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Basic fibroblast growth factor-mediated overexpression of vascular endothelial growth factor in 1F6 human melanoma cells is regulated by activation of PI-3K and p38 MAPK

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Abstract. *Background:* 1F6 human melanoma xenografts overexpressing either the 18 kD (18kD) form or all (ALL) forms of human basic fibroblast growth factor (bFGF) demonstrate an abundant number of microvessels and accelerated growth. We now examined whether bFGF mediates vascular endothelial growth factor (VEGF) expression.

Methods: Quantitative RT-PCR was used to determine bFGF and VEGF mRNA, VEGF protein secretion was measured by ELISA and VEGF promoter activation was assessed by a dual luciferase activity assay. Western blot was carried out to detect phosphorylation of bFGF-regulated target proteins.

Results: In 1F6-18kD and 1F6-ALL clones VEGF mRNA was increased 4- to 5-fold and VEGF protein secretion was highly stimulated due to activation of the VEGF promotor. PI-3K, p38 MAPK and ERK1/2 MAPK pathways were activated, while inhibition of PI-3K or p38 resulted in, respectively, 55% and up to 70% reduction of VEGF mRNA overexpression. A concurrent 60% decrease in VEGF protein secretion was mostly apparent upon inhibition of PI-3K. Inhibition of ERK1/2 hardly affected VEGF mRNA or protein secretion. Two unselected human melanoma cell lines with high metastatic potential contained high bFGF and VEGF, while three non- or sporadically metastatic cell lines displayed low bFGF and VEGF.

Conclusion: These data indicate that stimulation of VEGF protein secretion in response to bFGF overexpression may contribute to increased vascularization and enhanced aggressiveness in melanoma.

Keywords: Basic fibroblast growth factor, vascular endothelial growth factor, melanoma, PI-3K, p38 MAPK, ERK1/2 MAPK

1. Introduction

Melanoma cells express a wide variety of growth factors, such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). bFGF is considered to be a key protein involved in melanoma growth. In contrast to melanocytes, almost all melanomas produce bFGF. Since melanoma cells express bFGF receptors, bFGF can act as a paracrine and autocrine growth stimulator in each sequential step of melanoma tumor progression [15,18]. In addition, bFGF can have profound paracrine effects on melanoma growth by stimulating angiogenesis [22,40]. VEGF is another important growth factor in melanoma development, since it is upregulated during the course of melanoma progression and will stimulate angiogenesis as well [22,23,30,40]. The VEGF receptors Kinase Domain Region (KDR; VEGFR-2) and fms-related tyrosine kinase 1 (Flt-1; VEGFR-1) are not widely distributed on melanoma cells [12,19] Graeven et al. [13] have suggested that, in contrast to bFGF, VEGF expression has a beneficial, but non-essential role in melanoma development and progression. Birck et al. [1] have described in human melanoma primary tumors and metastases that bFGF was more often ex-

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pressed than VEGF, which might indicate that VEGF expression is a later event in melanoma growth. This group has suggested that bFGF expression may be required before VEGF expression can be accomplished.

VEGF upregulation in tumor cells is mainly mediated under hypoxic conditions by activation of hypoxia-responsive elements in the VEGF promotor (HREs) through the transcription factor HIF-1 [8,10, 24]. Overexpression of VEGF in melanoma cells may thus be caused by areas of low oxygen in the microenvironment in later stages of melanoma growth. Apart from HRE, however, the VEGF promotor region contains other potential binding sites for transcription factors, such as SP-1, AP-1, AP-2 and NF-1 [8,38]. These transcription factors can be activated by several growth factors, among which are bFGF, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). bFGF-mediated upregulation of VEGF has been described in a number of cell types, including rabbit vascular smooth muscle cells [36], human glioma cells [28], bovine aortic and capillary endothelial cells [32], rat osteoblastic cells [29] and mouse embryonic fibroblasts [3]. Experiments to elucidate the mechanism behind this upregulation have indicated that VEGF mRNA stability was not increased after bFGF stimulation, suggesting transcriptional upregulation [28, 29]. In human glioma cells, it was demonstrated that for bFGF-enhanced VEGF expression the presence of the SP-1 binding sites in the proximal VEGF promotor was essential [28]. Since bFGF is a key protein in melanoma development, we hypothesized that this growth factor may also be responsible for upregulation of VEGF in melanoma cells.

bFGF consists of different polypeptides of which the 18 kD form is mainly localized in the cytoplasm, but can also be secreted in biological fluids and in plasma. It is bound to heparan sulfate proteoglycans on the cell membrane and can activate cell surface FGF receptors. Therefore, it is the 18 kD protein that can exert autocrine effects on cell growth in addition to inducing paracrine effects, such as stimulation of endothelial cells. Owing to the expression of a nuclear localization sequence, the high molecular weight forms of 22, 22.5, 24 and 34 kD are localized in the nucleus and are thought to be involved in transcriptional regulation [33]. Previously, we have transfected 1F6 melanoma cells with the 18 kD and all forms of bFGF to determine whether bFGF overexpression was associated with increased aggressiveness and whether there was a difference in behaviour between the bFGF-overexpressing clones [7]. Indeed, bFGF-overexpressing 1F6 cells showed a significantly increased proliferation rate *in vitro* as well as grown as xenografts for which overexpression of the 18 kD form was sufficient. Since xenografts contained an abundant number of small blood vessels, we determined the amount of VEGF produced by the different clones. Here, we report that VEGF mRNA is upregulated by activation of the VEGF promoter and that VEGF protein secretion is increased. Furthermore, we demonstrate that activation of the PI-3K and p38 MAPK signal transduction pathways are involved in the increased VEGF production in bFGF-overexpressing melanoma clones. We observed that two human melanoma cell lines with simultaneous expression of high bFGF and VEGF are known to have a more aggressive behaviour, while three others known for low to moderate aggressiveness displayed both low bFGF and VEGF.

2. Materials and methods

2.1. Cell culture

The human melanoma cell lines BLM, Mel57, M14 and 1F6 have been established from surgically removed melanoma metastases [39]. The BRO cell line has been derived from a highly malignant and aggressive primary melanoma [20]. All cell lines contain mRNA transcripts for FGFR-1, -3 and -4 [7]. Cell lines and 1F6 clones overexpressing the 18 kD (18kD) form of human bFGF, all (ALL) forms of bFGF, or control empty vector cells (1F6-pcDNA3) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Bio Whittaker, Verviers, Belgium) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS; Gibco/Invitrogen, Breda, The Netherlands), 50 units/ml penicillin (ICN Biochemicals, Zoetermeer, The Netherlands) and 50 µg/ml streptomycin (ICN Biochemicals). In addition, bFGF-overexpressing 1F6 clones were cultured in the presence of 500 µg/ml geneticin (Gibco/Invitrogen) to maintain selection of bFGF overexpression. Cells were grown at 37°C in humidified air containing 5% CO_2.

