Overproduction of $VEGF_{165}$ Concomitantly Expressed with its Receptors Promotes Growth and Survival of Melanoma Cells through MAPK and PI3K Signaling

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Vascular endothelial growth factor (VEGF) is an important mediator of tumor-associated angiogenesis, and consequently it has been associated with metastasis. We report here that the overexpression of $VEGF_{165}$ in melanoma xenografts promotes an acceleration of tumor growth and an increase in angiogenesis as well as the spontaneous metastasis formation. In addition, VEGF receptors (VEGFR)1, VEGFR2 and neurophilin-1 are expressed in A375 melanoma cells. Forced overexpression of VEGF in these cells induces cell growth and triggers survival activity in serum-starved cultures, by a mechanism dependent on the mitogen-activating protein kinase signaling pathway. Furthermore, these effects are dependent MEK 1/2 activity. Kinase domain region-specific tyrosine kinase inhibitors dramatically reduced DNA synthesis to 20% with respect to the controls, although they did not completely suppress either the p44 or p42-phosphorylated forms of extracellular signal-regulated protein kinase. These inhibitors also provoked a decrease in Akt phosphorylation. We observed a dramatic reduction in survival after treatment with phosphatidylinositol 3′-kinase (PI3K)-specific inhibitor in the presence of specific tyrosinase inhibitors. We suggest that the overproduction of $VEGF_{165}$ concomitantly expressed with its receptors favors cell growth and survival of melanoma cells through MAPK and PI3K signaling pathways. These data support the involvement in melanoma growth and survival of a VEGF-dependent internal autocrine loop mechanism, at least *in vitro*.

Key words: angiogenesis/autocrine growth/ERK/Flk/KDR/melanoma/metastasis/NRP-1/proliferation/VEGF/VEGFR
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Cutaneous melanoma can develop *de novo* either from normal melanocytes or from precursor lesions such as atypical dysplastic nevi or congenital nevi (Clark *et al*, 1984; Herlyn *et al*, 1987; Albino, 1995). Using clinical and histopathological criteria, the melanoma can progress from radial growth phase (RGP) lesions without competence for metastasis to vertical growth phase (VGP), which does have such competence (Meier *et al*, 1998). Despite significant efforts to identify the genetic alterations responsible for this progression, there are still no clear predictors for the outcome of melanoma. To gain an insight into the cellular and molecular pathways involved in the transition from RGP to VGP and metastatic melanoma, we explored the role played by the proangiogenic factor vascular endothelial growth factor (VEGF) in this context. This approach was based on our previous results in which we demonstrated that the microvessel densities in <1.00 mm thick melanomas were in concordance with the aggressiveness of tumors (Marcoval *et al*, 1996, 1997). Indeed, angiogenesis has been put forward as a prognostic parameter in a variety of tumors such as breast (Gasparini *et al*, 1997), colon (Bossi *et al*, 1995), lung (Yuang *et al*, 1995), and prostate (Latil *et al*, 2000) among others, although its value remains controversial in melanoma (Busam *et al*, 1995). In melanoma, a number of putative angiogenic factors including VEGF, basic fibroblastic growth factor (bFGF), platelet-derived growth factor, IL-8, and transforming growth factor β are expressed by melanoma cells (Westphal *et al*, 2000). Of these factors, VEGF is one of the most potent inducers of angiogenesis, affecting endothelial cell proliferation, motility, and vascular permeability (Ferrara and Alitalo, 1999).

VEGF (or VEGF-A) is the founder member of a family of growth factors, including VEGF-A, B, C, D, E, and placental growth factor. It is a dimeric, disulfide-bonded protein with molecular weights between Mr 25.000 and Mr 42.000. Alternative splicing of the mRNA encoding VEGF generates six different isoforms (206, 189, 183, 165, 145, 121 amino acids) with varying heparin affinities (Robinson and Stringer, 2001). VEGF exerts its cellular functions by interacting with two tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (kinase domain region (Flk-1/KDR)), which are expressed

Abbreviations: bFGF, basic fibroblastic growth factor; ERK, extracellular signal-regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KDR/Flk-1, kinase domain region; MEK, MAP/ERK kinase; NRP-1, neuropilin 1; PI3-kinase, phosphatidylinositol 3′-kinase; RGP, radial growth phase; rh, recombinant human; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptors; VGP, vertical growth phase

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almost exclusively in endothelial cells (Neufeld *et al*, 2001). In addition, endothelial cells express the neuropilin-1 (NRP-1) and NRP-2 coreceptors, which bind selectively to the 165 amino acid form of VEGF ($VEGF_{165}$) (Miao and Klagsbrun, 2000). Recently, it has been reported that VEGFR-1 and VEGFR-2 are also expressed by the tumor cells of Karposi's sarcoma (Masood *et al*, 1997), ovarian, and breast cancer cell lines (de Jong *et al*, 1998), in choriocarcinoma (Charnock-Jones *et al*, 1994), and in prostate cell lines (Jackson *et al*, 2002) suggesting that the physiological role of the VEGF signaling pathway extends beyond angiogenesis in solid tumors. Melanoma cell lines are well adapted to growth in culture because of their low dependence on exogenous growth factors (Kath *et al*, 1991) but in spite of their tumorigenicity they are not always able to develop metastasis when injected into nude mice. Because of this, we have chosen the A375P cells in which the VEGF levels are undetectable, to study whether the forced expression of this factor might influence the metastatic behavior by directly evoking angiogenesis. Accordingly we set out to determine how signaling by VEGF protein enables cells to achieve this phenotype. As described below, overexpression of $VEGF_{165}$ promotes tumor growth and metastasis without an increase of vascular density in the primary tumor. VEGF-overexpressing tumors, however, accumulated slightly but consistently higher hemoglobin concentration on whole-tumor samples than controls. This new observation indicates that $VEGF_{165}$ also affects the angiogenic function. In so doing, in serum-starved cultures we found a high level of Flk-1 tyrosine phosphorylation and we demonstrated that its inhibition provokes an increase in apoptosis. Furthermore, we show that $VEGF_{165}$ may operate in melanoma cells as a survival factor through phosphoinositide 3-kinase (PI3K) activity. We thus provide evidence that VEGF signaling plays a different role from its traditional one as a vascular inducer and that this might contribute to the metastatic progression of these tumors.

Results

High levels of secreted $VEGF_{165}$ protein reduces the latency of tumor formation and accelerates growth kinetics without an increase in angiogenesis Earlier studies have demonstrated that VEGF production is beneficial but not required for melanoma tumorigenicity *in vivo* although it does accelerate tumor expansion when overexpressed (Graeven *et al*, 2001). Indeed, VEGF protein was undetectable in SK-Mel23, SK-Mel6, and parental A375 cells growing *in vitro* in which VEGF expression was only visualized by RT-PCR. We speculate whether the overexpression of VEGF might favor melanoma progression by tumor cell dissemination through induction of new blood vessels. To test this hypothesis, we ectopically expressed VEGF in the A375P cell line with relatively low levels of VEGF (only detected by RT-PCR) but able to form tumors that rarely develop metastasis. Expression of transgenes was evaluated in clones from exogenous $VEGF_{165}$ in sense orientation in which the vector was kept episomic (clone S4A-A375) or integrated into genomic DNA (clone S7B-A375). We also included clones with exogenous antisense $VEGF_{165}$-cDNA (AS7B and AS15A). The VEGF protein was detected in heparin-serum-free media of cultured clones by western blot and the amount of protein was quantified by ELISA (as shown in Fig 1*A* and *B*, respectively).

It is important to note that all five transfected cell lines grew *in vitro* at equivalent rates (without statistically significant differences) for up to 66 h in the presence of 5% fetal calf serum (FCS) (Fig 1*C*). In contrast, and as expected, the overexpression of $VEGF_{165}$ resulted in a robust tumorigenic growth *in vivo* with a significant reduction both in latency of tumor formation and in growth kinetics compared with the controls (Fig 2, *panels A* and *B*). In addition, the average size of tumors and kinetics of growth were tremendously reduced in the antisense $VEGF_{165}$ transfectants. At day 6 after inoculation, the average diameter of lesions in groups that received A375-$VEGF_{165}$ cells (S4A and S7A) was increased $\sim$ 4.5-fold as compared with those arising from control neo-A375 cells, whereas those groups with AS7B cells was $<$2-fold lower than those of A375P neo-1 cells (Fig 2, *panel A*). All mean values of tumors achieved statistically significant differences in comparison with controls as measured by the Mann–Whitney *U* test: A375P neo-1 *versus* A375P S4A, $p = 0.0177$; A375P neo-1 *versus* A375P S7A, $p = 0.0046$.

Accordingly, the differences in tumor volume doubling time were found to be significant between the groups of animals (A375P neo-1 *vs* A375P S4A (sense), $p = 0.010$; CI 95% (5.65; 23.95); A375P neo-1 *vs* A375P S7A (sense), $p = 0.018$; CI 95% (20.46; 39.14)). In contrast, there were no differences when comparing A375P neo-1 control group and A375P antisense $VEGF_{165}$ groups (Student's *t* test). Is important to note that in spite of the delay in tumor formation and kinetics, no spontaneous regression occurred in the antisense $VEGF_{165}$ group.

