Fibroblast growth factor-2-induced host stroma reaction during initial tumor growth promotes progression of mouse melanoma via vascular endothelial growth factor A-dependent neovascularization

Satoshi Tsunoda,[1,2,4] Toshiyuki Nakamura,[1] Hiroaki Sakurai[2,3] and Ikuo Saiki[2,3]

[1]Safety Research Department, Central Research Laboratories, Kaken Pharmaceutical Co., 301, Gensuke, Fujieda, Shizuoka, 426-0088; [2]Division of Pathogenic Biochemistry, Institute of Natural Medicine; [3]21st Century COE Program, University of Toyama, 2630, Sugitani, Toyama 930-0194, Japan

(Received August 29, 2006/Revised December 13, 2006/Accepted December 18, 2006/Online publication March 16, 2007)

Fibroblast growth factor (FGF)-2 has been considered to play a critical role in neovascularization in several tumors; however, its precise role in tumor progression is not fully understood. In the present study, we have characterized the role of FGF-2 in B16-BL6 mouse melanoma cells, focusing on effects during the initial phase of tumor growth. FGF-2 was injected at the tumor inoculation site of dorsal skin during the initial phase. FGF-2 induced marked tumor growth and lymph node metastasis. This was well correlated with an increase in neovascularization in the host stroma. FGF-2 also recruited inflammatory and mesenchymal cells in host stroma. Marked tumor growth, pulmonary metastasis and intensive neovascularization in tumor parenchyma were also observed after a single injection of FGF-2 into the footpad inoculation site. In contrast, repeated injections of FGF-2 at a site remote from the footpad tumor were ineffective in promoting tumor growth and metastasis. These promoting activities of FGF-2 were blocked by local injections of a glucocorticoid hormone, suggesting that host inflammatory responses induced by FGF-2 are associated with FGF-2-induced tumor progression. In addition, although FGF-2 did not promote cellular proliferation and vascular endothelial growth factor A (VEGFA) mRNA expression in B16-BL6 cells *in vitro*, FGF-2 induced VEGFA expression in host stroma rather than tumor tissue, and local injections of a neutralizing antibody against VEGFA inhibited these activities of FGF-2 *in vivo*. These results indicate that abundant FGF-2 during the initial phase of tumor growth induces VEGFA-dependent intensive neovascularization in host stroma, and supports marked tumor growth and metastasis. (*Cancer Sci* 2007; 98: 541–548)

The neovascularization and inflammatory reaction in the stromal microenvironment surrounding a solid tumor is closely associated with tumor growth and metastasis.[1–5] Among angiogenic growth factors and inflammatory cytokines, fibroblast growth factor (FGF)-2 has not only a potent mitogen-like effect on vascular endothelial cells and smooth muscle cells,[6,7] but also synergistic potential for the recruitment of mesenchymal and inflammatory cells in response to inflammatory cytokines.[8] Vascular endothelial growth factor (VEGF) A has also been identified as an essential growth factor in the proliferation of endothelial cells.[9] Furthermore, exogenous FGF-2 induces VEGFA expression in vascular endothelial cells[10] and stroma cells[11,12] through autocrine and paracrine mechanisms.

The blockage of FGF-2 and VEGFA function results in a marked inhibition of tumor growth *in vivo*.[13–16] FGF-2 and VEGFA synergistically stimulate tumor-induced neovascularization but have distinctive effects on vessel functionality and tumor survival.[17] Thus, FGF-2 and VEGFA have been generally considered to play a critical role in tumor-induced neovascularization, subsequent tumor growth and metastasis. In the growth of cutaneous malignant melanomas, the consecutive expression of endogenous FGF-2 enhances tumor growth directly in an autocrine and paracrine manner as well as indirectly by stimulating tumor-induced neovascularization.[18–22] Furthermore, inhibition of endogenous FGF-2 originating from human melanoma also results in the suppression of tumor-induced neovascularization and subsequent tumor growth and metastasis.[13,15] Thus, FGF-2 is regarded as a critical growth factor in tumor development, especially melanoma-induced neovascularization.

Various angiogenic growth factors play an important role in the initial phase, when a growing tumor is still seceding from dormancy.[3,23] Giavazzi *et al*. show that endogenous FGF-2 expression deeply affects the initial stage of tumor growth and tumor angiogenesis in human endometrial adenocarcinoma with a conditional endogenous FGF-2 expression system.[24] However, little is known about the precise role of angiogenic growth factors in tumor cells and/or host stroma during the initial phase of growth when a tumor is still seceding dormancy. In the present study, we investigated the role of FGF-2 during the initial phase of growth of B16-BL6 mouse melanoma cells,[25,26] and found that abundant FGF-2 during the initial stage supports subsequent tumor growth and metastasis. In addition, the effects of FGF-2 are dependent on the marked tumor-induced neovascularization mediated by endogenous VEGFA.

Materials and Methods

Antibodies and reagents. Recombinant human FGF-2 was obtained from Scios (Mountain View, CA, USA). A phospho-ERK1/2 (Thr202/Thy204) antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against ERK1 (C-16) and ERK2 (C-14) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Anti-von willebrand factor (vWF) rabbit polyclonal antibody was obtained from Dako Cytomaton (Kyoto, Japan). Anti-mouse VEGFA goat polyclonal antibody (anti-VEGFA ab; AF-493-NA) and normal goat IgG were obtained from R&D Systems (Minneapolis, MN, USA). Anti-VEGFA rabbit polyclonal antibody was obtained from Lab Vision (Fremont, CA, USA). Prednisolone sodium

[4]To whom correspondence should be addressed.
E-mail: tsunoda_satoshi@kaken.co.jp

doi: 10.1111/j.1349-7006.2007.00432.x

succinate (PDN) was obtained from Shionogi & Co. (Osaka, Japan).

Cell culture. B16-BL6 mouse melanoma cells with highly metastatic potential were used in the present study.[25,26] Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/mL of penicillin and 100 μg/mL streptomycin, and kept at 37°C in 5% CO_2/95% air. For serum starvation, cells were cultured in medium supplemented with 10% FBS for 24 h and subsequently maintained in medium supplemented with 0.5% FBS for 24 h.

Animals. Female C57BL/6 J mice (7 or 17 weeks of age) were purchased from CREA Japan (Tokyo, Japan). Mice were provided with a commercial diet (CRF-1; Charles River Japan, Kanagawa, Japan) and tap water *ad libitum*, kept in an animal room maintained under specific pathogen-free conditions at 20–26°C and 50–70% humidity, and housed individually with wood shavings in plastic cages. All experimental procedures were in accordance with the Guide for Care and Use of Experimental Animals of Kaken Pharmaceutical Co.