2.2. Isolation of RNA and RT-PCR

Total RNA of cell lines was isolated using Trizol reagent (Gibco/Invitrogen) according to the manufacturer's protocol. 3 µg RNA was reverse-transcribed with 50 Units MMLV reverse transcriptase (Invitro-

gen) in the presence of 1.2 μl 0.5 μg/ml random primers.

Quantitative light-cycler RT-PCR was used to determine bFGF and/or VEGF mRNA expression in human melanoma cell lines, bFGF-transfected 1F6 clones, and in 1F6 cells treated with exogenous recombinant human bFGF (rhbFGF; R&D systems/ITK diagnostics, Uithoorn, The Netherlands). mRNA expression was analyzed with the LightCycler-Faststart DNA Master Hybridization Probes kSYBR Green 1 kit (Roche Diagnostics, Mannheim, Germany). The human bFGF (bFGF sense primer, 5′-TGTGCTAACCGTTACGTG GC-3′ and bFGF antisense primer, 5′-ATAGCTTTCT GCCCAGGTCC-3′), the human VEGF ($VEGF_{165}$ sense primer, 5′-CCCTGATGAGATCGAGTACATC TT-3′ and $VEGF_{165}$ antisense primer, 5′-AGCAAGGC CCACAGGGATTT-3′) and, as an internal control, the human β2-microglobulin (β2-microglobulin sense primer, 5′-GATGAGTATGCCTGCCGTGTG-3′ and β2-microglobulin antisense primer, 5′-CAATCCAAA TGCGGCATCT-3′) or PGDB (PGDB sense primer, 5′-TCCAAGCGGAGCCATGTCTG-3′ and PGDB antisense primer, 5′-AGAATCTTGTCCCCTGTGGTG GA-3′) gene were amplified according to the manufacturer's protocol. In short, the reaction mix contained 4 mM $MgCl_2$, 0.5 μM sense and antisense primer, 1 × FastStart DNA Master SYBR Green 1 mix (containing LightCycler Faststart Enzyme, FastStart Taq DNA polymerase, SYBR green dye, dNTPs and reaction buffer) and 2 μl cDNA. PCR conditions were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 22 s. cDNA was replaced by PCR-grade water as a negative control. Relative expression levels of different samples were calculated from bFGF crossing points normalized to β2-microglobulin or PGDB. For each experiment, melting curve analysis was performed. Primer efficiencies were determined using pooled cDNAs.

In experiments designed to investigate the effect of bFGF on VEGF mRNA stability, cells were made quiescent in serum-free medium for 24 h. Next, cells were pre-incubated with 1 μg/ml actinomycin D (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 1 h in serum-free medium and subsequently (designated as $t = 0$ h) incubated with 1 μg/ml actinomycin D for 1, 2, 3 and 5 h in serum-free medium. After each period of actinomycin D treatment, VEGF mRNA levels were determined by light-cycler RT-PCR.

2.3. Western blot

Melanoma cell lines and bFGF-overexpressing clones were lysed in ice-cold FOS-RIPA lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.1% NP-40 and 0.1% sodium deoxycholate) supplemented with 0.5 mM trypsin inhibitor (Sigma-Aldrich), 0.5 μg/ml leupeptin (Sigma-Aldrich), 1 mM PMSF (Merck, Amsterdam, The Netherlands), 0.1 mM sodium ortho vanadate (Sigma-Aldrich) and 50 mM sodium fluoride (Baker Chemicals, Deventer, The Netherlands) for 10 min on ice. Lysates were centrifuged for 15 min at 13,000 rpm at 4°C to remove debris. Protein concentrations of cell lysates were measured according to Bradford [2].

Proteins were denatured by addition of sample buffer containing β-mercapto ethanol and incubation at 95°C for 5 min. Proteins were subjected to SDS-PAGE on 12% polyacrylamide gels and electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Etten-Leur, The Netherlands). Membranes were blocked for 1 h in TBST (10 mM Tris pH 8, 150 mM/l NaCl and 0.025% Tween 20)/5% milk and incubated overnight with 0.2 μg/ml rabbit polyclonal human bFGF-directed antiserum (sc-79; Santa Cruz Biotechnology, Heerhugowaard, The Netherlands). For determination of induction of PI-3K (Akt), p38 MAPK and p42/p44 (ERK1/2) MAPK, activity in bFGF-overexpressing 1F6 clones, 2.0×10^6 cells were plated in 10 cm^2 dishes. Activity of Akt, p38 and ERK1/2 was determined with rabbit polyclonal anti-phospho Akt antibody, rabbit polyclonal anti-phospho p38 or rabbit polyclonal anti-phospho ERK1/2 antibody (all from Cell Signaling Technology, Heerhugowaard, The Netherlands), respectively. Total levels of Akt, p38 and ERK1/2 were assessed after stripping membranes with strip buffer (10 ml 10% SDS, 0.347 ml β-mercapto ethanol, 3.125 ml Tris 0.1 M at pH 8 and 36.5 ml H_2O), washing and blocking with TBST/5% milk and incubation with rabbit polyclonal antibodies against Akt, p38 and ERK1/2 (Cell Signaling Technology).

For determination whether the PI-3K, p38 and ERK1/2 pathways were involved in the induction of VEGF mRNA expression and protein secretion in bFGF-overexpressing 1F6 cells, cells were incubated in the presence or absence of 30 μM LY294002 (inhibitor of PI-3K activity; Sigma-Aldrich), 10 μM SB202190, 10 μM SB203580 (both inhibitors of p38 activity; Sigma-Aldrich), 50 μM PD98059, or 10 μM U0126 (both inhibitors of ERK1/2 activity; Sigma-

Aldrich), respectively, in serum-free medium for 24 h. After this incubation period, cells for preparation of lysates and conditioned media (ELISA) were collected. Western blot was performed with the anti-phospho Akt antibody, a rabbit monoclonal antibody against the phosphorylated downstream target of p38 (p-MAPKAPK-2; Cell Signaling Technology) and the anti-phospho ERK1/2 antibody. Total levels of proteins were assessed after stripping membranes with strip buffer, washing and blocking with TBST/5% milk and incubation with rabbit polyclonal antibodies against Akt, MAPKAPK-2 (Cell Signaling Technology) and ERK1/2. As a loading control for the detection of the different proteins, blots were incubated with 40 ng/ml mouse monoclonal antibody against β-actin (Sigma-Aldrich).

