

The influence of neuropilin-1 silencing on semaphorin 3A and 3C activity in B16(F10) murine melanoma cells

A. M. MAZUREK*, M. OLBRYT

Center for Translational Research and Molecular Biology of Cancer, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Wybrzeże Armii Krajowej 15, 44-101 Gliwice, Poland

*Correspondence: amazurek@io.gliwice.pl

Received May 9, 2011 / Accepted August 1, 2011

Neuropilin-1 (Nrp1), originally characterized as an adhesion molecule in the nervous system, is a co-receptor for class-3 semaphorins. Neuropilins and semaphorins are highly expressed in a wide spectrum of tumors and have been shown to influence their growth and vascularization. Despite the growing body of data on neuropilin/semaphorin regulation of tumor growth, still the exact mechanism of their activity remains to be elucidated. Previously published data suggests that Nrp1 has both anti- and promigratory characteristics in different tumor types, although no data is available on its role in melanoma cells. In this paper, we studied the effect of Nrp1 downregulation on B16(F10) melanoma cells migration. Our results show that the silencing of Nrp1 significantly increases the overall mobility of B16(F10) cells and changes their morphology. Moreover, Nrp1-silenced B16(F10) cells show a decreased response to Sema3A. We also observed reduced binding of Sema3A to these cells. Contrarily, no changes were observed in the binding of Sema3C to Nrp1-silenced B16(F10) cells, nor in its chemorepellent activity. Our results suggest that modulation of B16(F10) cells migratory ability by semaphorin 3A can be preferentially mediated by Nrp1, while the contribution of semaphorin 3C in this process is less evident. In addition, silencing of Nrp1 did not change the migratory ability of B16(F10) cells towards VEGF.

Key words: neuropilin-1, semaphorin 3A, semaphorin 3C, melanoma, B16(F10), siRNA

Neuropilins (Nrp1 and Nrp2) were originally characterised as receptors for the axon guidance class-3 semaphorin family, the signal molecules which regulate cell migration, axon outgrowth and lead to the collapse of pseudopodia and growth cones [1, 2]. It was later discovered that neuropilins are also expressed by many invasive malignancies (e.g. astrocytoma, neuroblastoma, glioma, melanoma, osteosarcoma, ovarian cancer), influencing angiogenesis and tumor progression [reviewed in 3].

The overexpression of Nrp1 in prostate tumors correlated with the advanced stage, grade and metastatic potential of the tumor [4]. The expression of Nrp1 in stroma significantly increased with the malignant phenotype of the tumor cells [5]. In the same way, co-expression of Nrp1 and Nrp2 correlated with worse prognosis' for non-small cell lung carcinoma patients. Increased vessel numbers in the tumors expressing both neuropilins, as compared to those without co-expression, suggested that both Nrp1 and Nrp2 can be key molecules for tumor vascularization [6]. It was also found that a high

expression level of Nrp1 in tumor-associated endothelial cells correlated with an aggressive, angiogenic phenotype of melanoma [7]. So far, it has not been fully elucidated whether expression of Nrp1 in invasive carcinomas has a direct role in malignant transformation and/or metastatic potential of the tumor.

Previous results showed that Nrp1 has both pro- and anti-migratory activities in tumor cells. Overexpression of Nrp1 increased migration of prostate cancer cells AT2.1 [8] and colon cancer cells KM12SM/LM2 [9]. However, the opposite effect was observed in breast cancer MDA-435, MFCF7, MDA-231 cells [10] and pancreatic cancer Panc-1 cells [11], suggesting that Nrp1 may act in a cell-type dependent manner. Nrp1 was also reported to enhance the survival of breast cancer MDA-MB-231 [12] and colon cancer WiDR cells [13].