We next investigated whether the levels of $VEGF_{165}$ protein affected the latency and growth of tumors by increasing angiogenesis. On day 6 after the injection we excised very small tumors growing in the dermis of the animals to evaluate vascular density. Our previous experience has taught us that differences in vascular density should be evaluated at a very early stage of tumor development because, once angiogenesis is under way, new blood vessels form at comparable rates and tumors grow to similar sizes.

The sections were stained with anti-vWF VIII antigen and examined for numbers of intra/peritumoral vessels in 10 high power fields ($\times$ 200). It is noteworthy that no significant differences were found in vessel count between tumors overexpressing and underexpressing VEGF (Fig 2, *panel C*).

In addition, tumors harvested at days 10, 14, and 20 were assayed for hemoglobin content by Drabkin's method. We determined hemoglobin concentration on whole-tumor samples and then normalized to tumor weight. Our results show that the VEGF-overexpressing tumors accumulated slightly but consistently higher hemoglobin concentration on whole-tumor samples than controls at very early tumor development. The significative differences are found at day 14 after implantation of S4A cells into nude mice in comparison with controls as measured by the Mann–Whitney *U* test: A375P neo-1 *vs* A375P S4A, $p = 0.019$. In the antisense $VEGF_{165}$ tumors the hemoglobin content using

Drabkin's method was measurable from day 20 after implantation. In contrast, the $VEGF_{165}$ overexpression had no significant effect on hemoglobin content when the weight of tumors is 0.07 g or more. These data indicate that the accelerated S4A tumorigenesis, relative to AS7B, correlates with $VEGF_{165}$ production that might enhance tumorigenesis by promoting angiogenesis. Since no evidence of an increased vessel density was observed in S4A tumors, we suggest that $VEGF_{165}$ overexpression might cause architectural and functional changes on the initiated tumor vasculature, such as large lumina or dilation of the blood vessels that allow the hemoglobin accumulation.

To further investigate whether low levels of $VEGF_{165}$ provoked the tumor cell death that caused this delay in tumor formation or not, we analyzed apoptosis in tumors excised 15 d after inoculation. None of the tumors showed the pattern of DNA-ladder characteristic of apoptotic cells (results not shown).

These observations suggested that $VEGF_{165}$ overexpression clearly benefits the early expansion of tumors by contributing to angiogenesis.

Overexpression of $VEGF_{165}$ confers an advantage for metastatic development To assess potential changes in tumor progression mediated by $VEGF_{165}$ overexpression, tumors were removed when they reached 0.4 cm^3 in volume and animals were kept alive and followed until they became moribund or killed after 261 d. We found that differences in tumor growth rates were maintained as described above, between the S4A and S7B and control or antisense VEGF transfectants. The vessel density was examined in all these large tumors and again no differences were found between the tumors (results not shown).

After 70–80 d, 100% of $VEGF_{165}$ overexpression tumor-bearing mice (A375P S4A cells) had developed lung metastasis, as compared with only 20% of control mice. In contrast, animals that received cells in which VEGF production was ablated did not reveal any organ metastasis at autopsy (Table I).

$VEGF_{165}$ is a survival factor for melanoma cells through the Flk-1/KDR, extracellular signal-regulated protein kinase (ERK) 1/2 and PI3K signaling pathway To account for the possible autocrine effects of VEGF, we explored the expression of its receptors in melanoma cell lines. VEGF exerts its biological functions through interaction with its cognate receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), as well as a $VEGF_{165}$-specific receptor NRP-1 recently described (Miao *et al*, 2000). Using quali-

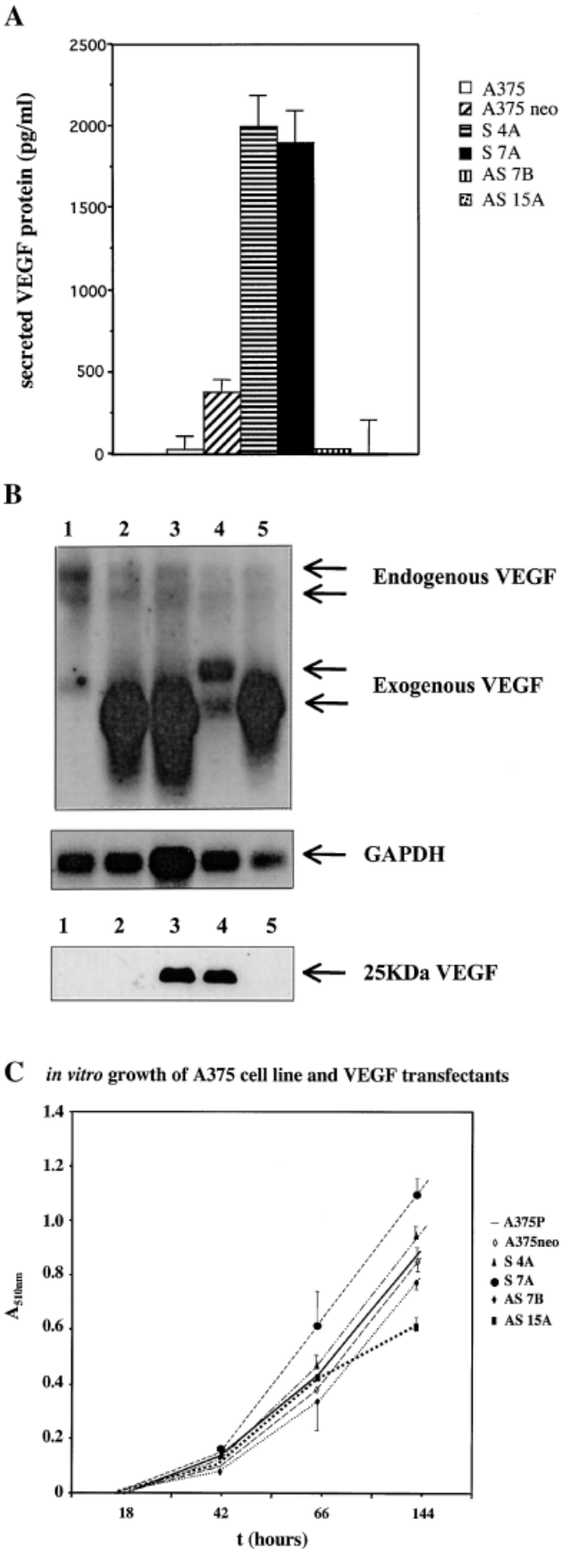

Figure 1
Expression of vascular endothelial growth factor $(VEGF)_{165}$ affects the proliferation of A375 melanoma cells *in vitro*. (*Panel A*) The histogram corresponds to the quantification of VEGF by ELISA in serum-free conditioned media of A375P cell line or the $VEGF_{165}$ transfectants (clones S4A and S7A and $VEGF_{165}$ clones in antisense orientation AS7B and AS15A). Bars represent values of ELISA measurements ± SEM of three independent experiments. (*B*, *top panel*) VEGF northern blot analysis of $VEGF_{165}$ in A375 cells grown *in vitro*. (*B*, *middle panel*) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript as control for RNA loading. (*B*, *bottom panel*) Western blot analysis of $VEGF_{165}$ in the serum-free conditioned media of A375 cells. *Lane 1*, A375P neo; *lane 2*, S4A; *lane 3*, S7A; *lane 4*, AS7B; and *lane 5*, AS15A. (*Panel C*) Cell proliferation of A375 cells and the selected clones of $VEGF_{165}$ transfectants growing *in vitro* in the presence of 5% fetal calf serum. The biomass was measured by SRB staining and values corresponds to the mean of absorbance ($A_{510\ nm}$) ± SEM of three independent experiments assayed in quadruplicate.

tative RT-PCR, we analyzed the expression of all three receptors in melanoma cell lines. We only observed the expression of VEGFR in the most aggressive cell lines SK-Mel6 and A375, which are highly tumorigenic and able to develop metastases in immunodeficient mice. The A375 cell line and $VEGF_{165}$ transfectants growing *in vitro* all showed similar levels of KDR/Flk-1, Flt-1, and NRP-1 expression evaluated by RT-PCR (Fig 3). KDR protein was slightly increased in sense $VEGF_{165}$-A375 cells, suggesting an upregulation of this receptor as a consequence of $VEGF_{165}$ overexpression (Fig 4). This observation is in agreement with the previous reports indicating that overexpression of VEGF led to post-transcriptional upregulation of the KDR receptor in endothelial cells (Weisz *et al*, 2001).