After incubation with specific antisera, membranes were washed with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated mouse anti-rabbit IgG (Cell Signaling Technology) or rabbit anti-mouse IgG (DAKO, Heverlee, Belgium) in TBST/5% milk. Membranes were washed and proteins were visualized by electro-chemiluminescence. Densitometry was performed with the use of ImageJ software. Intensities of phosphorylated proteins were normalized to the respective β-actin loading control.

2.4. ELISA

For quantification of VEGF protein secretion by parent 1F6 cells and bFGF-overexpressing clones, conditioned media were collected. Conditioned media were obtained 24 h after plating 1×10^6 cells in 10 cm culture dishes. Conditioned media were also collected from 1F6 cells treated with rhbFGF for a period of 72 h. In separate experiments, the medium was replaced after 16 h by serum-free medium in the presence or absence of 30 μM LY294002, 10 μM SB202190, 10 μM SB203580, 50 μM PD98059 and 10 μM U0126. After another 24 h, the conditioned media were collected, cells were detached and counted. ELISA was performed in duplicate using the reagents and protocol supplied with the Quantikine Human VEGF Immunoassay kit (R&D systems/ITK diagnostics). VEGF protein content in conditioned medium was expressed as pg/ml/10^6 cells.

2.5. Transient transfection and dual luciferase activity assays

The VEGF promoter-luciferase reporter construct pVEGF-Kpn I was a kind gift from Dr. G. Semenza, Johns Hopkins University School of Medicine, Baltimore, USA, and has been described before [10]. pGL2 basic and pRL-TK were purchased from Promega (Leiden, The Netherlands). Exponentially growing 1F6 and bFGF-overexpressing clones (2×10^5) were plated in 6-well plates. After 24 h, cells were transiently transfected with 0.5 μg of reporter plasmid for 48 h using Fugene 6 transfection reagent (Roche Diagnostics) following the manufacturer's protocol. Fifty ng pRL-TK was co-transfected to correct for transfection efficiency. The activities of firefly and Renilla luciferase were determined with the dual luciferase reporter assay (Promega) following the manufacturer's protocol. In short, cells were lysed by scraping in the presence of 250 μl 1 × passive lysis buffer. Twenty μl of cell lysate was transferred into the luminometer tube containing 100 ml LAR II (Promega), and firefly luciferase activity was measured. Thereafter, Renilla luciferase activity was measured after adding 100 ml of Stop & Glo Reagent (Promega). Relative VEGF promoter activity in 1F6 and bFGF-transfected clones was calculated by the formula:

$$\frac{\text{VEGF} - \text{Kpn I firefly luciferase value}}{\text{pRL} - \text{TK Renilla luciferase value}} \Big/ \frac{\text{pGL2} - \text{basic firefly luciferase value}}{\text{pRL} - \text{TK Renilla luciferase value}}.$$

2.6. Statistical analysis

Data presented as mean ± SD were analyzed using Student's t-test. $p < 0.05$ was considered significant.

3. Results

3.1. VEGF expression is increased in 1F6 cells overexpressing 18kD or ALL forms of bFGF

Recently, we have characterized 1F6 melanoma cells stably transfected with either the 18 kD (18kD) form or all (ALL) forms of bFGF [7]. bFGF protein expression in control empty vector cells 1F6-pcDNA3 and 1F6 clones highly (H) and lowly (L) overexpressing the 18kD form or ALL forms of bFGF is shown in Fig. 1A. High endogenous bFGF re-

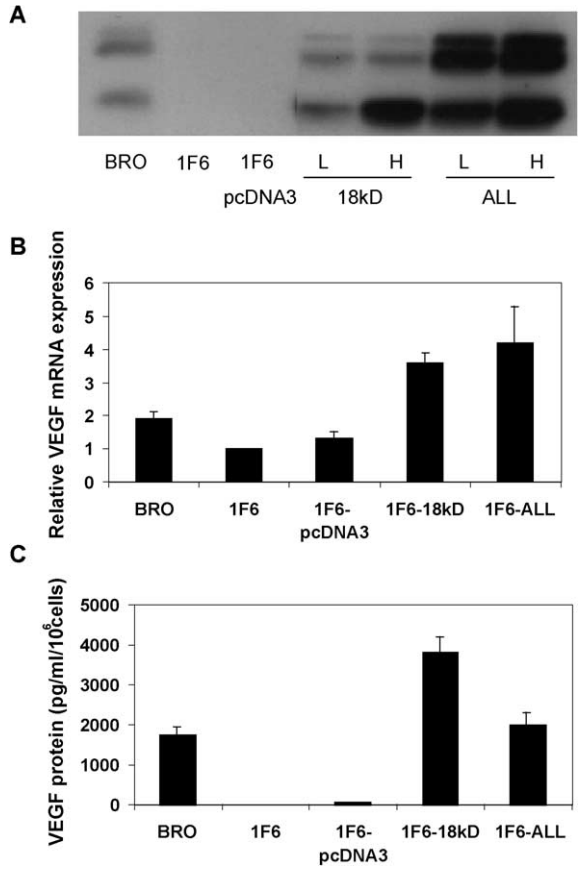


Fig. 1. bFGF overexpression in 1F6 cells and the effect of bFGF overexpression on VEGF mRNA expression and protein secretion in BRO, 1F6, 1F6-pcDNA3 and clones highly (H) or lowly (L) overexpressing the 18 kD form or all forms of bFGF. (A) bFGF protein expression was determined by subjecting 25 μg protein of each cell line to Western blot. (B) Cells were serum-starved for 24 h, total RNA was isolated and VEGF mRNA levels were determined by light cycler RT-PCR. Relative VEGF expression in 1F6 cells was set at 1. (C) VEGF protein expression in conditioned medium was determined by ELISA. BRO was included as a positive control cell line. Light cycler and ELISA results are shown as mean values ± range of duplicate samples. The experiments were performed three times with similar outcome.

sulted in only little amounts of bFGF on the cellular membrane and in conditioned medium. Since we detected enhanced angiogenesis in bFGF-overexpressing 1F6 xenografts [7] we here determined the VEGF mRNA and protein expression in 1F6 clones highly overexpressing bFGF. VEGF mRNA expression was upregulated 4- and 5-fold in clones 1F6-18kD and 1F6-ALL, respectively, as compared to that in parent 1F6 cells (Fig. 1B). High production of VEGF by bFGF-transfected 1F6 clones was confirmed by ELISA of conditioned media (Fig. 1C). While VEGF was not detectable in conditioned media of 1F6 and 1F6-pcDNA3 cells collected for a period of 24 h, VEGF protein secretion of 4000 and 2000 pg/ml/10^6 cells was measured in clones 1F6-18kD and 1F6-ALL, respectively. VEGF mRNA expression and protein secretion of bFGF-overexpressing 1F6 clones was slightly higher than that of the BRO cell line, which contains high endogenous VEGF.