The function of Nrp1 in tumor cells is mainly regulated by its interaction with semaphorins and VEGF (vascular endothelial growth factor), including Sema3A and VEGF₁₆₅

(isoform of vascular endothelial growth factor A). Previous results showed that semaphorin 3A (Sema3A) and VEGF₁₆₅ compete for the Nrp1 binding site [14, 15]. It seems that the Nrp1 effect on cancer cells' migration highly depends on its ligands accessibility. For example, cells with a high Sema3A/VEGF protein ratio exhibited a low rate of chemotaxis, whereas cells with low ratio of these proteins were more chemotactic. [10].

The role of Sema3A in Nrp1-mediated migration of tumor cells is suggested also by the observation that its expression in ovarian cancer decreased as the disease progressed and inversely correlated with the microvessel density of the tumor [16]. Antimigratory effect of Sema3A was also observed in MDA-435 and MDA-231 breast cancer cells [10] and PC3 prostate cancer cells [17]. The role of class 3 semaphorins on tumor growth seems to vary between the various members of this protein family. While it is generally accepted that the expression of Sema3A slows down the tumor growth, another member of the semaphorin family, namely semaphorin 3C (Sema3C), appears to exert the opposing effect [18]. Increased expression of Sema3C characterizes cancers with higher invasive and metastatic potential [19, 20].

The differential activity of semaphorins is regulated by their binding to specific neuropilin/plexin complexes, which restrict the signal semaphorin specificity and allows the receptor complex to discriminate between different semaphorins. It was shown that Sema3A binds with high affinity to Nrp1 but not to Nrp2 [21, 22]. Additionally, signal transduction by Sema3A involves a functional complex of Nrp1 and various types of Plexins: A1 [23], A2 [24], A3 [25], A4 [26] or D1 [27]. In contrast, Sema3C shows a similar affinity to both Nrp1 and Nrp2 [28]. The molecular mechanism of neuropilin-mediated semaphorins activity seems to be regulated by various complexes of neuropilins, plexins and other proteins (e.g. L1CAM: [29, 30]), presumably acting in a context-dependent manner.

In this paper, we studied the influence of Nrp1 silencing on B16(F10) melanoma cells migration and its effect on activity of Sema3A and Sema3C. We observed that the depletion of Nrp1 markedly enhanced the chemotactic ability of these cells and reduced binding ability and chemorepellent activity of Sema3A, whereas it had no influence on the activity of Sema3C in B16(F10) cells deprived of Nrp1.

Materials and methods

Cells lines. B16(F10) murine melanoma cells purchased from American Type Culture Collection (ATCC) were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in 95% air and 5% CO₂.

RNAi Transient Transfection. The target sequence for silencing of Nrp1 (5'-GTGGATGATATCAGTATTA-3') was chosen by using a free website to design siRNA (Dharmacon, Inc). Selected siRNA, which we called siRNA#460 matched the

nucleotides of 15th exon of *Nrp1* gene (GeneBank Acc. No. NM_008737). Transfection of B16(F10) cells was performed at about 50% cell culture confluence with the above mentioned siRNA duplex (siRNA#460), using DharmaFECTTM1 at 100 nM concentration. The transfection was repeated two times at an interval of 12 hours. The transfection with control siRNA (GFP DuplexIII; Dharmacon, Inc.) was performed under the same conditions. RNA analysis was performed after 36 h of cell growth. Protein detection and migration assay were carried out after 63 h of cell growth.

Reverse-transcription polymerase chain reaction. Total RNA was isolated from cells after 36 h of exposure to siRNA using the RNeasy Mini Kit (Qiagen), followed by DNaseI digestion and repurification. The following specific primers were used: Nrp1: 5'-GTGAAATCGGAAAAGGAAACCTTG-3', 5'-ATATCCTGGAGTGCTCCCTGTTTC-3'; Nrp2: 5'-AAGCAAACCTCTTTGGGTC-3', 5'-CAAAAGCTGATATGGGTTCCA-3'. Ribosomal RNA (18S) fragment was amplified as a loading control using the following primers: 5'-GTTGGTGGAGCGATTTGTCTGG-3', 5'-GCAGCCCCG-GACATCTAAGG-3'. RT-PCR was performed using the following conditions: 50°C for 10 min; 94°C for 3 min; 2 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 30 s; 2 cycles at 94°C for 30 s, 60°C for 30 s; and 72°C for 30 s; 31 cycles at 94°C for 30 s, 58°C for 30 s and 72°C for 30 s; 1 cycle at 72°C for 7 min. PCR products were separated by electrophoresis in 2% agarose gel.