Immunoblotting. The cells were lysed with sampling buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% (w/v) sodium dodecylsulfate, 10% glycerol, 50 mM dithiothreitiol and 0.01% (w/v) bromophenol. The cell lysates were resolved with sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidine difluoride (PVDF) membranes (immobilom-P; Millipore, Billerica, MA, USA). The membranes were incubated in blocking buffer (Block Ace; Dainippon Sumitomo Pharma Co., Osaka, Japan) for 2 h at room temperature and subsequently reacted with the primary antibodies for 1 h. The primary antibodies were detected using horseradish peroxidase (HRP)-conjugated antirabbit or antigoat antibodies. Target proteins were imaged with the ECL system (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Cell proliferation assay. The B16-BL6 melanoma cells (2.0×10^4 cells/35-mm well) were cultured in medium supplemented with 10% FBS for 24 h.

Subsequently, the medium was replaced with one containing 0.5% serum, and cultured for another 24 h. Then, cells were treated with FGF-2 (10 and 100 ng/mL) and heparin sulfate (10 μg/mL). The cells were suspended with 0.25% trypsin and stained with trypan blue at 24 h after the treatment with FGF-2. The number of living cells was counted with a hemocytometer.

Quantitative real-time reverse transcription–polymerase chain reaction. Total RNA from cultured cells and tissue samples was extracted using the RNeasy Mini Kit and RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden Germany), respectively, according to the manufacturer's directions. First-strand cDNA was synthesized with random primers and SuperScript II reverse transcriptase (Invitrogen). The reverse transcription (RT) reaction was carried out at 42°C for 50 min, then at 70°C for 15 min. The real-time polymerase reaction (PCR) was conducted using SYBR Green I (SYBR Premix Ex *Taq*; Takara Bio, Shiga, Japan) and an Applied Biosystems 7300 Real-Time PCR System (Foster, CA, USA) for one cycle of 10 s at 95°C, and subsequently 40 cycles of 5 s at 95°C and 32 s at 62°C, according to the manufacturer's instructions. The level of mRNA expression was determined with a standard curve and normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were obtained from Takara Bio, and the sequences optimized for real-time PCR were as follows: VEGFA sense 5′-GTGCACTGGACCCTGGCTTTA-3′ and antisense 5′-GGTCTCAATCGGACGGCAGTA-3′, GAPDH sense 5′-AAATGGTGAAGGTCGGTGTG-3′ and antisense 5′-TGAAGGGGTCGTTGATGG-3′.

Angiogram. The mice were infused with 145% (w/v) barium sulfate in two-fold its volume of a 0.5% gelatin solution dissolved in phosphate-buffered saline into the left ventricle. The dorsal skin was collected and fixed with a 10% formalin solution. The vessels oriented toward the tumor mass or injection site in the dorsal skin were imaged by contrast X-ray angiography and were enumerated as described by Kreisele and Ershler with some modifications.[27]

Immunohistochemistry and analysis of microvessel density. After the preparation of paraffin-embedded sections (4-μm thick) of inoculation sites fixed in 10% formalin solution, the tumor parenchyma and the host stroma surrounding the tumor were immunohistochemically stained using antivWF rabbit polyclonal antibody and anti-VEGFA rabbit polyclonal antibody with the labeled streptavidin–biotin method according to the manufacturer's instructions (Histofine SAB-PO kit; Nichirei, Tokyo, Japan).

The high density of microvessels stained positively with vWF was observed with a microscope at ×400 power and analyzed as described by Weidner with some modifications.[28]

Spontaneous metastasis. B16-BL6 cells ($\sim 5 \times 10^5$) were suspended in DMEM supplemented with 10% FBS and inoculated into subcutaneous tissue of the dorsal skin (dorsal skin model) or the right footpad (footpad model) of mice using a Hamilton syringe and 27-gauge needle. The major and minor axes of the tumor mass as well as tumor thickness in the footpad model were measured, using a digital gauge (500-401 type; Mitsutoyo Co., Kanagawa, Japan) under anesthesia with ether to calculate the tumor volume using the following formulas: (large diameter × short diameter^2/2) for the dorsal skin model, (large diameter/2 × short diameter/2 × thickness/2 × circle ratio × 4/3) for the footpad model.[29] The tumor mass in the dorsal skin and footpad was excised surgically under anesthesia with ether. The number of metastatic nodules in axillary and inguinal lymph nodes (the dorsal skin model) and the lungs (the footpad model) was counted macroscopically; in addition, the lymph nodes with metastasis were also weighed after autopsy.

Injection of FGF-2 into the inoculation site in the dorsal skin model. In the dorsal skin model, FGF-2 (40 μg/mouse) was injected into the inoculation site of 17-week-old female mice once only on day 1 or once daily from days 1 to 4 after the inoculation. A dose of 40 μg/mouse was the minimum needed to enhance primary tumor growth in preliminary studies (data not shown). Saline was injected into the respective control animals once daily from days 1–4. On day 18, the primary tumor was excised surgically and on day 40 all mice were killed and lymph node metastasis was evaluated.

To investigate the effects of FGF-2 on the tumor parenchyma and the host stroma surrounding the tumor mass, 40 μg/mouse of FGF-2 was injected into the inoculation site of 7-week-old female mice once daily from days 1 to 4 after the inoculation. Tumor tissue attached to the host stroma was collected on day 5 after the inoculation, which is considered to be when the inoculated tumor secedes dormancy. The tumor parenchyma and the host stroma in the collected tissue were examined histopathologically using hematoxylin–eosin staining. On day 11, considered to correspond to the active tumor growth phase, the number of vessels oriented toward the tumor mass was determined by contrast X-ray angiography and the density of microvessels stained positively with the ant-ivWF antibody in the tumor parenchyma and host stroma was analyzed. In order to examine its neovascular potency and the modulation of host stroma in mice without tumor inoculation, FGF-2 was injected subcutaneously into the intact dorsal skin and the modification of host stroma and vessel formation was evaluated as described above.

To investigate the effects of FGF-2 on the expression of VEGFA in the tumor tissue and host stroma surrounding the tumor, the tumor tissue and host stroma were collected on the days 5, 7 and 11 after tumor inoculation. The VEGFA mRNA level in tumor tissue and host stroma was determined by quantitative real-time RT-PCR. The localization of cells producing

doi: 10.1111/j.1349-7006.2007.00432.x

VEGFA in the tumor tissue and host stroma was analyzed by immunohistochemical staining.

Injection of FGF-2 into the inoculation site in the footpad model. A single dose of FGF-2 (4 μg/mouse) was injected into the inoculation site on day 1 after inoculation. This dose was the minimum needed to enhance primary tumor growth in a preliminary study (data not shown). Control mice were injected with saline at the site using the same procedure. Tumor volume was measured on day 15 after inoculation and the footpad was excised surgically on day 16. Microvessels in the tumor parenchyma were stained immunohistochemically and enumerated as described above. Mice were killed on day 33 and the number of metastatic nodules in the lungs was counted.