To provide functional evidence of the role of VEGF in the acquisition of metastatic phenotype, we investigated the signaling pathways between VEGFR and $VEGF_{165}$ in autocrine cell proliferation. To this purpose, we plated the cells in the presence of 5% FCS and then switched to serum-free media and incubated the cells for an additional 72 h. For the last 24 h, cultures were incubated with and without specific inhibitors of MAPK, PI3K, or exogenous $VEGF_{165}$. Firstly, we observed that all A375 cells responded to the addition of 2% FCS by increasing their DNA synthesis up to 2.2-fold with respect to the starved cultures. In contrast, the addition of recombinant human VEGF ($rhVEGF_{165}$) had no obvious effect on DNA synthesis (shown in Fig 5, *panel A*). Taken together, these results suggested that supplementary addition of this cytokine was not necessary for cell proliferation. In order to study the signaling pathway through $VEGF_{165}$ and VEGFR-2/KDR, we incubated cells with SU 1498, a specific inhibitor of KDR receptor phosphorylation. We found that SU 1498 (12.8 μM) reduced the incorporation of [methyl ^{3}H]thymidine into all A375 cell lines by $>70\%$ with respect to their respective baseline levels. PD 98059, a specific inhibitor of MEK1, also reduced the DNA synthesis up to $>80\%$ with respect to the controls. In contrast, SB 203580, a specific inhibitor of p38 MAPK, only had a moderate but not significant inhibitory effect on AS7B cell proliferation (Fig 5, *panel A*).

Previous studies have shown that the PI3K-specific inhibitor wortmannin is able to block the mitogenic action of nerve growth factor and platelet-derived growth factor in serum-deprived PC12 cells (Yao and Cooper, 1995). To explore whether PI3K was involved in the autocrine growth pathway of VEGF/VEGFR, we incubated serum-deprived $VEGF_{165}$-A375 cells with wortmannin (30–200 nM). We observed that in the presence of this inhibitor, cells appeared as healthy and well attached to the plate as the untreated controls. Moreover, incorporation of [methyl ^{3}H]thymidine into cells was not affected by wortmannin (data not shown).

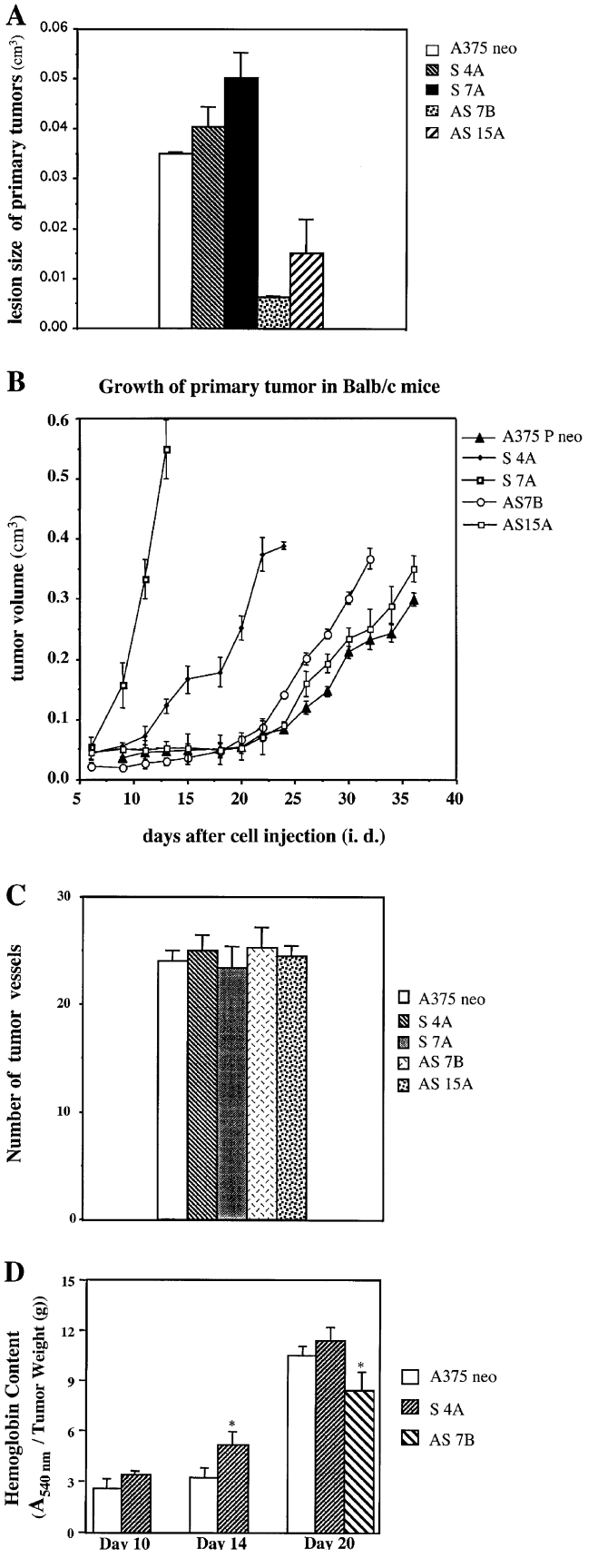

Figure 2
Effect of vascular endothelial growth factor $(VEGF)_{165}$ expression on tumor growth and angiogenesis. (*A–C*) 10^6 A375 cells and $VEGF_{165}$ transfectants were injected into the dermis of BALB/c nude mice. (*A*, *top panel*) The histogram shows the mean of lesion size expressed as volume (cm^3) of tumors from eight mice at day 6 after injection; *bars*, SD. (*Panel B*) The growth rate of i.d. tumors implanted in nude mice. Data is presented as the mean of volume (cm^3) from 10 tumors per group; *bars*, SE. (*C*, *top panel*) Microvessel density analysis of tumors at day 6 after injection. The mean number of blood vessels/field for eight individual tumors derived from each of the cell lines; *bars*, SD. (*D*, *bottom panel*) Mean hemoglobin content of whole tumors at days 10, 14, and 20; *bars*, SD. *Statistically significant increase ($p<0.05$).

Table I. Incidence of spontaneous and experimental metastasis *in vivo* by A375 human melanoma cells and vascular endothelial growth factor $(VEGF)_{165}$ A375 transfectants

Experiment	Cell line	Tumorigenicity	Tumor latency	Pulmonary metastasis				Day of sacrifice[a]
				Spontaneous metastasis[a]	Experimental metastasis[a]	Lung foci/mouse[b]	Weight of lungs (g)[c]	
I[d]	A375 P-neo	10/10	27 ± 4	0/10		0	ND	261
	A375 S4A	10/10	10 ± 2	8/10		3–11	ND	60–72
	A375 AS7B	8/8	30 ± 5	0/8		0	ND	261
II[e]	A375 P-neo				5/6	3–9	0.35 ± 0.26	75–85
	A375 S4A				7/7	1–22	0.45 ± 0.19	35–75
	A375 AS7B				0/5	0	0.2 ± 0.05	85
III[d]	A375 P-neo	5/5	23 ± 3	1/5		3	0.2 ± 0.05	200
	A375 S4A	6/6	14	6/6		2–16	0.35 ± 0.1	57–90
	A375 AS7B	6/6	34 ± 2	0/6		0	0.2 ± 0.05	200

[a]The mice were killed at the indicated days after tumor cell implantation in each experiment and pulmonary foci were examined.
[b]The incidence of tumors and metastasis is expressed as the number of mice that developed tumors or lung metastatic foci/total number of inoculated mice.
[c]The data indicate the weight (g) of lungs removed when mice were sacrificed at the days indicated above.
[d]Groups of BALB/c nude mice were inoculated with 1×10^6 cells intradermally in the left flank and tumors were excised when they reach 0.4 cm^3 diameter.
[e]Groups of BALB/c nude mice were inoculated with 1×10^6 cells intravenously (caudal vein tail).

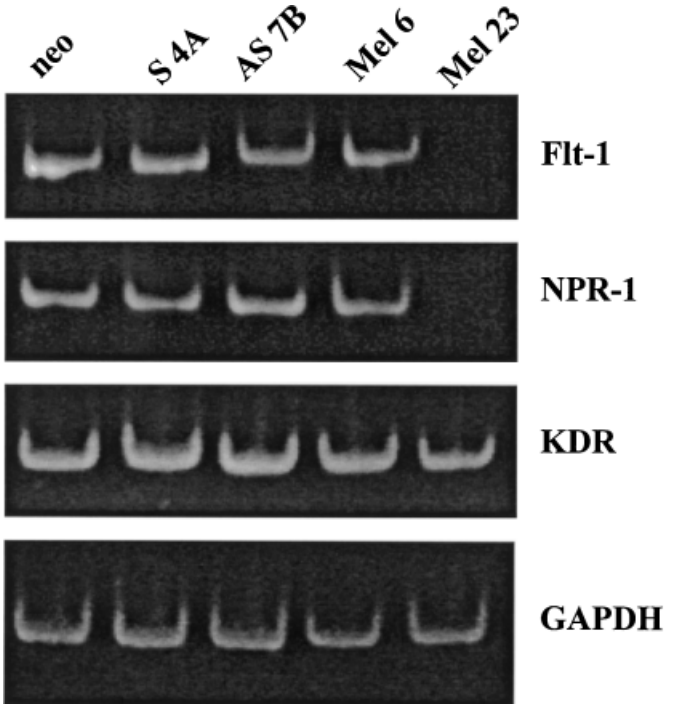

Figure 3
Detection of vascular endothelial growth factor (VEGF) receptors in human melanoma cell lines. The presence of Flt-1, neuropilin-1 (NRP-1), and kinase domain region (KDR) transcripts in neo- and $VEGF_{165}$-transfected A375 clones (S4A and AS7B), in highly invasive Mel-6 and in non-invasive Mel 23 melanoma cell lines was analyzed by RT-PCR (see Material and Methods). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed in the same samples as a control for loading.