Protein detection. Cell lysates were obtained 67 hours after transfection. Cells were solubilized in lysis buffer (50mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1% NP-40, 1mM DTT, 1mM PMSF) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Diagnostic). After centrifugation at 15000 g for 10 minutes at 4°C, supernatants were frozen and total protein content was determined using Protein Assay Kit (BioRad); 30 µg of protein extracts were separated by SDS-PAGE in 8% polyacrylamide gel and immobilized on a nitrocellulose membrane (Whatman). Blots were blocked in 5% skimmed milk in TTBS (0.25 M Tris-HCl (pH 7.5), 0.1% Tween-20, 0.15 M NaCl) for 1 hour and incubated overnight at 4°C with polyclonal anti-Nrp1 antibody (1:2000; Santa Cruz Biotechnology), followed by incubation with secondary anti-rabbit-HRP antibody (1:5000; Pierce). Labelled protein were visualized using Chemiluminescence Reagent Detection System (Pierce).

Conditioned medium (CM). Cells (3×10⁵) were seeded using 20 cm² plates. After 24 hours the cells were transfected using LipofectamineTM2000 (Invitrogen) with the following plasmids: pAG-3/AP, pAG-3/AP/Sema3A [31], pSec/VEGF₁₆₅ [32] and pAG-3/AP/Sema3C (a gift from dr J. Solowska-Baird). The control plasmid pAG-3/AP contains the signal sequence and the coding sequence of human alkaline phosphatase (AP). pAG-3/AP/Sema3A or pAG-3/AP/Sema3C plasmids contain the signal sequence and the coding sequence of alkaline phosphatase fused in frame with mouse semaphorin 3A or 3C, respectively. pSec/VEGF₁₆₅ contains the signal sequence and the coding sequence of human VEGF₁₆₅. After transfection

(24 h) the medium containing semaphorins-AP or VEGF₁₆₅ and alkaline phosphatase (control conditioned medium) was collected, spun down, frozen and used for migration and binding assays. Plasmids pAG-3/AP, pAG-3/AP/Sema3A and pAG-3/AP/Sema3C all had alkaline phosphatase (AP) as a reporter protein and plasmid pSec/VEGF₁₆₅ did not have a reporter protein. Alkaline phosphatase was measured in the following way: after heat inactivation of endogenous alkaline phosphatase, 5 µl of CM was incubated for 1 h with 200 µl of pNPP (p-nitrophenyl phosphate, Sigma) and assessed by measuring absorbance changes at 405 nm using a 96-microplate reader EL_x800 (Bio Tek Instruments, Inc); 1 pM AP fusion protein corresponds to approximately 30 OD₄₀₅ units per hour [33]. The presence of VEGF₁₆₅ protein in CM was confirmed using Western Blot analysis and antibody against VEGF (RD Systems) (data not shown).

Semaphorin binding assay. B16(F10) cells were cultured in 8-well chamber slides. Cells were incubated with conditioned medium containing AP/Sema3A or AP/Sema3C or control CM for 90 minutes at 37°C in 95% air and 5% CO₂. Cells were then washed with PBS, fixed with 4% PFA and heated for 15 minutes at 65°C to inactivate endogenous alkaline phosphatase. Bound heat-stable alkaline phosphatase was detected by incubation with substrates: BCIP (5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt) and NBT (nitro-blue tetrazolium chloride) (Zymed) for 14 h at room temperature (RT). To exclude non-specific binding of alkaline phosphatase to cells, several testing experiments were performed using control conditioned medium (control CM). Control CM bound neither to wild type B16(F10), control siRNA transfected cells, nor to Nrp1-specific siRNA#460 transfected cells (data not shown).