Influences of PDN and anti-VEGFA ab on FGF-2-induced neovascularization. The influences of PDN and anti-VEGFA ab on the neovascularization after FGF-2 treatment were examined in the dorsal skin. One day after the FGF-2 injection, PDN or anti-VEGFA ab was injected into the site daily from days 2 to 5. The normal goat IgG and saline were injected into the control animals using the same experimental procedure. On day 6, the number of vessels oriented toward the injection site was determined by contrast X-ray angiography.

Influences of PDN and anti-VEGFA ab after the injection of FGF-2 in the footpad model. The influence of PDN and anti-VEGFA ab on primary tumor growth and pulmonary metastasis after FGF-2 treatment were observed using the footpad model. One day after tumor inoculation, a single dose of FGF-2 (4 μg/mouse) was injected into the inoculation site. After the FGF-2 treatment, PDN (900 μg/mouse) or anti-VEGFA ab (50 μg/mouse) was injected into the sites daily from days 2 to 4 and then every other day from days 5 to 14 after the inoculation. Normal goat IgG and saline were injected into the respective control animals using the same experimental procedure. After the measurement of tumor volume on day 15, tumors developed in the footpad were excised surgically on day 16 and microvessels in the tumor parenchyma were enumerated. The number of pulmonary metastatic nodules was counted on day 33 after the inoculation.

Injection of FGF-2 at a site remote from the primary tumor in the footpad model. FGF-2 (20 μg/mouse) was injected subcutaneously into the dorsal skin every other day from days 2 to 10 after the inoculation in the footpad model. The footpad was excised surgically on day 21 and mice were killed on day 39. Lungs with metastasis were weighed and the number of metastatic nodules was counted.

Statistical analysis. Statistical differences for the *in vitro* study of the dorsal skin model and a site remote injection experiment were analyzed with the Dunnett type multiple comparison test. The Turkey type multiple comparison test was carried out for the other experiments. *P*-values of less than 0.05 were considered statistically significant.

Results

Effects of FGF-2 on primary tumor growth and lymph node metastasis in the dorsal skin model. To investigate the effect of FGF-2 at the initial phase, 40 μg of FGF-2 was injected at the site of tumor inoculation only on day 1 or daily from days 1 to 4. The local single or repeated injections of FGF-2 significantly increased tumor volume on days 10, 13, 15 and 17 compared with the saline-injected control group (Fig. 1a). On day 17, the single and repeated injections of FGF-2 increased tumor volume 2.0-fold and 2.5-fold, respectively. The swelling of axillary and inguinal lymph nodes was also observed macroscopically from day 30 (data not shown). The lymph nodes with metastatic tumor nodules were found at autopsy. The repeated injection of FGF-2 caused a significant increase in the number of metastatic nodules and weight of the lymph nodes with metastasis (Fig. 1b,c).

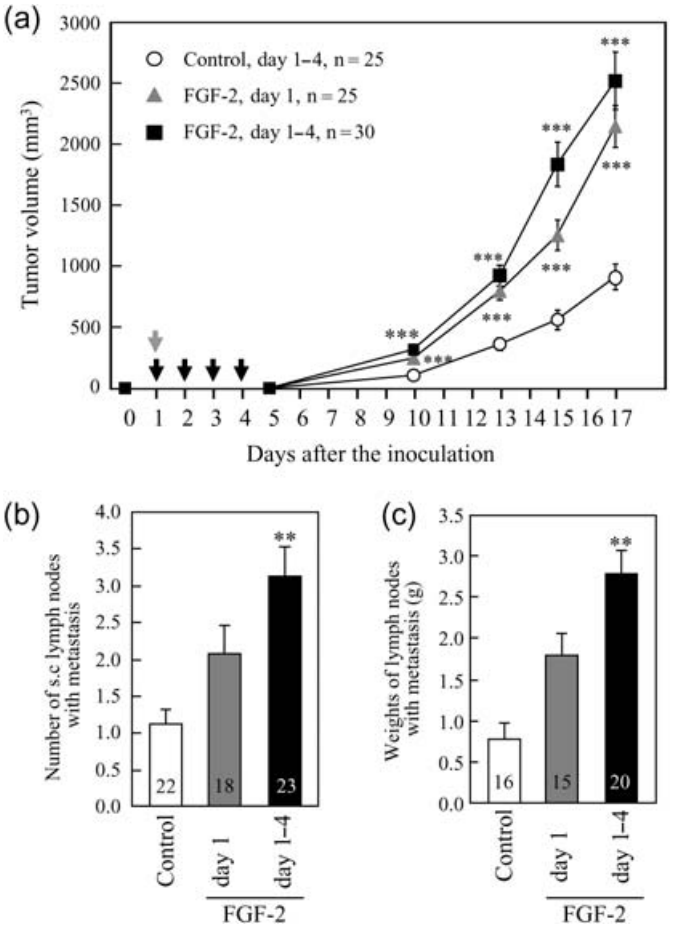

Fig. 1. Effects of fibroblast growth factor (FGF)-2 on B16-BL6 tumor growth and metastasis in axillary and inguinal lymph nodes in the dorsal skin model. C57BL/6 female mice (17 weeks old) were inoculated with B16-BL6 melanoma cells in the dorsal skin. Then, 40 μg/mouse of FGF-2 was injected at the inoculation site on day 1 (gray arrow) or from days 1 to 4 (black allow) after the inoculation. (a) Tumor volume was measured on days 5, 10, 13, 15 and 17. The (b) number and (c) weight of axillary and inguinal lymph nodes with metastasis were measured on day 40 after the inoculation. The number of mice examined is shown in each column. Each value represents the mean ± SEM and significant differences from the control group are shown as ***$P < 0.001$.

Effects of FGF-2 on neovascularization in tumor parenchyma and host stroma surrounding the tumor. Histopathological observation revealed that the repeated injections of FGF-2 caused mesenchymal cells, neutrophils, T cells and monocytes (inflammatory cells) to be recruited to the host stroma surrounding the tumor mass as well as in the subcutaneous tissue without tumor inoculation on day 5 (Fig. 2a). A central necrotic area in the tumor parenchyma was observed in the control group, but not in the FGF-2-injected group (Fig. 2a), suggesting that nutritional starvation in the center of the inoculated tumor was improved by FGF-2.