3.2. *bFGF-induced VEGF expression is not due to increased VEGF mRNA stability*

One possible mechanism for the increased VEGF expression observed in bFGF-overexpressing 1F6 cells could be increased VEGF mRNA stability. To that end, we determined the turnover of VEGF mRNA in 1F6-pcDNA3 and bFGF-transfected 1F6 clones by incubation with the transcription inhibitor actinomycin D for different time-periods. The reduction in VEGF mRNA levels was determined by light-cycler RT-PCR. VEGF mRNA was degraded with similar half-lives of approximately 3 h in 1F6-pcDNA3 and both bFGF-overexpressing 1F6 clones (Fig. 2). Thus, overexpression of 18kD form or ALL bFGF forms did not alter the stability of VEGF mRNA, suggesting that VEGF upregulation by bFGF is transcriptionally mediated.

3.3. *bFGF overexpression increases VEGF promoter activity*

Based on the previous results, we anticipated that the increased VEGF mRNA expression in 1F6 clones overexpressing bFGF was the result of enhanced promoter activity. Cells were transiently transfected with a VEGF promoter-luciferase reporter construct to determine the VEGF promoter activity in bFGF- or pcDNA3-transfected clones. To correct for possible differences in transfection efficiency between the cell lines, 50 ng pRL-TK was co-transfected. While both 1F6 and 1F6-pcDNA cells showed low VEGF promoter activity, increased VEGF promoter activity up to 13-fold was observed in 1F6-18kD and up to 7-fold in 1F6-ALL cells (Fig. 3). These results confirm the hypothesis that bFGF-induced upregulation of VEGF is transcriptionally mediated.

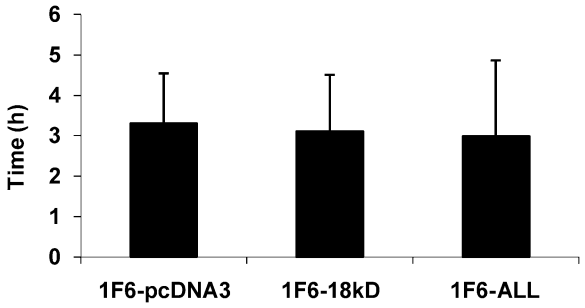


Fig. 2. Determination of VEGF mRNA half-life in 1F6-pcDNA3, 1F6-18kD and 1F6-ALL clones. Cells were treated with the transcription inhibitor actinomycin D after an initial exposure time of one h (from $t = -1$ h till $t = 0$). Thereafter, VEGF mRNA levels were determined by light-cycler RT-PCR at 1 h, 2 h, 3 h, 4 h and 5 h. Half-lives were calculated in h. Bars represent mean values $\pm$ range of two independent experiments.

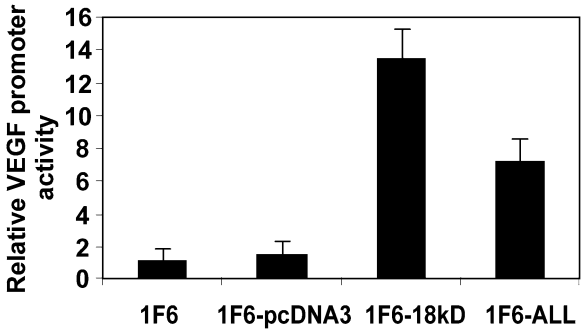


Fig. 3. VEGF promoter activity in 1F6 parent cells, 1F6-pcDNA3 and bFGF-overexpressing 1F6 clones. Cells were transiently transfected for 48 h with either the VEGF promoter luciferase construct VEGF-Kpn I or the promotor-less construct pGL2-basic. All cells were co-transfected with the Renilla luciferase reporter construct pRL-TK to correct for differences in transfection efficiency. Promoter activities are expressed relative to VEGF promoter activity in 1F6, which was set at 1. Bars represent mean values $\pm$ range of duplicate samples. The experiment was performed three times with similar outcome.

3.4. *Exogenous recombinant human bFGF slightly stimulates VEGF in 1F6 cells*

Earlier, we have shown that exogenous rhbFGF can stimulate 1F6 cell growth [7]. We here determined whether VEGF production would be stimulated as well. Since VEGF secretion by 1F6 cells is extremely low (Fig. 1C), we treated cells with rhbFGF 12.5 or 25 ng/ml for a period of 72 h. Thereafter, conditioned media were collected and cells were counted. Conditioned media were concentrated 8-fold to be able to measure VEGF protein by ELISA. Cells were subjected to light-cycler RT-PCR to assess the amount

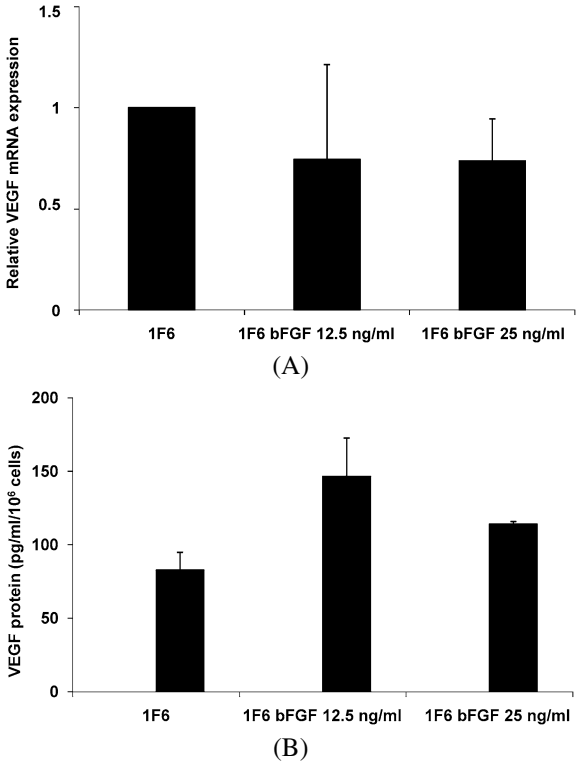


Fig. 4. VEGF mRNA expression and VEGF protein production by 1F6 cells treated with exogenous rhbFGF 12.5 or 25 ng/ml for 72 h. (A) Total RNA was isolated and VEGF mRNA levels were determined by light-cycler RT-PCR. Relative expression in 1F6 was set at 1. (B) Conditioned media was 8 times concentrated and VEGF secretion was measured by ELISA. After exposure to rhbFGF, cells were counted. Results were expressed in pg/ml/10^6 cells. Bars represent mean values $\pm$ range of duplicate samples.