In agreement with the previous reports (Gerber *et al*, 1998), these results indicate that PI3K was not involved in the proliferation of these melanoma cells.

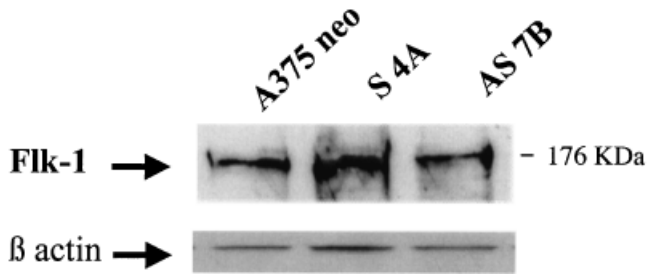

Figure 4
Kinase domain region (KDR) protein was detected in A375 transfectants. The expression of Flk-1/KDR protein was analyzed on lysates from untreated cultures of A375-neo and vascular endothelial growth factor 165-transfectant cells by western blot. The bands at the *top* represent an immunoblot with an antibody specific for phosphorylated Flk-1 protein. The *bottom* panel represents an immunoblot with an antibody for β-actin.

Additionally, it was intriguing that in these previous experiments, done under starvation conditions, we did not observe a significant increase in the number of apoptotic cells in any of the A375 cultures. We suggest that VEGF, as well as other autocrine factors (such as bFGF or ILGF among others), might render the cells more resistant to apoptosis. Whether this is a consequence of increased survival activity or attributable to ongoing proliferation, or both, remains to be determined. Thus, we focused on our previous observation of induction of cell death by KDR/VEGF selective signaling inhibitors added to serum-starved S4A cultures. Treatments with 12.8 μM or low concentrations of SU 1498 maintained 80% of the cells alive for the following 48 h. To extend these observations, we incubated serum-starved S4A cultures with 100 μM SU 1498 and they underwent apoptotic cell death starting 18 h after the addition of the inhibitor. We consistently observed membrane

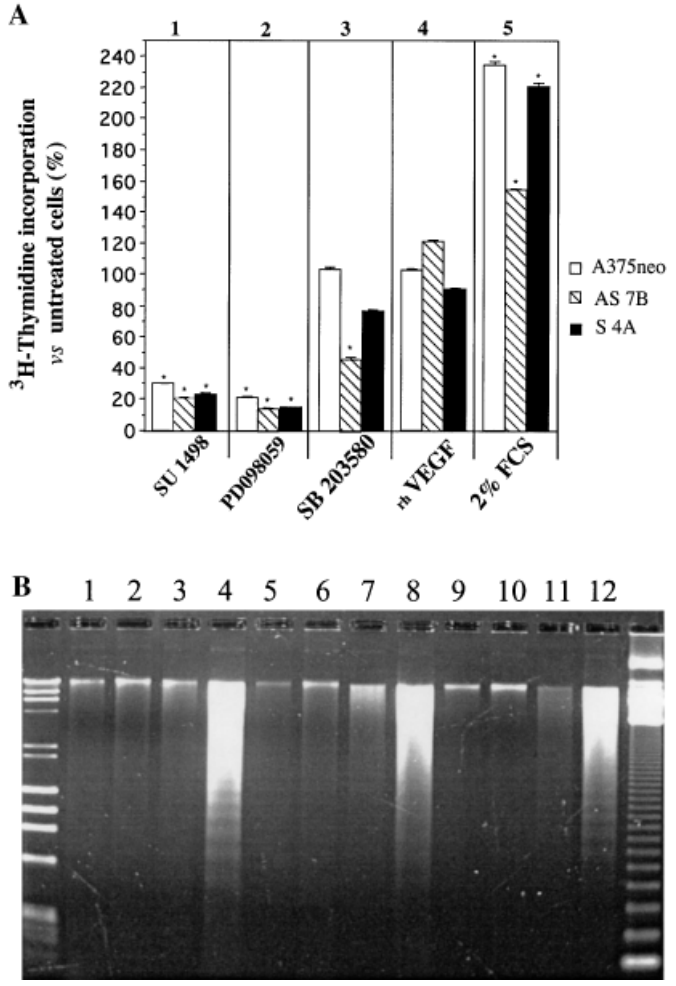

Figure 5
Different effects of specific signaling inhibitors (kinase domain region phosphorylation, MAPK, p38 MAP kinase, phosphatidylinositol 3′-kinase) or exogenous vascular endothelial growth factor (VEGF)$_{165}$, on DNA synthesis and survival in cultured A375P and VEGF$_{165}$-transfected clones. (*Panel A*) A375P neo (*white bars*) and VEGF$_{165}$ transfectants (antisense VEGF$_{165}$ clone 7B (*hatched bars*) and sense VEGF$_{165}$ clone 4A (*solid bars*)) were treated with the inhibitors SU 1498, PD 098059, SB 20380 as well as with 50 ng per mL recombinant human (rh)VEGF or complete media containing 2% fetal calf serum (FCS) (five different treatments are shown). DNA synthesis was assessed by addition of 0.5 μCi per mL [methyl ^{3}H]thymidine (see Material and Methods). Values (c.p.m. per well) are expressed as the percentage of controls (serum-free media containing equivalent concentration of DMSO was considered the 100%); *bars*, SD. *Statistically significant increase ($p < 0.05$). Here reproducible data is shown of one of three independent experiments assayed in triplicate. (*Panel B*) Agarose gel electrophoresis of DNA fragmentation in neo- and VEGF$_{165}$-transfected A375 clones (S4A and AS7B) under serum-free media (*lanes 1*, *5*, and *9*) or in the presence of 50 ng per mL rhVEGF (*lanes 2*, *6*, and *10*), SU 1498 (12.8 μM) (*lanes 3*, *7*, and *11*), and PD 098059 (25 μM) (*lanes 4*, *8*, and *12*). Treatments of A375P neocells are shown in *lanes 1–4*, the VEGF$_{165}$-antisense AS7B cells are shown in *lanes 5–8* and VEGF$_{165}$-sense S4A cells are shown in *lanes 9–12*. On the left and right, migration of DNA size markers in the same gel is shown. The data shown is representative of three similar and reproducible experiments.

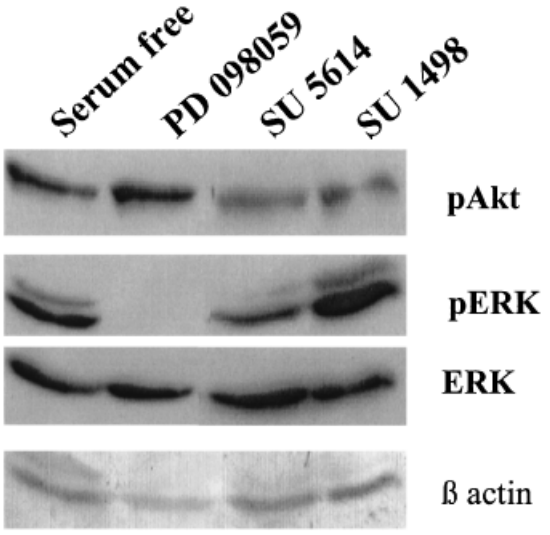

Figure 6
Effect of kinase domain region (KDR)-phosphorylation inhibition on extracellular signal-regulated protein kinase (ERK) and Akt activation in A375 overexpressing vascular endothelial growth factor (VEGF)$_{165}$ cultured cells. Representative western blotting with anti-dually phosphorylated ERK 1/2 Ab (αp-ERK 1/2) or total ERK in VEGF$_{165}$-A375P-transfected cell lysates (clone S4A) exposed to KDR-phosphorylation inhibitor SU 5416 or SU 1498 (25 μM) in comparison with the untreated cultures. Addition of PD 098059 was included as a control for total abrogation of the p-ERK 1/2. p-Akt was analyzed in the same blot using a specific antibody (see Material and Methods).

blebbing, loss of contact with neighboring cells, shrinkage of the cytoplasms, and disintegration into small vesicles (not shown). Similar results were obtained with treatments with SU 5614 (a different inhibitor of VEGFR tyrosine kinases), indicating that both inhibitors provoked cell-death in a concentration-dependent manner. These results confirmed the role and specificity of VEGF/KDR signals sustaining the survival of melanoma cells under adverse culture conditions such as serum starvation.

A convergent pathway in the mitogenic action of many growth factors, including VEGF, is the MAPK cascade. We found that incubation with PD 98059 induced the cell death morphology described above. These results were corroborated by the observation of a DNA ladder pattern characteristic of apoptosis, which resulted from internucleosomal cleavage of the genomic DNA, (Fig 5, *panel B*).

We next examined whether the MAPK cascade was influenced by the treatment of cells with SU 1498 in starved cultures. Western blot was performed using extracts from overexpressing VEGF$_{165}$ and a specific antibody for the activated Thr^{183}- and Tyr^{185}-phosphorylated form of MAPK. Our results revealed that both p42 and p44 forms of ERK, remained phosphorylated both in the presence and absence of SU 1498. Treatment with SU 5614 inhibitor showed a significant reduction mainly in the phosphorylated p44 form of ERK. Neither SU 1498- nor SU 5614-specific inhibitors, however, completely abrogate the phosphorylation of ERK proteins. In contrast, treatment with PD 098059 completely suppressed the phosphorylation of ERK proteins although there was no significant change in the total MAP/ERK protein (as seen by reprobing the membranes using a specific antibody against total ERK1 and ERK2 proteins; Fig 6). These results demonstrated that direct and selective activation of ERK 1/2, at least in part, mediates the survival of these melanoma cells.