A soft X-ray angiogram was recorded to investigate the formation of new vessels directed toward the tumor mass. The repeated injections of FGF-2 promoted the formation of new blood vessels that sprouted and branched from existing ones, oriented toward the tumor mass in the host stroma (Fig. 2b). New vessels were also observed in the subcutaneous tissue without tumor inoculation (Fig. 2b). Immunohistochemical analysis with anti-vWF antibody showed that FGF-2 stimulated the formation of a high density of microvessels not only in the host stroma surrounding the tumor but also in the presence of tumor parenchyma. In addition, FGF-2-induced neovascularization

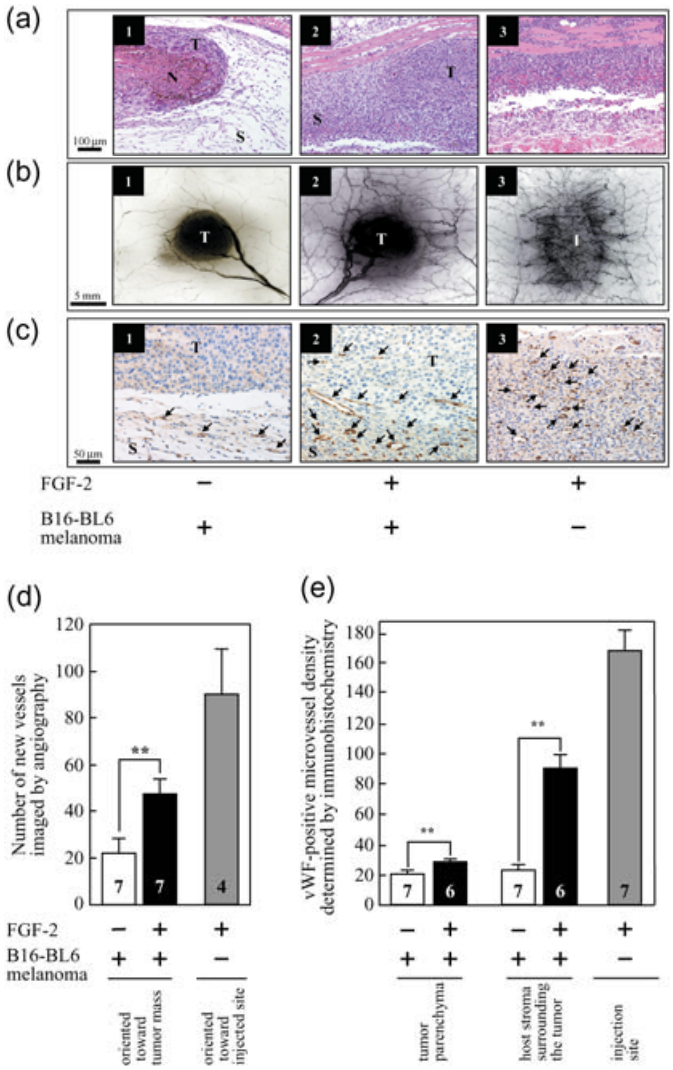

Fig. 2. Effects of fibroblast growth factor (FGF)-2 on the host stroma surrounding the tumor mass in the dorsal skin model. Mice (7 weeks old) were inoculated with B16-BL6 melanoma cells in the dorsal skin. A dose of 40 μg/mouse of FGF-2 was injected repeatedly at the inoculation site from days 1 to 4 after inoculation. FGF-2 was also injected daily into the dorsal skin without the tumor inoculation for 4 days. (a) Tissue section of host stroma surrounding the tumor mass on day 5 after the tumor inoculation (panels 1 and 2) and site of injection of FGF-2 (panel 3) stained with hematoxylin–eosin. (b) Soft X-ray angiogram of host stroma surrounding the tumor mass (T) (panels 1 and 2) and site of injection (I) of FGF-2 (panel 3). (c) Tissue section of the host stroma surrounding the tumor mass (panels 1 and 2) and site of injection of FGF-2 (panel 3) stained immunohistochemically with antihuman von willebrand factor (vWF) antibody and observed with a microscope. Highly dense microvessels (panels 1–3, arrows) were formed after the FGF-2 treatment. N, necrotic area in tumor tissue; S, host stroma; T, tumor tissue. (d) The number of new vessels oriented toward the tumor mass on day 11 or toward the injection site of intact skin imaged with the angiogram was counted. (e) The number of vWF-positive microvessels was counted in the tumor parenchyma and the host stroma as well as at the injection site without the inoculation. The number of mice is shown in each column. Each value is represented as the mean ± SEM and significant differences from the control values are shown as $^{**}P < 0.01$.

accompanied the migration of mesenchymal and inflammatory cells (Fig. 2c). Moreover, the repeated injections of FGF-2 alone enhanced the tumor-independent formation of microvessels (Fig. 2c). The number of new vessels oriented toward the tumor mass (Fig. 2d), and microvessels formed in the tumor parenchyma and host stroma surrounding the tumor (Fig. 2e), was significantly increased. A small but significant increase in the number of microvessels was also observed in tumor parenchyma after treatment with FGF-2 (Fig. 2d). The ratio of neovascularization, which indicated the degree of microvessel density between the host stroma and tumor parenchyma, showed a significant increase in the FGF-2-treated group (Table 1).

Table 1. Effects of FGF-2 on the ratio of microvessels formed in the dorsal skin model

Group	The ratio of microvessels (in the host stroma/in the tumor parenchyma)
Control	1.2 ± 0.2 (7)
FGF-2	3.2 ± 0.3*** (6)

The ratio of microvessels was calculated with the following formula:

$$\text{The ratio} = \frac{\text{Value}^{\#} \text{ in the high density of microvessel formation in the host stroma}}{\text{Value}^{\#} \text{ in the high density of microvessel formation in tumor parenchyma}}$$

#Number of microvessels.
The values are represented as the mean ± SEM and a significant difference from the control value is shown as ***P < 0.001. The number of mice examined is given in parentheses.

Table 2. Effects of FGF-2 injected as a site remote from the tumor mass in the footpad model

Group	Turnor volume on day 20 (mm^3)	Number of pulmonary metastases		Lung weights (g)
		0.5#	Total	
Control	510.5 ± 88.9 (15)	33.4 ± 7.0 (14)	78.7 ± 27.7 (14)	0.539 ± 0.03
FGF-2	598.0 ± 80.2 (15)	38.8 ± 4.5 (15)	88.4 ± 24.0 (15)	0.523 ± 0.03

#0.5 mm or more in diameter. The values are represented as the mean ± SEM. The number of mice examined is given in parentheses.