of VEGF mRNA. Figure 4A and B demonstrate that although exogenous rhbFGF did not increase VEGF gene expression, protein excretion was slightly stimulated up to approximately 2-fold. VEGF protein levels, however, were far less than those calculated for clones 1F6-18kD and 1F6-ALL (Fig. 1C). This observation suggests that VEGF upregulation by endogenous bFGF may be caused by activation of the membrane-associated FGFR-1 signaling route, but an intracellular mechanism should also be taken into consideration.

3.5. *Activity of Akt, p38 and ERK1/2 is increased in bFGF-overexpressing 1F6 cells*

To acquire more insight into the signal transduction pathways activated in the bFGF-transfected 1F6 clones, the phosphorylation status of PI-3K-activated Akt, p38 and ERK1/2, was examined. BRO was in-

cluded as a cell line with high endogenous bFGF expression. As shown in Fig. 5A and B, phosphorylated Akt and ERK1/2 could be detected in 1F6 parent cells. Increased phosphorylation of Akt, p38 and ERK1/2 was observed in bFGF-overexpressing clones when compared to levels in 1F6 and 1F6-pcDNA3 cells, while no clear changes in total protein levels were seen. Together, these experiments suggest that PI-3K, p38 as well as ERK1/2, could possibly be involved in the upregulation of VEGF in bFGF-overexpressing 1F6 clones.

3.6. *PI-3K and p38 MAPK are involved in bFGF-mediated VEGF overexpression*

Since bFGF-overexpressing 1F6 cells contained increased activity of Akt, p38 and ERK1/2, we determined whether LY294002 (a specific inhibitor of PI-3K, the upstream kinase of Akt), SB202190 and SB203580 (both specific inhibitors of p38), PD98059 and U0126 (both inhibitors of ERK1/2) at concentrations derived from literature were sufficient to abrogate protein signaling activities. Figure 6A and B shows that LY294002 almost completely inhibited the increased Akt activity in 1F6-18kD and 1F6-ALL cells, while total Akt and β-actin levels remained constant. Both SB022190 and SB203580 resulted in almost complete inhibition of the increased activity of the downstream target of p38, MAPKAPK-2 in 1F6-18kD and 1F6-ALL cells, while total MAPKAPK-2 and β-actin levels were not affected. Further, PD98059 and U0126 almost completely abrogated the increased p-ERK1/2 levels in 1F6-18kD and 1F6-ALL clones without affecting total ERK1/2 and β-actin.

To investigate whether the PI-3K, p38 and ERK1/2 pathways were involved in the bFGF-induced VEGF expression, the effects of the various inhibitors on the increased VEGF mRNA expression levels and the amount of protein secretion in conditioned media were examined. Treatment with LY294002 (55%), SB202190 (70%) and SB203580 (35%) significantly inhibited VEGF mRNA expression in both 1F6-18kD and 1F6-ALL cells, while PD98059 and U0126 hardly reduced VEGF mRNA (Fig. 7A). Inhibition of VEGF protein secretion in both 1F6-18kD and 1F6-ALL cells was mostly apparent (60%) after treatment with LY294002 (Fig. 7B). Treatment with SB202190 and SB203580 resulted in a 35–50% reduction of VEGF secretion in 1F6-18kD cells. PD98059 and U0126 hardly affected secretion of VEGF.

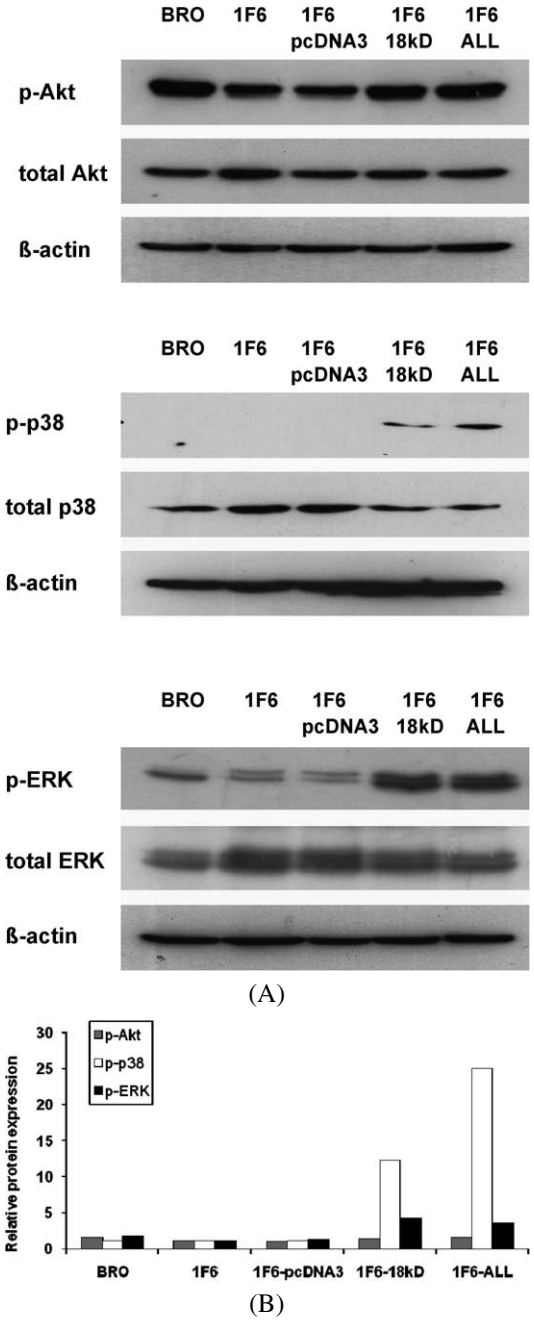
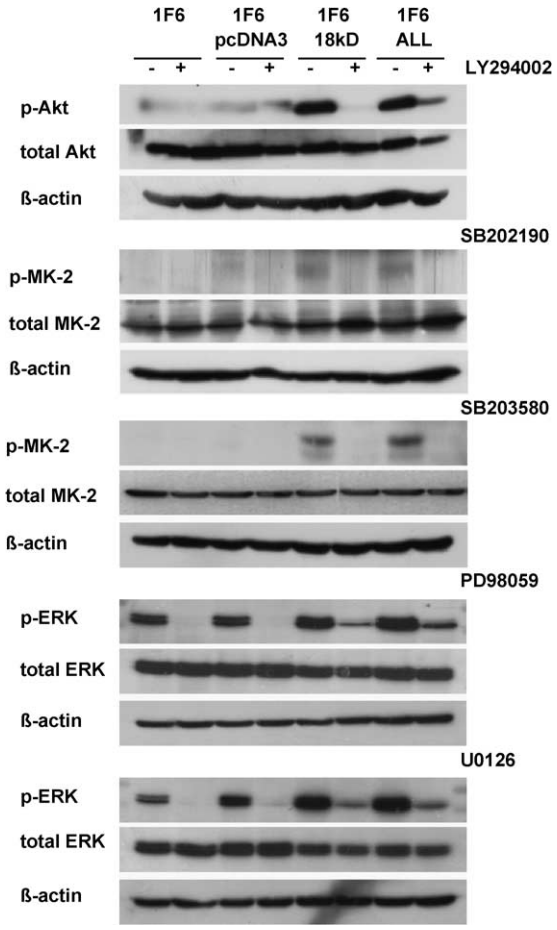
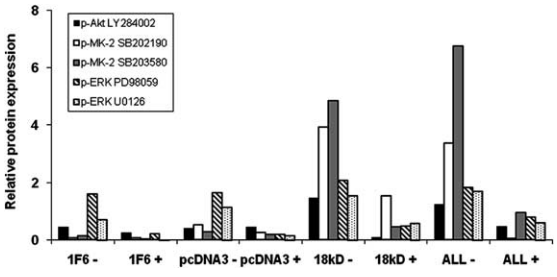