Cell migration assay. Chemotactic activity of semaphorins was measured by a migration assay performed on a Boyden chamber system with 8 µm membranes pores (BD Biosciences transwell system). Prior to performing the migration assay, inserts were coated by immersing in a fibronectin solution (4 µg/ml of fibronectin, 138 mM NaCl, 2.7 mM KCl, 11 mM glucose, 3.6 mM NaH₂PO₄, 2 mM KH₂PO₄, 0.47 mM NaHCO₃, 0.00025% Phenol Red, pH 7.2) for 1 h. Control siRNA- or Nrp1 specific siRNA#460-transfected B16(F10) cells (5×10⁴) were suspended in 0.3 ml RPMI/0.1 % BSA and placed in the top chamber; conditioned medium (0.8 ml of control CM, Sema3A-AP or Sema3C-AP conditioned medium) was added to the bottom chamber. After 4 hours, non-migrating cells were scraped from the top compartment; cells that had migrated to the bottom compartment were fixed (10 minutes) with methanol, stained with 5% Giemsa and counted in four observation fields (20x magnification, Nikon microscope). The experiment was repeated four times. Statistical analysis was performed using the unpaired t-test (Statistica Software).

Results

Nrp1 silencing increases migration and alters morphology of B16(F10) cells. To determine Nrp1 influence

on migration of B16(F10) cells we downregulated Nrp1 expression in these cells using Nrp1 specific siRNA oligonucleotide called by us siRNA#460. Although both neuropilins (Nrp1 and Nrp2) were expressed in B16(F10) cells (Fig. 1A), transfection of the cells with siRNA#460 resulted in specific silencing of Nrp1 (Fig. 1A). The effect of Nrp1 downregulation in B16(F10) on the migration ability of B16(F10) cells was assessed after silencing procedure using the Boyden chamber system. The migration ability of cells transfected with siRNA#460 (155.7 cells/field) significantly increased ($p < 0.001$), as compared to the mobility of the cells transfected with control siRNA (43.2 cells/field) (Fig. 1B). We also observed that silencing of the *Nrp1* gene expression induced significant changes in cell morphology. As shown in Fig. 1C, control B16(F10) cells contain long microvilli and tend to pile up on each other, whereas B16(F10) cells transfected with siRNA#460 were deprived of microvilli and show a tendency to attached to each other.

Semaphorin 3C inhibits migration rate of Nrp1-silenced B16(F10) cells. In order to find out whether Sema3A and Sema3C would affect the Nrp1-dependent migrating ability of B16(F10) melanoma cells, we performed migration assays using the Boyden chamber system. We analyzed the migration activity of the cells towards the bottom compartment filled with conditioned medium containing Sema3A-AP or Sema3C-AP. The presence of Sema3A-AP and Sema3C-AP in the conditioned medium was expressed by molar concentration of alkaline phosphatase and amounted to 2 nM and 2.3 nM, respectively. Chemorepellent activity of semaphorins was referred to as the number of cells that had migrated across the membrane to the bottom compartment. As shown in Fig. 2A, Sema3A-AP inhibited migration of the cells by 43%, and Sema3C-AP by 47%, compared to the control conditioned medium, confirming chemorepellent activity of these semaphorins. To investigate the influence of Nrp1 silencing on semaphorin activity, we performed a migration assay for cells transfected with siRNA#460. It can be seen from the Fig. 2B that although both semaphorins exhibited an inhibitory effect on Nrp1-downregulated B16(F10) cells, Sema3A-AP reduced the number of migrating cells only by 27%, and Sema3C-AP by 60%, compared to the control conditioned medium. To investigate whether the observed differences may result from the changes in Nrp1/semaphorins interaction, we evaluated the binding ability of Sema3A-AP and Sema3C-AP to B16(F10) cells. As shown in Fig. 3A, both semaphorins bind to B16(F10) control cells, whereas downregulation of Nrp1 resulted in a reduction of Sema3A-AP binding without influencing the binding ability of Sema3C-AP (Fig. 3B).