Effects of PDN on FGF-2-induced neovascularization and tumor progression. A single injection of FGF-2 into the tumor on day 1 induced significant and pronounced increases in microvessel density in the tumor parenchyma (Figs 3b,6b), tumor volume (Figs 3c,6c), and the number of pulmonary metastatic nodules (Figs 3d,e,6d,e). In contrast, repeated subcutaneous injections of FGF-2 into the dorsal skin were ineffective against primary tumors in the footpad on day 20 after inoculation (Table 2). The number and size of pulmonary metastases and weight of the lungs on day 39 were not affected by FGF-2 (Table 2). To evaluate the influence of FGF-2-induced inflammatory reactions in host stroma on subsequent neovascularization, tumor growth and metastasis, we initially tested the effects of PDN, a synthetic glucocorticoid, on FGF-2-induced neovascularization in dorsal skin. The repeated injection of PDN after the single treatment of rhFGF-2 decreased significantly the number of new vessels oriented toward the injection site in the dorsal skin without tumor inoculation (Fig. 3a). We next tested the effects of PDN on FGF-2-induced tumor growth and metastasis. PDN significantly inhibited microvessel formation in the tumor parenchyma (Fig. 3b), tumor growth (Fig. 3c) and pulmonary metastases (Fig. 3d,e).

Effects of FGF-2 on endogenous VEGFA expression in tumor tissue and host stroma. VEGFA is a well-known angiogenic factor that is induced by FGF-2 in various tissues. Therefore, we first tried to investigate the effects of FGF-2 directly on VEGFA mRNA expression in B16-BL6 cells *in vitro*. Although B16-BL6 cells treated with FGF-2 showed induced phosphorylation of ERK mitogen-activated protein kinases *in vitro* (Fig. 4a), cellular proliferation (Fig. 4b) and VEGFA mRNA expression (Fig. 4c)

doi: 10.1111/j.1349-7006.2007.00432.x

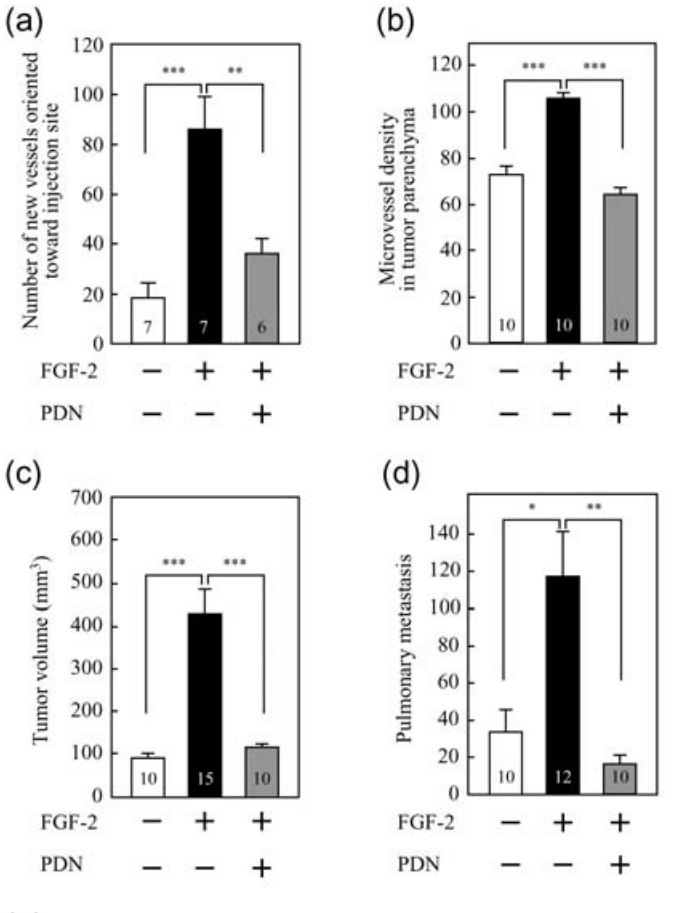

Fig. 3. Effects of prednisolone (PDN) on the marked neovascularization, tumor growth and metastasis stimulated by local injection of fibroblast growth factor (FGF)-2. FGF-2 (40 μg/mouse) was injected into the dorsal skin without the tumor inoculation, and then PDN (900 μg/mouse) was injected into the site daily for 4 days after the FGF-2 treatment. Vessels in the dorsal skin were imaged by soft X-ray angiogram. (a) The number of vessels oriented toward the injection site was evaluated after the PDN treatment using the same procedure described in Fig. 2. A single dose of 4 μg/mouse of FGF-2 was injected into the tumor on day 1 after the inoculation and then PDN (900 μg/mouse) was injected daily into the sites from days 2 to 4 and then every other day from days 5 to 14 after the inoculation. (b) The high density of microvessels in tumor parenchyma on day 16 was stained immunohistochemically with anti-von willebrand factor (vWF) antibody and the number of vWF-positive microvessels was counted. (c) Tumor volume was measured on day 15 after the tumor inoculation. (d, e) Pulmonary metastatic nodules in mice treated with PDN after the injection of FGF-2. The number of pulmonary metastatic nodules was counted on day 33. The number of mice examined is shown in each column. Each value is represented as the mean ± SEM and significant differences between groups are shown as $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$.

were not affected. We next investigated the effects of FGF-2 on VEGFA production in tumor tissue and host stroma *in vivo*. The endogenous VEGFA mRNA expression was not enhanced in the tumor tissue treated with FGF-2 (Fig. 5a). In contrast, FGF-2

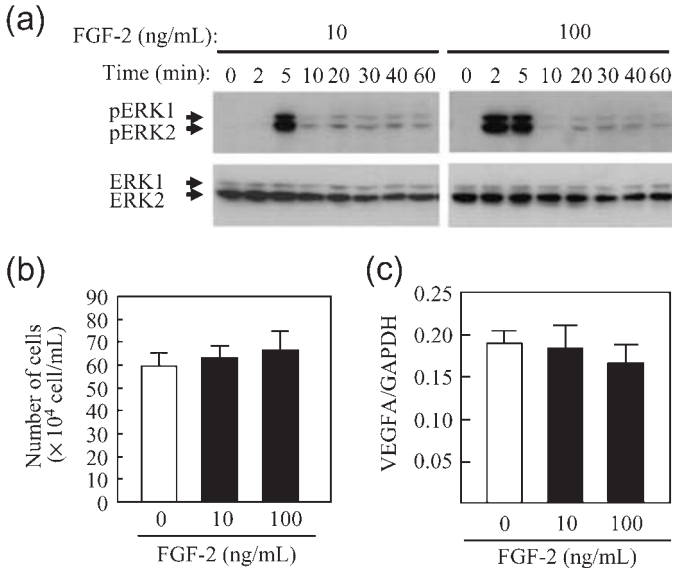

Fig. 4. The effect of fibroblast growth factor (FGF)-2 on intracellular signaling, cellular proliferation, and vascular endothelial growth factor (VEGF) A mRNA expression in B16-BL6 melanoma cells cultured with 0.5% fetal bovine serum. B16-BL6 melanoma cells (2.0×10^5) were seeded in 6-cm dishes, and then treated with FGF-2 (10 and 100 ng/mL). (a) Whole cell lysates were collected and immunoblotted with antibodies against phospho-ERK and ERK1/2. (b) The number of B16-BL6 melanoma cells counted at 24 h after the FGF-2 treatment. Each value represents the mean ± SEM for five samples. (c) Total RNA was collected at 24 h after the FGF-2 treatment. The relative levels of endogenous VEGFA mRNA were measured with real-time reverse transcription–polymerase chain reaction. Each value represents the mean ± SEM for five samples.