Fig. 5. Expression of Akt, p38 and ERK1/2 in BRO, 1F6 parent cells, 1F6-pcDNA3 and bFGF-overexpressing 1F6 clones. (A) Cell lysates were prepared and Western blot was performed with antibodies specific for the phosphorylated state of Akt, p38 and ERK1/2. After electro-chemiluminescence detection, blots were stripped and re-probed with antibodies against total Akt, p38 and ERK1/2. As a loading control, blots were probed for ß-actin. (B) Quantification of band intensities of phosphorylated proteins when normalized to their respective β-actin loading control. Normalized protein intensities were expressed relative to protein expression in 1F6 which was set at 1.

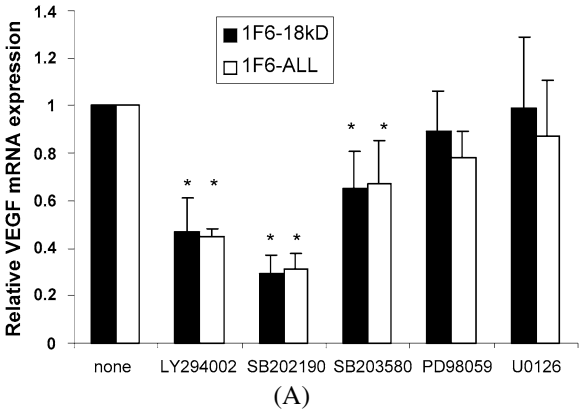


(A)

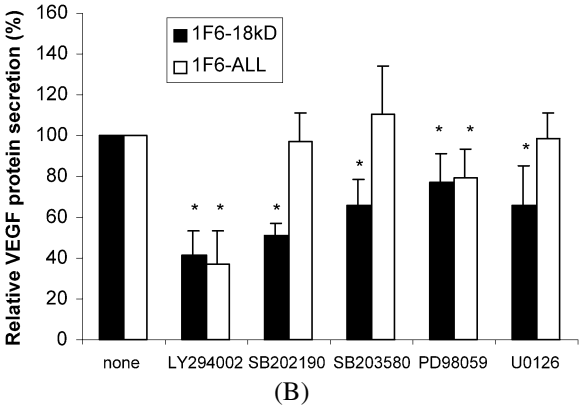


(B)

Fig. 6. Efficiency of PI-3K, p38 and ERK1/2 inhibition. 1F6 parent cells, 1F6-pcDNA3, 1F6-18kD and 1F6-ALL clones were incubated in serum-free medium for 24 h in the absence or presence of 40 μM LY294002, 10 μM SB202190, 10 μM SB203580, 50 μM PD98059 or 10 μM U0126. (A) Inhibition of PI-3K, p38 and ERK1/2 was determined by Western blots for the downstream targets, p-Akt, p-MAPKAPK-2 (p-MK-2) and p-ERK1/2, respectively. Blots were stripped and re-probed with antibodies against total Akt, MAPKAPK-2 (MK-2) and ERK1/2. As a loading control, blots were probed for β-actin. (B) Quantification of band intensities of phosphorylated proteins when normalized to their respective β-actin loading controls.



(A)



(B)

Fig. 7. Effect of PI-3K, p38 and ERK1/2 inhibitors on VEGF mRNA production (A) and protein secretion (B). Cells were incubated in serum-free medium for 24 h in the absence or presence of 40 μM LY294002, 10 μM SB202190, 10 μM SB203580, 50 μM PD98059 or 10 μM U0126 for 24 h. VEGF mRNA production was determined by light-cycler RT-PCR and relative expression in untreated cells was set at 1. VEGF protein secretion in conditioned medium was determined by ELISA, cells were counted and results were expressed in pg/ml/10^6 cells. Values in untreated cells were set at 100%. The experiments were carried out at least three times. Bars represent mean values $\pm$ SD. *Indicates a significant difference ($p < 0.05$) as compared to untreated cells.

Together, these findings indicate that VEGF upregulation in bFGF-overexpressing clones is mediated mainly through induction of the PI-3K and, to a lesser extent, the p38 signaling routes.