Furthermore, the survival of A375 cells in our conditions of normoxia, was not affected by wortmannin treatment (30 nM) as assessed by the integrity of the DNA (Fig 7).

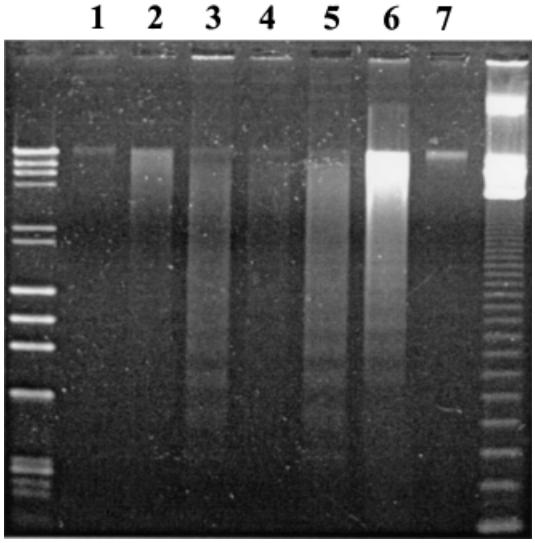

Figure 7
Induction of DNA fragmentation by kinase domain region (KDR)-phosphorylation inhibition and wortmannin in A375 overexpressing vascular endothelial growth factor (VEGF)$_{165}$ *in vitro*. The induction of DNA fragmentation by MAPK/extracellular signal-regulated protein kinase inhibitor PD 098059 and SU 1498 a specific inhibitor of VEGFR-2 receptor phosphorylation was analyzed alone or in combination with other inhibitors on serum-starved cultures of S4A cells. *lane 1*, untreated cells; *lane 2*, cells treated with SU 1498 (12.8 μM); *lane 3*, cells treated with SU 1498 (25 μM); *lane 4*, cells treated with Wortmannin (30 nM); *lane 5*, cells treated with SU 1498 (12.8 μM) and Wortmannin (30 nM); *lane 6*, cells treated with PD 098059 (25 μM); *lane 7*, cells treated with SB 20380 (3 μM). On the left and right are shown the migration of DNA size markers in the same gel. The representative data of one of three similar and reproducible experiments are shown.

Under double treatment with Wortmannin and SU 1418 (12.8 μM) kinase inhibitor, however, cells rapidly underwent apoptotics as shown by the DNA ladder pattern (Fig 7, *lane 5*). Such results indicate that a VEGF$_{165}$-dependent autocrine survival loop may operate in melanoma cells in a PI3K activity-dependent manner, as demonstrated by the inhibitory effect of the wortmannin.

Finally, we investigated whether the treatment with SU 1498 might influence the phosphorylation of Ser^{473}-Akt or not. Western blot using a specific antibody demonstrated that this form, although reduced, was not completely abrogated (shown in Fig 6), suggesting that the signaling through other receptors present in such melanoma cells may converge in this pathway.

Discussion

It has been reported that expression of VEGF correlates with patient outcome (Straume and Akslen, 2001) and aggressiveness of human melanoma xenografts (Claffey *et al*, 1996; Pötgens *et al*, 1996). Although these studies demonstrated that overexpression of VEGF promotes tumor growth, angiogenesis and metastasis *in vivo*, therefore, shed no light on the mechanisms, whereby these cells acquired an advantadge for progression covered up by the belief that VEGF exclusively influenced angiogenesis. To this effect, the expression of functional VEGFR on human melanoma cells suggests the intriguing possibility that VEGF might also exert autocrine effects on the tumor cells themselves (Gitay-Goren *et al*, 1993; Liu *et al*, 1995). Indeed, a potential autocrine role for VEGF$_{165}$ and VEGFR has been suggested for prostate cancer (Jackson *et al*, 2002) and angiosarcoma (Arbiser *et al*, 2000) as was previously described for bFGF in melanoma cell lines (Halaban *et al*, 1991).

To assess an autocrine role of VEGF on melanoma cells we developed an experimental system using stable down- or overexpression of VEGF$_{165}$ in A375 human melanoma cells, which normally express only low baseline VEGF levels. Based on our previous results showing the functional role of Flk-1/KDR receptors in this cell line (Liu *et al*, 1995), we explored whether the VEGF overexpression stimulates tumor growth via the VEGFR-2/KDR-dependent autocrine mechanism. Our findings *in vitro* provide evidence that under serum-starved conditions, cell proliferation is, at least in part, mediated by an autocrine loop involving the Flk-1/KDR receptor and secreted VEGF$_{165}$, as demonstrated by the inhibitory effect of the specific inhibitor of VEGFR-2 receptor phosphorylation SU 1498. Moreover, the activation of ERK 1/2 was the key for DNA synthesis, as we demonstrated by the treatment of cells with the specific inhibitor PD 098059. These results bring us to the conclusion that activation of ERK is required for autocrine proliferation of these melanoma cells. Our results are consistent with recent findings (Vega-Diaz *et al*, 2001) showing the existence of an autocrine loop for VEGF in human dermal microvascular endothelial cells. While this paper was in progress, another study reported that the MAPK activation functionally contributes to the development of melanoma, as the introduction of a constitutively active MAPKK into melanocytes leads to transformation *in vivo* (Govindarajan *et al*, 2003). Furthermore, we demonstrated that the activation of ERK 1/2 in melanoma cells also works as a survival pathway as other authors reported in endothelial cells (Takahashi *et al*, 1999; Kanno *et al*, 2000; Shan *et al*, 2004) or cortical neurons *in vitro* (Ogunshola *et al*, 2002).

The levels of the activated Thr^{183} and Tyr^{185} (phosphorylated) form of MAPK were, however, not completely suppressed after the treatment with either SU 1498 or SU 5614, suggesting that other autocrine signaling converges with the activation of the MEK-ERK pathway. Interestingly, the exogenously added VEGF did not lead to either a significant increase of ^{3}H-thymidine incorporation or affect survival. Although not studied here, this observation suggests that the endogenous ligand produced by these cells at sufficiently high levels might saturate the available binding sites of the receptor. Similar effects of intracrine stimulation in cells expressing a growth factor and its cognate receptors have been described before (Valgeirsdottir *et al*, 1995). Therefore, our results investigating phospho-Akt demonstrate that disruption of the KDR pathway does not completely inhibit all VEGF$_{165}$-mediated signal transduction. This suggests that there are divergent signal transduction pathways activated by VEGF$_{165}$.

In addition to the functional VEGFR-2/KDR, we have shown here the expression of VEGFR-1/Flt-1 and NRP-1 receptors in A375 melanoma cells. With regard to VEGFR-1/Flt-1, our results do not support the idea that this receptor controls the DNA synthesis of melanoma cells by activation

of p38. Furthermore, SB 203580, a specific inhibitor of p38 MAPK, did not abrogate the synthesis of DNA in serum-starved A375 melanoma cells. Thus, we consider the possibility that VEGF/Flt-1 might activate p38 MAK kinase in melanoma cells and transduce the signal for other cell functions such as cell migration, as has been demonstrated in endothelial cells (Kanno *et al*, 2000).

Little is known about the role of NRP-1 in melanoma cells and its contribution to this autocrine loop, but it does not appear to be a growth-signaling factor by itself (Miao *et al*, 2000). NRP-1 appears to function in endothelial cells as a coreceptor that enhances the binding of $VEGF_{165}$ to KDR and its subsequent bioactivity (Bachelder *et al*, 2001; Soker *et al*, 1998). The importance of this receptor in maintaining breast carcinoma (Bachelder *et al*, 2001) or hematopoietic stem cell survival has been recently reported (Gerber *et al*, 2002). In spite of the fact that such a mechanism has not yet been elucidated, these studies suggest that NRP supports the $VEGF_{165}$ survival function in cells lacking KDR expression by stimulating the PI3K pathway. In agreement with these findings we attribute a promoting survival role to the PI3K contributing to the signaling elicited by KDR. This data indicates that PI3K may prolong cell survival independently of high-affinity receptor phosphorylation. Whether or not NRP-1 is mediating this survival signal through PI3K remains to be investigated. It is conceivable that survival pathways mediated by NRP-1 might play a role in progression until either growth factor supply *in vitro* is provided, or endogenous $VEGF_{165}$ protein levels are sufficient to upregulate KDR or angiogenesis is initiated *in vivo*.