significantly enhanced the endogenous VEGFA mRNA in host stroma on days 5 and 7 after inoculation (Fig. 5b). Immunohistochemical analysis with the anti-VEGFA antibody showed that a high density of VEGFA-positive fibroblastic and vascular endothelial cells was observed in the host stroma treated with FGF-2 on days 5, 7 and 11 (Fig. 5c).

Effects of anti-VEGFA antibody on FGF-2-induced neovascularization and tumor progression. In the present study, FGF-2 directly induced the expression of endogenous VEGFA in host stroma surrounding the tumor tissue. Therefore, we tried to investigate the effects of FGF-2-induced intensive neovascularization, tumor growth and metastasis on endogenous VEGFA produced by host stromal cells. The repeated injection of antimouse VEGFA antibody also significantly decreased the number of new vessels oriented toward the injection site of FGF-2 in the dorsal skin without the tumor (Fig. 6a). It is particularly noteworthy that repeated injections of anti-VEGFA antibody after the single injection of FGF-2 on day 1 caused complete inhibition of FGF-2-induced intensive microvessel formation in the tumor parenchyma (Fig. 6b), tumor growth (Fig. 6c) and pulmonary metastasis (Fig. 6d,e) in the foot pad model, indicating that VEGFA-dependent neovascularization is one of the critical pathways for FGF-2-induced tumor growth and metastasis.

Discussion

FGF-2 has been shown to be a potent angiogenic factor in tumor-induced neovascularization. However, its precise role during tumor growth is not fully understood. In the present study, we focused on the role of FGF-2 during the initial growth phase in the spontaneous metastasis of B16-BL6 mouse melanoma cells. The present study demonstrates that local

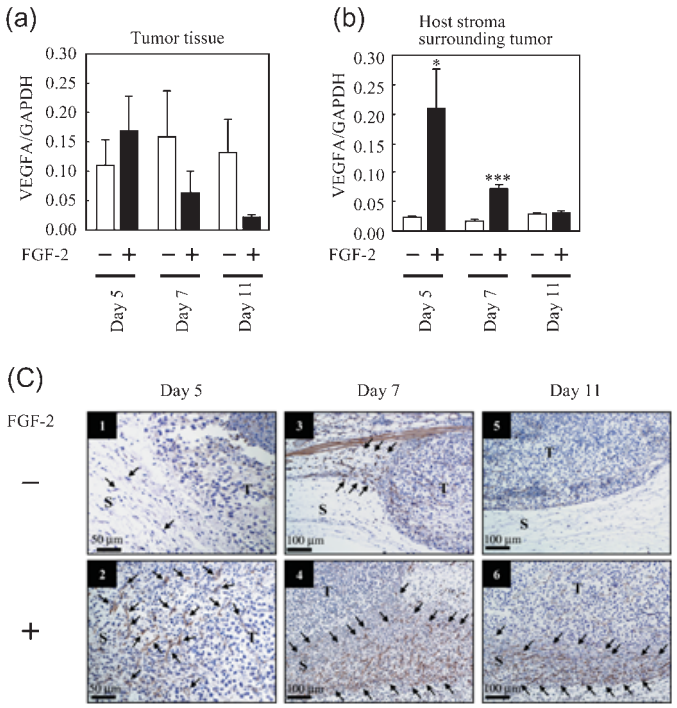

Fig. 5. The effect of fibroblast growth factor (FGF)-2 on vascular endothelial growth factor (VEGF) A expression in tumor tissue and host stroma *in vivo*. Mice were inoculated with B16-BL6 melanoma cells in the dorsal skin. FGF-2 (40 μg/mouse) was injected repeatedly at the inoculation site. Mice were killed on days 5, 7 and 11 after the inoculation. The relative levels of endogenous VEGFA mRNA in the (a) tumor tissue and (b) host stroma were measured by real-time reverse transcription–polymerase chain reaction. Each value represents the mean ± SEM for five samples. Significant differences between groups are shown as $***P < 0.001$ and $*P < 0.05$. (c) Tumor parenchyma and host stroma surrounding the tumor was stained immunohistochemically with anti-VEGFA antibody. A high density of VEGFA-positive endothelial and fibroblastic cells (panels 1–6, arrows) was found in the host stroma after FGF-2 treatment. S, host stroma; T, tumor tissue.