3.7. *VEGF and bFGF expression in a panel of human melanoma cell lines*

Since bFGF transfection in 1F6 cells resulted in VEGF upregulation, we determined the expression of both growth factors in a panel of human melanoma cell lines. In Fig. 8A, bFGF and VEGF mRNA expression relative to β2-microglobulin is plotted. As can be seen, the two cell lines with high bFGF expres-

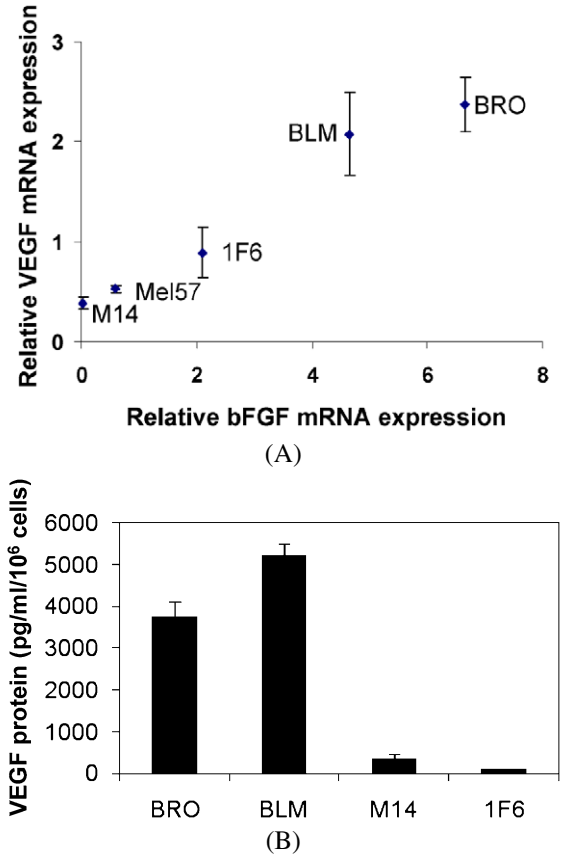


Fig. 8. bFGF and VEGF expression in a panel of human melanoma cell lines. (A) bFGF and VEGF mRNA levels of five different human melanoma cell lines: M14, Mel57, 1F6, BLM and BRO. mRNA expression was determined by light-cycler RT-PCR and expressed as a ratio relative to β2-microglobulin mRNA levels. (B) VEGF protein expression in conditioned medium was determined by ELISA. Bars represent mean values $\pm$ range of duplicate samples.

sion (BRO and BLM) also show high VEGF expression. Low to moderate VEGF expression was observed in cell lines Mel57, M14 and 1F6 expressing low levels of bFGF mRNA. In accordance with mRNA data, VEGF protein secretion in conditioned medium was significantly higher in BRO and BLM cells expressing high bFGF levels as compared to that in M14 and 1F6 cells expressing low to moderate bFGF protein levels (Fig. 8B).

4. Discussion

We demonstrate for the first time in human melanoma cells that overexpression of 18 kD form or all forms of bFGF results in increased VEGF mRNA expression and protein secretion. High VEGF secretion is not due to increased VEGF mRNA stability, but is rather the result of activation of the VEGF promoter. We further show that the PI-3K and p38 pathways, but not the ERK1/2 MAPK pathway, are involved in the bFGF-induced VEGF upregulation.

Our bFGF-overexpressing 1F6 cells grown as subcutaneous xenografts displayed enhanced angiogenesis [7]. bFGF is known to be a potent stimulator of angiogenesis as has been demonstrated *in vitro* and *in vivo* [26]. Experimental evidence suggests that in addition to its direct effect on endothelial cells, bFGF may exert its angiogenic effects indirectly through the induction of VEGF expression [26,32,36]. As an example, Seghezzi et al. [32] have shown that endothelial cell proliferation and neovascularization in the mouse corneal assay induced by exogenous bFGF was inhibited by addition of a neutralizing antibody against VEGF. These data indicate that VEGF can be an important paracrine mediator of bFGF-induced angiogenesis. In our bFGF-overexpressing 1F6 xenografts the increased angiogenesis could not be attributed to bFGF itself, but was the consequence of enhanced VEGF secretion [7].

We here report that high endogenous bFGF expression upregulates VEGF expression in human melanoma cells by an autocrine loop. Similar results have been observed in bovine capillary endothelial (BCE) cells [32]. In that study, medium from SK-Hep-1 hepatoma cells, which does not contain bFGF by itself [25], could induce the expression of 18 kD and HMW bFGF. In these BCE cells containing high bFGF expression mediated by SK-Hep-1 conditioned medium, a concomitant upregulation of VEGF was observed [32]. The authors have further shown that overexpression of 18 kD bFGF in NIH-3T3 cells resulted in high VEGF expression through extracellular interaction with membrane bFGF receptors, while high molecular weight (HMW) bFGF upregulated VEGF via intracellular mechanisms [32].

In addition to high endogenous bFGF levels, exogenous bFGF can also upregulate VEGF expression as described in a number of normal as well as malignant cell types. Exposure of rabbit vascular smooth muscle cells to 10 ng/ml rhbFGF resulted in a time-dependent increase of VEGF mRNA, starting within 1 h of incubation [36]. In human glioma cells, VEGF mRNA increased over 5-fold after incubation with 10 ng/ml bFGF for 30 min [28]. In rat osteoblastic cells addition of 12.5 ng/ml rhbFGF resulted in a 6-fold peak increase of VEGF mRNA after 6 h [29]. Seghezzi et al. [32] have stimulated BCE and bovine aortic endothelial (BAE) cells with 10 ng/ml rhbFGF for 4 h and measured increased VEGF mRNA expression up to 10- and

5- fold, respectively. VEGF protein secretion in conditioned medium was also increased in cells treated with bFGF. We did not find increased VEGF mRNA expression, but VEGF secretion was slightly increased in 1F6 cells treated with exogenous rhbFGF for a period of 72 h.