On the other hand, VEGF-overexpressing cells formed rapidly expanding tumors *in vivo* in comparison with the antisense cells. As in this study, earlier reports also demonstrated that *in vivo* expansion of melanoma cells expressing VEGF antisense was delayed but not abrogated (Graeven *et al*, 2001). Of note, when microvessel density was determined at day 6 after implantation we did not find a relationship between VEGF expression and vessel density in these early tumors. Since bFGF (Singh *et al*, 1995) and angiopoietins (Armstrong *et al*, 1992; Holash *et al*, 1999; Siddiqui *et al*, 2001) have been reported to be produced by these A375 melanoma cells themselves or induced by local host factors (Rofstad and Halsor, 2001; Li *et al*, 2003), we suspect that VEGF might not represent the exclusive angiogenic activity in these tumors (Dvorak *et al*, 1995; Streit and Detmar, 2003). Thus, we suggest that these proteins would provide the angiogenic stimulus to initiate sprouting angiogenesis and sustain blood vessel network *in vivo*, when VEGF is downregulated. It should, however, be noted that VEGF-overexpressing tumors accumulated slightly but consistently higher hemoglobin concentration on whole-tumor samples than controls at early tumor development (<0.07 g), indicating that $VEGF_{165}$ also affects the angiogenic function. Because microvessel densities were comparable with controls (at least at day 6 after implantation), we suggest that $VEGF_{165}$ overexpression might cause architectural and functional changes on the co-initiated tumor vasculature, such as large lumina or dilation of the blood vessels (Graeven *et al*, 2001; Kusters *et al*, 2002). On the other hand, we propose that the same causes for the delay of tumor growth rates observed in the $VEGF_{165}$-antisense clones might also influence the growth of metastasis at the secondary sites (doubly delayed by low angiogenesis and low cell proliferation rates).

In conclusion, we report here an autocrine loop between $VEGF_{165}$ and VEGFR-2 (when both ligand and receptors are coexpressed in the same tumor cells) that promotes, at least *in vitro*, the survival and proliferation of cells under conditions of stress or restricted growth factor supply. Taken together, our results highlight that overproduction of $VEGF_{165}$ besides its angiogenic function might confer an advantage to melanoma cells for progression through an autocrine signaling loop.

With regard to therapy, initial attempts by using specific inhibitors, humanized monoclonal antibodies, or by administering decoy-soluble receptors are beginning to show promise in human cancer patients, underscoring the importance of optimizing VEGF blockade (Holash *et al*, 2002; Wedge *et al*, 2002). Until now all these efforts were designed to fight tumor-induced neovascularization, but our results suggest that blockade of $VEGF_{165}$ functions *in vivo*, might provide a benefit in that tumor progression might be doubly down. These issues are currently under investigation in our laboratory.

Material and Methods

Cell and culture conditions The human melanoma cell line A375P (Kozlowski *et al*, 1984) was obtained from Dr Janet Price (MD Anderson Cancer Center, University of Texas). SK-Mel23 and SK-Mel6 human melanoma cell lines exhibiting low and high aggressiveness *in vivo* (Houghton *et al*, 1987) were generously provided by Dr Anna Bassols (Universitat Autonoma de Barcelona, Fac. Veterinaria, Spain). The cells were grown on plastic in an antibiotic-free mixture of Dulbecco's modified Eagle's and Ham's F12 (1:1 DMEM/F12) medium, supplemented with 10% (v/v) heat inactivated fetal bovine serum, 1 mM piruvate, and 2 mM L-glutamine and incubated in a humidified atmosphere of 5% CO_2 in air, subcultured twice a week after trypsinization (trypsin-EDTA). All solutions were purchased from Life Technologies (Life Technologies Inc., Gaithersburg, Maryland). Cultures were verified to be free of mycoplasma contamination using PCR methods (Minerva Biolabs, Berlin, Germany).

Plasmid and gene transfer The full-length coding region of the human $VEGF_{165}$ amino acid cDNA was kindly provided by Dr Len Seymour (CRC, Birmingham University, UK) and cloned in sense or antisense orientation into the expression vector pRC/cytomegalovirus (CMV) neo (Invitrogen, San Diego, California) under the transcriptional control of the CMV promoter.

For stable transfections A375P cells were seeded at a density of 1×10^5 to 2×10^5 into 35 mm dishes, and transfected with 2 μg of DNA by Lipofectamine Reagent (Life Technologies Inc.) according to the manufacturer's specifications. Stable transfectants were selected after 15–21 d growth in the presence of 500 μg per mL of G418 in media. Single colonies were isolated and plated into a 2 cm^2 well and then expanded.

Northern blot analysis and RT-PCR Approximately 50 μg of total RNA were applied to a 1.1% agarose gel and electrophoresed in the presence of 2.2 M formaldehyde as previously described in northern blot protocols. RNA was transferred to a nylon membrane (Amersham, Little Chalfont, UK) and hybridized with human $VEGF_{165}$-cDNA as a probe. Hybridization was performed at 42°C under high-stringency conditions using ^{32}P-labeled probe (4×10^8 c.p.m. per mg). After hybridization with the specific probe, the filters were stripped and rehybridyzed with glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) probe, as a control for RNA loading. Each relative RNA expression was compared with the intensities of transcripts and those of GAPDH in each lane.

mRNA was obtained from cultured cell lines with a Fast-track isolation kit (Invitrogen). Fifty nanograms of Poly $(A)^+$-RNA were reverse transcribed in a total reaction volume of 20 μL. This contained 1 × RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; and 3mM $MgCl_2$), 20 U RNase inhibitor (Promega, Madison, Wisconsin), 10 mM dithiothreitol, 50 μM deoxyribonucleoside triphosphates (dNTP), 200 U Moloney murine leukemia virus reverse transcriptase (M-MLV-RT; Life Technologies), and 0.5 μM of one or other of the following primers: KDR-R: 5′-CTTCATCAATCTTTACCCC-3′; or random hexamers. Oligodeoxynucleotides were purchased from Pharmacia Biotech (Genosys, Cambridge, UK). The mixture was incubated at 37°C for 60 min and 95°C for 5 min, using a programmable thermal cycler (PTC-100 TM; MJ Research, Watertown, Massachusetts) and stored at −80°C or used immediately.

One microliter of reaction mix was used for each PCR that included 1 pmol of each specific primer, 200 μM each dNTP, 2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1 U of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The reaction was performed in a thermal cycler for 35 high-stringency cycles (94°C for 30 s and 65°C for 45 s for high-stringency annealing of primer and 72°C for 45 s for extension).

The sequences of the oligonucleotides were as follows:

NRP-1 (designed from the GeneBank sequence no. AF 145712): NP1-F: 5′-AGGACAGAGACTGCAAGTATGAC-3′ and NP1-R: 5′-AACATTCAGGACCTCTC TTGA-3′ (amplifies a fragment of 210 bp).

VEGFR-1/Flt-1 (designed from the GeneBank sequence no. AF063658): Flt-1-F: 5′-GTCACAGAAGAGGATGAAGGTGTC-3′ and Flt-1-R: 5′-CACAGTCCGGCACGTAGGTGATT-3′ (amplifies a fragment of 335 bp) and *VEGFR-2/KDR*: KDR-F3: 5′-CCTGGCGGCACGAAATATCCTC-3′ and KDR-R4: 5′-GACCAGACGTCACTGTGGATTG-3′ (amplifies a fragment of 186 bp).

VEGF (designed from the GeneBank sequence no. AB 021221: VEGF F: 5′-TCCAGGAGTACCCTGATGAG-3′ and VEGF R: 5′-TCACCGCCTCGGCTTG TCACA-3′ (amplifies a fragment of 391 bp).

GAPDH (designed from the GeneBank sequence no. AF 261085): GAPDH F: 5′-CCATGGAGAAGGCTGGGG-3′ and GAPDH R: 5′-CAAAGTTGTCATGGATG ACC-3′ (amplifies a fragment of 175 bp).

Western blot hybridization Two microliters of fresh serum-free medium containing Heparin (100 μg per mL) (Sigma Chemical Co. St. Louis, Missouri) were incubated for 48 h with selected clones growing in 24-well plate (TPS, Trasadingen, Switzerland). The protein concentration was determined by BCA (Pierce, Pierce Europe, oud-Beijerland, Holland) using bovine serum albumin (BSA) as a standard. Samples containing 50 μg of total protein were mixed with Laemmli buffer sample, heated at 95°C for 5 min before carrying out SDS-PAGE (Bio-Rad Mini Vertical Electrophoresis standard protocol Bio-Rad, Hercules, California) using a 12% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Buckinghamshire, England).

Additionally, A375 cells and transfectants were treated with 12–100 μM SU 1498 or SU 5614 (Calbiochem, La Jolla, California) in DMSO or with DMSO alone for 24 h. Cells were lysed in lysis buffer containing 50 mmol per liter PBS, pH 7.2, 5 mmol per liter EDTA, 5% glycerol and 0.3 mmol per liter PMSF. The protein concentration was evaluated for cell extracts and samples were electrophoresed on SDS-PAGE as described above.