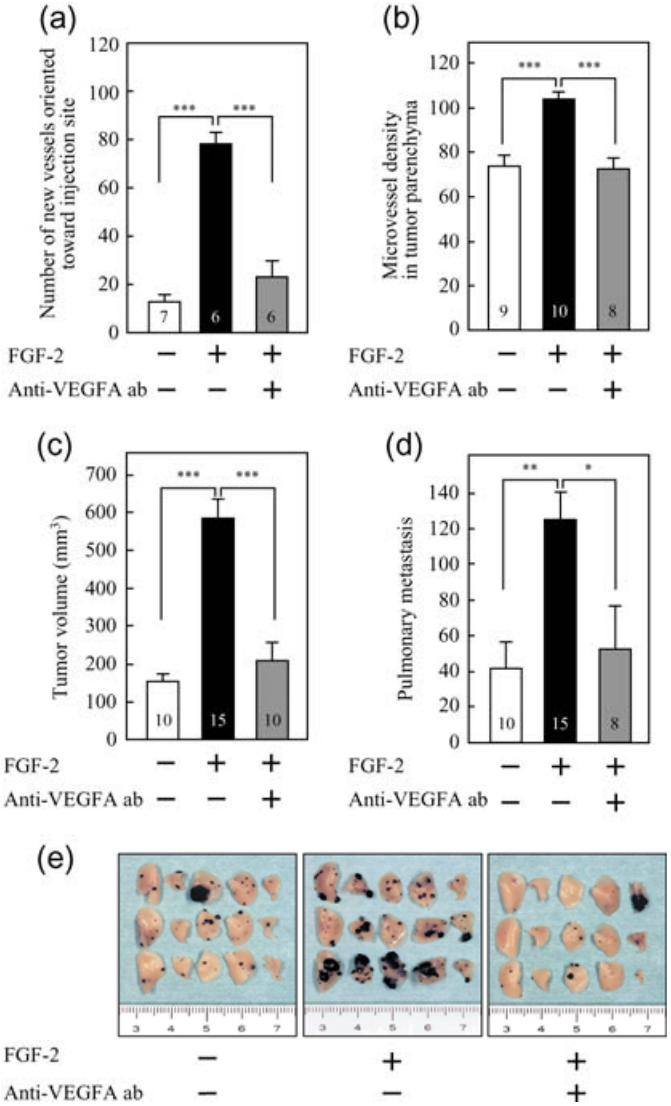

Fig. 6. Effects of vascular endothelial growth factor (VEGF) A antibody on the marked neovascularization, tumor growth and metastasis stimulated by local injection of fibroblast growth factor (FGF)-2. FGF-2 (40 μg/mouse) was injected into the dorsal skin without tumor inoculation, and then anti-VEGFA antibody (50 μg/mouse) was injected into the injection site of FGF-2 using the same procedure for prednisolone (PDN) (Fig. 3). Vessels in the dorsal skin were imaged by soft X-ray angiogram. (a) The number of vessels oriented toward the injection site was evaluated on day 6 after FGF-2 treatment using the same procedure described in Fig. 2. Mice were inoculated with B16-BL6 melanoma cells in the right footpad. A single dose of 4 μg/mouse of FGF-2 was injected on day 1 after the tumor inoculation, then 50 μg/mouse of anti-VEGFA antibody was injected into the tumor using the same administration period of PDN. (b) von willebrand factor (vWF)-positive microvessels in the tumor parenchyma, (c) tumor volume and (d, e) the number of pulmonary metastatic nodules were evaluated using the same procedure described in Fig. 3. The number of mice examined is shown in each column. Each value represents the mean ± SEM and significant differences between groups are shown as $***P < 0.001$, $**P < 0.01$ and $*P < 0.05$.

injection of FGF-2 into the inoculation site is sufficient for subsequent tumor-induced neovascularization, tumor growth and metastasis of the melanoma cells.

The analysis of host stroma surrounding the tumor mass clearly demonstrates that repeated local injections of FGF-2 into the inoculation site of dorsal skin significantly promotes the formation of new blood vessels oriented toward the tumor mass detectable by angiography, and the high density of vWF-positive microvessels in the host stroma detectable by immunohistochemical staining. Interestingly, the subcutaneous injection of FGF-2 alone generated new blood vessels oriented toward the injection site and a high density of microvessels, indicating that the neovascular potential of FGF-2 in the host stroma is independent of the inoculated tumor cells. A significant increase in the density of microvessels was also seen in the dorsal and footpad tumor parenchyma stimulated locally by FGF-2. This involved a small necrotic area in the tumor tissue during the initial phase and subsequent aggressive tumor growth after the local stimulation with FGF-2. These findings demonstrate that the abundant FGF-2 during the initial phase of tumor growth is important for intensive tumor-induced neovascularization during the subsequent phase of an active tumor.

Histopathological observation in the dorsal skin inoculated with the tumor revealed that the repeated local injections of FGF-2 into the tumor inoculation site during the initial phase induced an intense migration of mesenchymal cells and the recruitment of various inflammatory cells in the host stroma surrounding the tumor. The inflammatory and mesenchymal cells migrating in the host stroma surrounding the tumor are considered to act as positive modulators of tumor-induced neovascularization by producing various chemokines, cytokines and growth factors.[3–5] Moreover, local injection of the anti-inflammatory agent PDN completely inhibited FGF-2-induced

doi: 10.1111/j.1349-7006.2007.00432.x

neovascularization in host tissue without tumor inoculation and intensive formation of microvessels in the tumor parenchyma as well as marked primary tumor growth in the footpad and pulmonary metastasis. This result demonstrates that exogenous FGF-2 induces inflammatory reactions in the host stroma during the initial phase of tumor growth, which plays an especially critical role in intensive tumor-induced neovascularization during the active growing phase, supporting the subsequent tumor growth and metastasis.

Intense neovascularization in tumor parenchyma has been recognized as a risk factor for metastasis and results in a poor prognosis.[2,3,15,28] Vessels oriented toward the tumor mass have also been shown to be crucial for the volumetric growth of tumors in a xenograft model of human melanoma.[30] Highly aggressive and metastatic human malignant melanoma cells induce marked neovascularization via excessive expression of endogenous FGF-2 and VEGFA.[31] As described above, abundant FGF-2 during the initial phase induced subsequent intensive neovascularization in host stroma and tumor parenchyma. In addition, we showed that the progressive activity of FGF-2 was completely inhibited by the administration of anti-mouse VEGFA antibody during tumor growth in the present footpad metastasis model. These findings suggest a simple hypothesis that the local injection of FGF-2 acts directly on tumor cells to stimulate their proliferation and migration or the production of VEGFA. Exogenous FGF-2 was insufficient to directly induce cellular proliferation and VEGFA expression, even though cultured B16-BL6 melanoma cells are able to respond to exogenous FGF-2. FGF-2 did not affect the migration of B16-BL6 cells *in vitro*.[32] Accordingly, the marked tumor progression caused by the local injection of FGF-2 is likely to be attributable to neovascularization in the host stroma and tumor parenchyma rather than direct proliferative activity of B16-BL6 cells. In the present study, we demonstrated that FGF-2 enhances endogenous VEGFA expression in host stroma rather than tumor tissue, and induces intensive proliferation and migration of VEGFA-positive endothelial and fibroblastic cells in the host stroma surrounding the tumor. The administration of anti-VEGFA antibody in the host tissue without the tumor inoculation directly inhibited FGF-2-induced neovascularization. These results are supported by reports that FGF-2 upregulates the expression of VEGFA and VEGF receptors in vascular endothelial cells,[10] smooth muscle cells[11] and stroma cells,[12] which in turn stimulate synergistically the proliferation of vascular endothelial cells with VEGFA.[33,34] The local injection of FGF-2 also induced the recruitment of neutrophils, T cells and monocyes in the host stroma during the initial phase and these inflammatory cells are also sources of VEGFA.[35–39] Taken together, abundant FGF-2 during the initial phase recruits vascular endothelial cells and various inflammatory and mesenchymal cells in the host stroma surrounding the tumor, and stimulates endogenous VEGFA expression in these host cells but not the tumor cells.

In conclusion, the present study demonstrates for the first time that abundant FGF-2 during the initial phase of tumor growth induces not only the recruitment of various host cells to the stromal microenvironment surrounding the tumor but also intensive VEGFA-dependent neovascularization during the active growing phase, which is essential for the subsequent tumor growth and metastasis of B16-BL6 melanoma cells. The present results provide important information that the local effect of FGF-2 on host stroma during the initial phase of tumor growth significantly affects subsequent tumor-induced neovascularization and progression of the tumor.