Interestingly, overexpression of the 18 kD bFGF form alone in our 1F6 cells was sufficient for the induction of VEGF expression, since the presence of HMW bFGF proteins did not further increase VEGF production. Sheng et al. [33] have demonstrated that the endogenous 18-kD bFGF can also translocate to the nucleus. It harbours a C-terminal non-classical bipartite localization sequence of which part is responsible for nucleolar localization. Stachowiak et al. [34] have clearly shown the presence of FGFR-1 in the nucleus of U251MG glioma cells. Increased proliferation and FGFR-1 nuclear accumulation was demonstrated upon transfection of a pcDNA3.1-FGFR-1 expression vector in glioma cells that did not express FGFR-1. It is now believed that apart from the classical theory of bFGF-induced autocrine or paracrine signal transduction of membrane-associated FGFR-1 ('membrane pathway'), that nuclear FGFR-1 enables bFGF to act as an intracrine nuclear signaling molecule ('nuclear pathway') [35]. Nuclear FGFR-1 stimulates a variety of transcription factors, among which is AP-1. We hypothesize in 1F6 cells that endogenous overexpression of the 18 kD bFGF may be responsible for activation of membrane-associated FGFR-1 resulting in transcriptional upregulation of VEGF expression, but that activation of nuclear FGFR-1 should also be taken into consideration. Our hypothesis is strengthened by the recent finding of Go et al. [11] in human bone marrow-derived mesenchymal stromal cells in which transfected 18 kD bFGF, but not exogenous bFGF, was responsible for consistent proliferation capability. This group detected the protein mostly in the nucleus, suggesting the possibility that the shortest form is also involved in intracrine signaling events.

Two possible explanations for increased VEGF protein expression by bFGF include promoter activation through the induction of transcription factors or increased mRNA stability. We found that VEGF mRNA half-lives were similar in 1F6-pcDNA3, 1F6-18kD and 1F6-ALL cells, excluding that increased VEGF expression in bFGF-overexpressing clones was the result of increased mRNA stability. Instead, VEGF promoter activity in 1F6-18kD and 1F6-ALL cells was considerably higher than that in 1F6-pcDNA3 cells. These data are in agreement with studies in rat osteoblastic cells and human glioma cells in which exogenous bFGF-induced VEGF expression was not the result of increased VEGF mRNA stability suggesting transcriptional upregulation of VEGF [3,32]. In eight human glioma cell lines, cellular mRNA levels of transcription factors AP-1 and SP-1 were closely correlated with those of VEGF [32]. Incubation of U251 human glioma cells with bFGF resulted in increased VEGF and SP-1 mRNA. Mithramycin, an inhibitor of SP-1, abolished bFGF-mediated VEGF upregulation. In addition, studies with promoter deletion constructs provided further evidence that VEGF induction by bFGF is mediated in part through the transcription factor SP-1 [32]. It should be investigated whether SP-1 and AP-1 are also involved in the bFGF-induced transcriptional upregulation of VEGF expression in our 1F6 melanoma cells.

We detected high basal levels of phosphorylated ERK1/2 in the 1F6 parental melanoma cell line. In a study by Satyamoorthy et al. [31], constitutively active ERK was detected in almost all melanoma cell lines and tumor tissues tested. In addition, Ras was constitutively activated in the melanoma cell lines, but underlying mutations could not be detected. Activation of Ras is therefore probably due to excessive stimulation by autocrine growth factors, such as bFGF. Activating mutations of BRAF, the kinase downstream of Ras, have also been identified in the majority of melanoma cell lines tested [34]. These activating mutations also result in increased activity of ERK1/2. These and other data clearly suggest that melanoma growth, invasion, and metastasis are associated with constitutively activated ERK, mediated by excessive growth factors through (autocrine) stimulation of Ras and by autonomic BRAF kinase activation. Further activation of the ERK1/2 pathway was, however, not involved in VEGF upregulation in our bFGF-transfected 1F6 clones. Previously, we have shown that addition of exogenous bFGF to 1F6 cells resulted in increased proliferation, but not in increased ERK1/2 activity [7]. These data indicate that in 1F6 melanoma cells, bFGF-stimulated growth is also independent of ERK1/2 activation.

bFGF-overexpressing 1F6 clones show accelerated growth ($p < 0.05$) when compared to the proliferation rate of 1F6 and 1F6-pcDNA3 cells [7]. In both clones we observed activation of p38 and PI-3K expressed by increase of phosphorylated levels of p38 and Akt. Although p38 is thought to be primarily activated in response to inflammatory cytokines and cellular stress, recent evidence suggests that p38 can also be activated by growth factors, such as bFGF [16, 21,37]. Akt kinase activity can be induced via PI-3K

activation by various growth factors, which includes bFGF [5,9,14,17]. Recent studies have indicated that Akt plays a pivotal role in melanocyte survival and that Akt is associated with melanoma tumor progression [6,17]. In melanoma samples of 292 patients, activated Akt expression increased dramatically with invasion and progression and was inversely correlated with patient survival [4]. Both signaling routes point towards the presence of an intact 'membrane' pathway in 1F6 cells. When p38 activity was blocked, we measured a 35–70% reduction of VEGF mRNA in bFGF-overexpressing clones and inhibition of VEGF secretion in 1F6-18kD cells. Furthermore, inhibition of PI-3K activity resulted in 55% reduction of VEGF mRNA and up to 60% inhibition of VEGF secretion in both 1F6-18kD and 1F6-ALL cells. Thus, it appears that the PI-3K and p38 routes are involved in the bFGF-mediated transcriptional upregulation of VEGF in 1F6 cells.

Since bFGF overexpression in 1F6 cells resulted in increased VEGF expression, we determined the expression of both growth factors in five unselected human melanoma cell lines. Of interest, melanoma cell lines BRO and BLM are aggressive cell lines on the basis of a high *in vitro* and *in vivo* growth rate and in both cell lines high bFGF and VEGF mRNA and VEGF protein expression could be measured. Furthermore, BLM and BRO cells grown as subcutaneous xenografts in nude mice have a very high rate and early incidence of spontaneous lung metastasis [20, 39]. In contrast, 1F6 cells grown as xenografts are non-metastatic, while M14 and Mel57 cells only sporadically form spontaneous metastases after subcutaneous inoculation [39]. The functional relevance of coexpression of bFGF and VEGF for the aggressiveness of melanoma in the clinic remains to be investigated.

Human melanomas often express bFGF and VEGF, although VEGF is considered to be a later event in melanoma progression than bFGF expression [1]. Redondo et al. [27] have studied bFGF and VEGF expression by immunohistochemistry in biopsy samples from 42 patients with different melanoma stages. In primary melanomas bFGF was positive in 85% and VEGF in 47.5%. bFGF and VEGF levels in stage III and IV melanoma were significantly higher than in lower stages. Melanoma cell lines with high coexpression of bFGF and VEGF will be of use to assess the effect of inhibition of bFGF signaling on VEGF protein expression. Since bFGF and VEGF are important growth factors in melanomas, both signaling routes should be further explored as possible targets to treat melanoma metastases and to improve survival of melanoma patients.

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