Membranes were blocked with blocking buffer (5% dry milk, 10 mmol per liter Tris-HCl, pH 7.5, 10 mmol per liter NaCl, 0.1% Tween-20) and subsequently incubated with the appropriate antibodies. A mouse monoclonal anti-human VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, California) was used to detect VEGF production in conditioned media. The cell-extract membranes were incubated with a phospho-VEGFR-2 polyclonal antibody (Cell Signaling Technologies, New England BioLabs, Beverly, Massachussetts). p44/p42 MAPK antibody and a phosphoPlus p44/42 MAPK antibody were used to analyze the phosphorylation status of Erk1 and Erk2 (Cell Signaling Technologies). For detection of the levels of Akt phosphorylation we used the PhosphoPlus Akt (Ser 473) antibody (New England BioLabs, Beverly, Massachusetts). The blots were incubated first with the above antibodies and subsequently with an anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase (Amersham Corp., Arlington Heights, Florida) as a secondary antibody. Immunoreactive bands were visualized by using an enhanced chemiluminiscence kit ECL (Amersham Corp.).

Quantitation of $VEGF_{165}$ by ELISA Levels of $VEGF_{165}$ in conditioned media of A375 cell lines were quantified by a modified colorimetric sandwich ELISA (R&D Systems, Minneapolis, Minnesota). For the detection of $VEGF_{165}$, individual wells of a 96-well microtiter plate were coated with 100 μL of a 3 μg per mL solution of an anti-$VEGF_{165}$ polyclonal antibody (AF-293-NA, R&D Systems) in 50 mM sodium carbonate buffer, pH 9.6, overnight at 4°C. The wells were then washed with PBS containing 0.03% Tween-80 and blocked by 0.5% BSA in the same buffer for 1 h at room temperature. Aliquots of diluted samples (200 μL) and $VEGF_{165}$ standard (recombinant protein 293-VE supplied by R&D Systems, ranging from 15.6 to 1000 pg per mL) were added to each well and incubated for 2 h at room temperature. The supernatant was discarded and the wells washed. One hundred microliters of anti-VEGF murine monoclonal antibody (MAB293, R&D Systems) solution (1 μg per mL) was added to the microtiter plate and it was incubated for 1 h at room temperature, after which the supernatant was discarded and the wells were washed and incubated with horseradish peroxidase-conjugated goat IgG specific for murine IgG (Dakopatts, Glosrup, Denmark) at 1:250000 dilution. Finally, the wells were developed with orthophenylenediamine (0.04%), H_2O_2 in 50 mM citrate phosphate buffer, pH 5. The reaction was stopped by 4.5 N H_2SO_4 and the absorbance measured at 492 nm on a microplate reader (Titertek, Multiskan, Huntsville, Alabama). The concentrations of $VEGF_{165}$ in samples were calculated by interpolation of a standard curve using non-linear regression analysis. Samples from two independent collections of serum-free conditioned medium containing heparin were evaluated in two different ELISA assays in which measurements were made in triplicate.

Analysis of cell proliferation and DNA synthesis First we analyzed the cell proliferation of A375 cells and the selected clones of $VEGF_{165}$ transfectants growing *in vitro* in the presence of FCS. To this purpose, 10^3 A375 cells were plated onto 96-well culture plates in complete media containing 5% FCS and four wells were harvested daily and the biomass measured by SRB staining.

To evaluate DNA synthesis under serum-free medium conditions, cells were plated at a density of 2.7×10^5 cells per well in media containing 2% FCS onto 24-well culture plates, cultured for 24 h and then changed to serum-free media for 72 h prior to the addition of treatments. Medium was then replaced by fresh media containing one of the following reagents: rh$VEGF_{165}$ (50 ng per mL; R&D Systems), PD 098059 (25 μM), an inhibitor of ERK 1/2, SU 1498 (12.8 μM), a specific inhibitor of VEGFR-2 receptor phosphorylation, SB 203580 (3 μM), a specific inhibitor of p38 MAPK (Calbiochem), 30–100 nM Wortmannin, a PI3K-specific inhibitor or 2% FCS, cultured for a further 30 h. DNA synthesis was assessed by addition of 0.5 μCi per mL [methyl ^{3}H]thymidine (Amersham, Buckinghamshire, UK) for the last 18 h. Incorporation of [methyl ^{3}H]thymidine into cells was determined by liquid scintigraphy. Statistical analysis (paired sample *t* test) was performed using the software SPSS (SPSS, Chicago, Illinois).

Analysis of DNA fragmentation by agarose gel electrophoresis DNA was isolated from A375 and $VEGF_{165}$-transfectant cells growing in 10 cm Petri dishes in serum-free media with or

without the inhibitors and rhVEGF as described above. After incubation for 72 h cells were collected and then lysed using 0.6 mL lysis solution (10 mM Tris, pH 7.5, containing 1 mM EDTA and 0.2% Triton X-100) and treated with RNase. The protein was removed by phenol–chloroform extraction and DNA was precipitated using 100% isopropanol and washed with 70% ethanol. Samples containing equal numbers of cells were electrophoresed on a 1% agarose gel in TBE buffer in parallel with DNA molecular weight marker (123-bp DNA ladder; Life Technologies). Following electrophoresis, gels were stained with ethidium bromide and photographed on a UV transilluminator.

***In vivo* tumorigenesis and spontaneous metastasis** Athymic BALB/c nude mice were obtained from the Animal Production Area of Charles Rives (Lyon, France). The mice were maintained in pathogen-free conditions and used when they were 5 weeks old. For melanoma xenografts 1×10^6 cells were inoculated intradermally (or intravenously to explore experimental metastasis) in male mice. After 6 d of cell inoculation, the animals were anesthetized and tumors dissected, harvested, and immediately phormol included and paraffin embedded for vascular density studies. Additionally, the growth of tumors was monitored for 40 d. Tumor volumes were calculated from calliper measurements of two orthogonal diameters (x and y, larger and smaller diameters, respectively) by using the formula volume $= xy^2/2$. These tumors were excised on reaching 0.4 cm^3 and the animals were kept alive until they became moribund and then sacrificed and necropsed to investigate the presence of spontaneous metastasis. Tumor growth and doubling time of the volumes were compared between groups. All procedures and animal care were carried out in accordance with Institutional guidelines.

Mean times to reach a final target volume were compared between the groups with Student's *t* test for unpaired values. Significant differences were determined at $p < 0.05$. The mean values of volumes between groups were compared with the Mann–Whitney *U* test.

Vascular density and hemoglobin measurements Vascular counts were performed on paraffin-embedded sections of tumors (excised 6 d after implantation) by immunostaining for Factor VIII-related antigen (Dako, Carpenteria, California) and counterstaining with hematoxylin. Immunochemistry was done with the avidin–biotin–peroxidase kit with diaminobenzidine as the chromogen, according to the instructions of the manufacturer (Vector Labs, Burlingame, California).

Vascularity was quantified by two observers in a multiple-headed microscope in six fields at $\times$ 200 magnification, by selecting areas of higher vascularization. Any positively stained vessel, endothelial cell or endothelial cluster that was clearly separated from adjacent microvessels was included in the measurements (6).

The mean values of vascularity as measured by Factor VIII staining between groups were compared with the ANOVA test. $p < 0.05$ was considered statistically significant.

Hemoglobin content was measured on excised tumors growing for 10, 14, and 20 d in nude mice. Whole tumors were homogenized in 100 μL of double-distilled H_2O using disposable pellet pestles for microtubes. Homogenates were incubated in 500 μL of Drabkin's solution (Sigma Chemical Co.) for 15 min at room temperature (Drabkin and Austin, 1932). Samples were centrifuged to pellet cell debris. The absorbance was measured in the supernatants at 540 nm. Drabkin's solution was used as a blank. The absorption, which is proportional to the total hemoglobin concentration, was normalized to tumor weight. Six tumors derived from each of the cell lines were assayed and evaluated with the ANOVA test.

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References

Albino AP: Genes involved in melanoma susceptibility and progression. Curr Opin Oncol 7:159–161, 1995

Arbiser JL, Larsson H, Claesson-Welsh L, *et al*: Overexpression of VEGF 121 in immortalized endothelial cells causes conversion to slowly growing angiosarcoma and high level expression of the VEGF receptors VEGFR-1 and VEGFR-2 *in vivo*. Am J Pathol 156:1469–1476, 2000

Armstrong CA, Tara DC, Hart CE, Kock A, Luger TA, Ansel JC: Heterogeneity of cytokine production by human malignant melanoma cells. Exp Dermatol 1:37–45, 1992

Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G, Mercurio AM: Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. Cancer Res 61:5736–5740, 2001

Bossi P, Viale G, Lee AKC, Alfano RM, Coggi G, Bosari S: Angiogenesis in colorectal tumors: Microvessel quantitation in adenomas and carcinomas with clinicopathological correlations. Cancer Res 55:5049–5053, 1995

Busam KJ, Berwick M, Blessing K, *et al*: Tumor vascularity is not a prognostic factor for malignant melanoma of the skin. Am J Pathol 147:1049–1056, 1995

Charnock-Jones DS, Sharkey AM, Boocock CA, Ahmed A, Plevin R, Ferrara N, Smith SK: Vascular endothelial growth factor receptor localization and activation in human trophoblast and choriocarcinoma cells. Biol Reprod 51:524–530, 1994

Claffey KP, Brown LF, del Aguila LF, Tognazzi K, Yeo KT, Manseau EJ, Dvorak HF: Expression of vascular permeability factor/vascular endothelial growth factor by melanoma cells increases tumor growth, angiogenesis, an experimental metastasis. Cancer Res 56:172–181, 1996