Acknowledgments

We thank Kenji Minowa, Hajime Sugimoto, Yoshiko Nakano, Yoko Ueno and Yurika Saitoh for technical support.

References

1 Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000; **407**: 249–57.
2 Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995; **1**: 27–31.
3 Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003; **3**: 401–10.
4 Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860–6.
5 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **17**: 539–45.
6 Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G. Structural characterization and biological functions of fibroblast growth factor. *Endocr Rev* 1987; **8**: 95–113.
7 Bikfalvi A, Klein S, Pintucci G, Rifkin DB. Biological roles of fibroblast growth factor-2. *Endocr Rev* 1997; **18**: 26–45.
8 Zittermann SI, Issekutz AC. Basic fibroblast growth factor (bFGF, FGF-2) potentiates leukocyte recruitment to inflammation by enhancing endothelial adhesion molecule expression. *Am J Pathol* 2006; **168**: 835–46.
9 Veikkola T, Karkkainen M, Claesson-Welsh L, Alitalo K. Regulation of angiogenesis via vascular endothelial growth factor receptors. *Cancer Res* 2000; **60**: 203–12.
10 Seghezzi G, Patel S, Ren CJ *et al.* Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol* 1998; **141**: 1659–73.
11 Belgore F, Lip GY, Blann AD. Basic fibroblast growth factor induces thsecretion of vascular endothelial growth factor by human aortic smooth muscle cells but not by endothelial cells. *Eur J Clin Invest* 2003; **33**: 833–9.
12 Claffey KP, Abrams K, Shih SC, Brown LF, Mullen A, Keough M. Fibroblast growth factor 2 activation of stromal cell vascular endothelial growth factor expression and angiogenesis. *Lab Invest* 2001; **81**: 61–75.
13 Wang Y, Becker D. Anitsense targeting of basic fibroblast growth factor and fibroblast growth factor receptor-1 in human melanomas blocks intratumoral angiogenesis and tumor growth. *Nat Med* 1997; **3**: 887–93.
14 Prewett M, Huber J, Li Y *et al.* Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res* 1999; **59**: 5209–18.
15 Rofstad EK, Halsør 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* 2000; **60**: 4932–8.
16 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 fibroblast growth factor. *Cancer Res* 2001; **61**: 7282–90.
17 Giavazzi R, Sennino B, Coltrini D *et al.* Distinct role of fibroblast growth factor-2 and vascular endothelial growth factor on tumor growth and angiogenesis. *Am J Pathol* 2003; **162**: 1913–26.
18 Streit M, Detmer M. Angiogenesis, lymphangiogenesis and melanoma metastasis. *Oncogene* 2003; **22**: 3172–9.
19 Rodeck U, Becker D, Herlyn M. Basic fibroblast growth factor in human melanoma. *Cancer Cells* 1991; **3**: 308–11.
20 Shih IM, Herlyn M. Role of growth factors and their receptors in the development and progression of melanoma. *J Invest Dermatol* 1993; **100**: 196S–203S.
21 Lázár-Molnár E, Hegyesi H, Tóth S, Falus A. Autocrine and paracrine regulation by cytokines and growth factors in melanoma. *Cytokine* 2000; **12**: 547–54.
22 Hsu MY, Meier F, Herlyn M. Melanoma development and progression: a conspiracy between tumor and host. *Differentiation* 2002; **70**: 522–36.
23 Fidler IJ. The organ microenvironment and cancer metastasis. *Differentiation* 2002; **70**: 498–505.
24 Giavazzi R, Giuliani R, Coltrini D *et al.* Modulation of tumor angiogenesis by conditional expression of fibroblast growth factor-2 affects early but not established tumors. *Cancer Res* 2001; **61**: 309–17.
25 Poste G, Doll J, Hart IR, Fidler IJ. *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. *Cancer Res* 1980; **40**: 1636–44.
26 Hart IR. The selection and characterization of an invasive variant of the B16 melanoma. *Am J Pathol* 1979; **97**: 587–600.
27 Kreisle RA, Ershler WB. Investigation of tumor angiogenesis in an id mouse model: role of host–tumor interactions. *J Natl Cancer Inst* 1988; **80**: 849–54.

28 Weidner N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. *Breast Cancer Res Treat* 1995; **36**: 169–80.
29 Price JE. Xenograft Models in immunodeficient animals. In: Brooks SA, Schumacher U, eds. *Metastasis Research Protocols*, vol. II. Totowa, NJ: Humana Press, 2001: 205–13.
30 Danielsen T, Rofstad EK. VEGF, bFGF and EGF in the angiogenesis of human melanoma xeografts. *Int J Cancer* 1998; **76**: 836–41.
31 Westphal JR, Van't Hullenaar R, Peek R *et al*. Angiogenic balance in human melanoma: expression of VEGF, bFGF, IL-8, PDGF angiostatin in relation to vascular density of xeografts *in vivo*. *Int J Cancer* 2000; **86**: 768–76.
32 Saiki I, Murata J, Yoneda J, Kobayashi H, Azuma I. Influence of fibroblasts on the invasion and migration of highly or weakly metastatic B16 melanoma cells. *Int J Cancer* 1994; **56**: 867–73.
33 Mandriota SJ, Pepper MS. Vascular endothelial growth factor-induced *in vitro* angiogenesis and plasminogen activator expression are dependent on endogenous basic fibroblast growth factor. *J Cell Sci* 1997; **110**: 2293–302.
34 Koolwijk P, van Erck MG, de Vree WJ *et al*. Cooperative effect of TNFα, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J Cell Biol* 1996; **132**: 1177–88.
35 Pertovaara L, Kaipainen A, Mustonen T *et al*. Vascular endothelial growth factor is induced in response to transforming growth factor-β in fibroblastic and epithelial cells. *J Biol Chem* 1994; **269**: 6271–4.
36 Cursiefen C, Chen L, Borges LP *et al*. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatry neovasculation via macrophage recruitment. *J Clin Invest* 2004; **113**: 1040–50.
37 Taichman NS, Young S, Cruchley AT, Taylor P, Paleolog E. Human neutrophils secrete vascular endothelial growth factor. *J Leukoc Biol* 1997; **62**: 397–400.
38 Namiki A, Brogi E, Kearney M *et al*. Hypoxia induces vascular endothelial growth factor in cultured human endothelial cells. *J Biol Chem* 1995; **270**: 3189–95.
39 Freeman MR, Schneck FX, Gagnon ML *et al*. Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis. *Cancer Res* 1995; **55**: 4140–5.

doi: 10.1111/j.1349-7006.2007.00432.x