*, *19*: 1896, 2000.
  10. Bergsland, E., Fehrenbacher, L., Meropol, N. J., Novotny, W. F., Gaudreault, J., Lieberman, G., and F., K. A Randomized Phase II trial comparing rhuMAB VEGF (recombinant humanized monoclonal antibody to vascular endothelial cell growth factor) plus 5-fluorouracil/leucovorin (FU/LV) to FU/LV alone in patients with metastatic colorectal cancer. *Proc. Am. Soc. Clin. Oncol.*, *19*: 939, 2000.
  11. McMahon, G. VEGF receptor signaling in tumor angiogenesis. *Oncologist*, *5*: 3–10, 2000.
  12. Muller, Y. A., Chen, Y., Christinger, H. W., Li, B., Cunningham, B. C., Lowman, H. B., and de Vos, A. M. VEGF and the Fab fragment of a humanized neutralizing antibody: crystal structure of the complex at 2.4 Å resolution and mutational analysis of the interface. *Structure (Lond.)*, *6*: 1153–1167, 1998.
  13. Jain, R. K. Delivery of molecular medicine to solid tumors. *Science (Washington DC)*, *271*: 1079–1080, 1996.
  14. Chen, Y., Wiesmann, C., Fuh, G., Li, B., Christinger, H. W., McKay, P., de Vos, A. M., and Lowman, H. B. Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *J. Mol. Biol.*, *293*: 865–881, 1999.
  15. Tomayko, M. M., and Reynolds, C. P. Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother. Pharmacol.*, *24*: 148–154, 1989.
  16. Mordenti, J., Thomsen, K., Licko, V., Chen, H., Meng, Y. G., and Ferrara, N. Efficacy and concentration-response of murine anti-VEGF monoclonal antibody in tumor-bearing mice and extrapolation to humans. *Toxicol. Pathol.*, *27*: 14–21, 1999.
  17. Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., Winkler, M., and Ferrara, N. Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res.*, *57*: 4593–4599, 1997.
  18. Mordenti, J., Cuthbertson, R. A., Ferrara, N., Thomsen, K., Berleau, L., Licko, V., Allen, P. C., Valverde, C. R., Meng, Y. G., Fei, D. T., Fourre, K. M., and Ryan, A. M. Comparisons of the intraocular tissue distribution, pharmacokinetics, and safety of <sup>125</sup>I-labeled full-length and Fab antibodies in rhesus monkeys following intravitreal administration. *Toxicol. Pathol.*, *27*: 536–544, 1999.
  19. Nomura, M., Yamagishi, S., Harada, S., Hayashi, Y., Yamashita, T., Yamashita, J., and Yamamoto, H. Possible participation of autocrine and paracrine vascular endothelial growth factors in hypoxia-induced proliferation of endothelial cells and pericytes. *J. Biol. Chem.*, *270*: 28316–28324, 1995.
  20. Ferrara, N., and Davis-Smyth, T. The biology of vascular endothelial growth factor. *Endocr. Rev.*, *18*: 4–25, 1997.
  21. Cao, Y., Chen, H., Zhou, L., Chiang, M. K., Anand Apte, B., Weatherbee, J. A., Wang, Y., Fang, F., Flanagan, J. G., and Tsang, M. L. Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. *J. Biol. Chem.*, *271*: 3154–3162, 1996.
  22. Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., Orpana, A., Pettersson, R. F., Alitalo, K., and Eriksson, U. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc. Natl. Acad. Sci. USA*, *93*: 2576–2581, 1996.
  23. Park, J. E., Chen, H. H., Winer, J., Houck, K. A., and Ferrara, N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, *in vitro* and *in vivo*, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J. Biol. Chem.*, *269*: 25646–25654, 1994.
  24. Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E. C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R. K., and Seed, B. Tumor induction of VEGF promoter activity in stromal cells. *Cell*, *94*: 715–725, 1998.
  25. Kishimoto, J., Ehama, R., Ge, Y., Kobayashi, T., Nishiyama, T., Detmar, M., and Burgeson, R. E. *In vivo* detection of human vascular endothelial growth factor promoter activity in transgenic mouse skin. *Am. J. Pathol.*, *157*: 1–3, 2000.

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