Clark WH, Elder DE, Guerry D, Epstein MN, Greene MH, Van Horn M: A study of tumor progression: The precursor lesion of superficial spreading and nodular melanomas. Hum Pathol 15:1147–1165, 1984

de Jong JS, van Diest PJ, van der Valk P, Baak JP: Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol 184:53–57, 1998

Drabkin DS, Austin JH: Spectophotometric constants for common hemoglobin derivatives in human, dog, and rabbit blood. J Biol Chem 98:719–733, 1932

Dvorak HF, Detmar M, Claffey KP, Nagy JA, van de Water L, Senger DR: Vascular permeability factor/vascular endothelial growth factor: An important mediator of angiogenesis in malignancy and inflammation. Int Arch Allergy Immunol 107:233–235, 1995

Ferrara N, Alitalo K: Clinical applications of angiogenic growth factors and their inhibitors. Review. Nat Med 5:1359–1364, 1999

Gasparini G, Toi M, Gion M, *et al*: Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst 89:139–147, 1997

Gerber HP, Malik AK, Solar GP, *et al*: VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature 47:954–958, 2002

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N: Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem 273:30336–30343, 1998

Gitay-Goran H, Halaban R, Neufeld G: Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. Biochem Biophys Res Com 190:702–708, 1993

Govindarajan B, Bai X, Cohen C, *et al*: Malignant transformation of melanocytes to melanoma by constitutive activation of mitogen-activated protein kinase kinase (MAPKK) signaling. J Biol Chem 278:9790–9795, 2003

Graeven U, Rodeck U, Karpinski S, Jost M, Philippou S, Schmiegel W: Modulation of angiogenesis and tumorigenicity of human melanocytic cells by vascular endothelial growth factor and basic growth factor. Cancer Res 61:7282–7290, 2001

Halaban R, Funasaka Y, Lee P, Rubin J, Ron D, Birnbaum D: Fibroblast growth factors in normal and malignant melanocytes. Ann NY Acad Sci 638: 232–243, 1991

Herlyn M, Clark WH, Rodeck U, Mancianti ML, Jambrosic J, Koprowski H: Biology of tumor progression in human melanocytes. Lab Invest 56:461–474, 1987

Holash J, Davis S, Papadopoulos N, *et al*: VEGF-Trap: A VEGF blocker with potent antitumor effects. Proc Natl Acad Sci USA 99:11393–11398, 2002

Holash J, Maisonpierre PC, Compton D, *et al*: Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284: 1994–1998, 1999

Houghton AN, Real FX, Davis LJ, Cordon-Cardo C, Old LJ: Phenotypic heterogeneity of melanoma. J Exp Med 64:812–829, 1987

Jackson MW, Roberts JS, Heckford SE, Ricciardelli C, Stahl J, Horsfall DJ, Tilley WD: A potential autocrine role for vascular endothelial growth factor in prostate cancer. Cancer Res 62:854–859, 2002

Kanno S, Oda N, Abe M, *et al*: Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. Oncogene 19:2138–2146, 2000

Kath R, Jambrosic JA, Holland L, Rodeck U, Herlyn M: Development of invasive and growth factor-independent cell variants from primary human melanomas. Cancer Res 51:2205–2211, 1991

Kozlowski JM, Hart IR, Fidler IJ, Hanna N: A human melanoma line heterogeneous with respect to metastatic capacity in athymic nude mice. J Natl Cancer Inst 72:913–917, 1984

Kusters B, Leenders WP, Wesseling P, *et al*: Vascular endothelial growth factor-A(165) induces progression of melanoma brain metastases without induction of sprouting angiogenesis. Cancer Res 62:341–345, 2002

Latil A, Bieche I, Pesche S, Valeri A, Fournier G, Cussenot O, Lidereau R: VEGF overexpression in clinically localized prostate tumors and neuropilin-1 overexpression in metastatic forms. Int J Cancer 89:167–171, 2000

Li G, Satyamoorthy K, Meier F, Berking C, Bogenrieder T, Herlyn M: Function and regulation of melanoma-stromal fibroblast interactions: When seeds meet soil. Oncogene 22:3162–3171, 2003

Liu B, Earl HM, Baban D, Shoaibi M, Fabra A, Kerr DJ, Seymour LW: Melanoma cell lines express VEGF receptor kdr and respond to exogenously added VEGF. Biochem Biophys Res Commun 217:721–727, 1995

Marcoval J, Moreno A, Graells J, Vidal A, Escribà JM, Garcia-Ramírez M, Fabra A: Angiogenesis and malignant melanoma. Angiogenesis is related to the development of vertical (tumorigenic) growth phase. J Cutan Pathol 24: 212–218, 1997

Marcoval J, Moreno A, Graells J, Vidal A, Escriba JM, Peyri J, Fabra A: Vascular density and survival in cutaneous melanoma. Br J Dermatol 134:809–810, 1996

Masood R, Cai J, Zheng T, Smith DL, Naidu Y, Gill PS: Vascular endothelial growth factor/vascular permeability factor is an autocrine growth factor for AIDS-Kaposi sarcoma. Proc Natl Acad Sci USA 94:979–984, 1997

Meier F, Satyamoorthy K, Nesbit M, Hsu MY, Schittek B, Garbe C, Herlyn M: Molecular events in melanoma development and progression. Front Biosci 3:1005–1010, 1998

Miao HQ, Klagsbrun M: Neuropilin is a mediator of angiogenesis. Review. Cancer Metastasis Rev 19:29–37, 2000

Miao HQ, Lee P, Lin H, Soker S, Klagsbrun M: Neuropilin-1 expression by tumor cells promotes tumor angiogenesis and progression. FASEB J 15: 2532–2539, 2000

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z: Vascular endothelial growth factor (VEGF) and its receptors. Review. FASEB J 1:9–22, 1999

Ogunshola OO, Antic A, Donoghue MJ, *et al*: Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system. J Biol Chem 277:11410–11415, 2002

Pötgens AJG, van Altena MC, Lubsen NH, Ruiter DJ, de Waal RMW: Analysis of the tumor vasculature and metastatic behavior of xenografts of human melanoma cell lines transfected with vascular permeability factor. Am J Pathol 148:1203–1217, 1996

Robinson CJ, Stringer SE: The splice variants of vascular endothelial growth factor (VEGF) and their receptors. Review. J Cell Sci 114:853–865, 2001

Rofstad EK, Halsor EF: Vascular endothelial growth factor, interleukin 8, platelet-derived endothelial cell growth factor, and basic fibroblast growth factor promote angiogenesis and metastasis in human melanoma xenografts. Cancer Res 60:4932–4938, 2000

Shan S, Robson ND, Cao Y, *et al*: Responses of vascular endothelial cells to angiogenic signaling are important for tumor cell survival. FASEB J 18: 326–328, 2004

Siddiqui FA, Amirkhosravi A, Amaya M, Desai H, Meyer T, Francis JL: Purification and properties of human melanoma cell tissue factor. Clin Appl Thromb Hemost 7:289–295, 2001

Singh RK, Gutman M, Reich R, Bar-Eli M: Ultraviolet B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8. Cancer Res 55:3669–3674, 1995

Socker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M: Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745, 1998

Soker S, Miao HQ, Nomi M, Takashima S, Klagsbrun M: VEGF(165) mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF(165)-receptor binding. J Cell Biochem 85:357–368, 1998

Straume O, Akslen LA: Expression of vascular endothelial growth factor, its receptors (Flt-1, KDR) and TSP-1 related to microvessel density and patient outcome in vertical growth phase melanomas. Am J Pathol 159:223–235, 2001

Streit M, Detmar M: Angiogenesis, lymphangiogenesis, and melanoma metastasis. Oncogene 20:3172–3179, 2003

Takahashi T, Ueno H, Shibuya M: VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene 18:2221–2230, 1999

Valgeirsdottir S, Eriksson A, Nister M, Heldin CH, Westermark B, Claesson-Welsh L: Compartmentalization of autocrine signal transduction pathways in *Sis*-transformed NIH 3T3 cells. J Biol Chem 270:10161–10170, 1995

Vega-Diaz B, Herron GS, Michel S: An autocrine loop mediates expression of vascular endothelial growth factor in human dermal microvascular endothelial cells. J Invest Dermatol 11:525–530, 2001

Wedge SR, Ogilvie DJ, Dukes M, *et al*: ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. Cancer Res 16:4645–4655, 2002

Weisz A, Koren B, Cohen T, Neufeld G, Kleinberger T, Lewis BS, Flugelman MY: Increased vascular endothelial growth factor 165 binding to kinase inser domain-containing receptor after infection of human endothelial cells by recombinant adenovirus encoding the Vegf (165) gene. Circulation 103:1887–1892, 2001

Westphal JR, Van't Hullenaar R, Peek R, *et al*: Angiogenic balance in human melanoma: Expression of VEGF, bFGF, IL-8, PDGF and angiostatin in relation to vascular density of xenografts *in vivo*. Int J Cancer 86:768–776, 2000

Yao R, Cooper GM: Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267:2003–2006, 1995

Yuang A, Yang PC, Yu CJ, *et al*: Tumor angiogenesis correlates with histologic type and metastasis in non-small-cell lung cancer. Am J Respir Crit Care Med 152:2157–2162, 1995