VEGF₁₆₅ has no effect on B16(F10) migration activity. It has been shown that VEGF₁₆₅ promoted membrane ruffling activity and migration of breast cancer cells [34 – 36]. To find out whether VEGF₁₆₅ could influence the migratory activity of B16(F10) melanoma cells we performed migration assays. The conditioned medium containing VEGF₁₆₅ (as confirmed by Western blot; data not shown), or control medium was placed

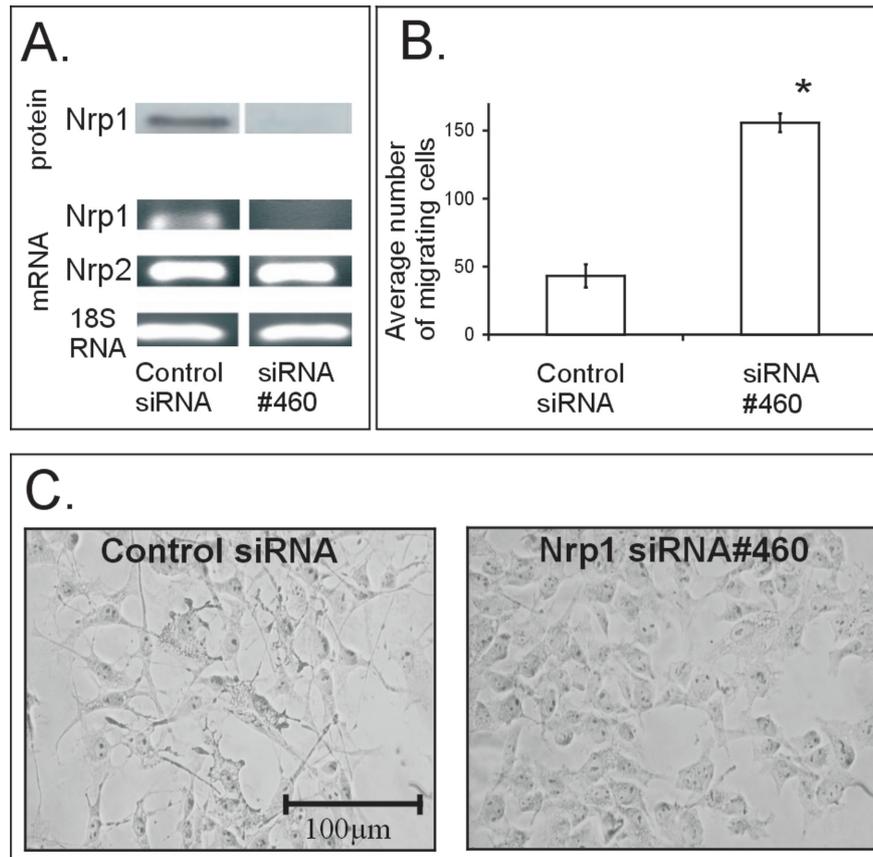


Fig. 1. Influence of Nrp1 downregulation on migration and morphology of melanoma B16(F10) cells. B16(F10) melanoma cells were transfected with either control siRNA or Nrp1-specific siRNA#460. (A) Downregulation of Nrp1 in B16(F10) cells transfected with Nrp1-specific siRNA#460 was proved by RT-PCR and Western blotting. The protein level of Nrp1 was determined at the same time when migration assay was performed. (B) The migration assay of Nrp1-downregulated B16(F10) cells toward the control conditioned medium. Migration assay was performed for 4 hours using the Boyden chamber system, and then the mean number of migrated cells from 4 non-overlapping microscope fields was calculated. Data represents the mean values of 4 separate experiments \pm SE. Statistical significance was evaluated using an unpaired t-test (* - $p = 0.001$ vs. control siRNA). Error bars represent the standard error of the mean. Statistical significance is presented when $p < 0.05$. (C) Morphology of control and Nrp1-lacking cells. Microvilli were present in control cells (treated with control siRNA) and were absent in siRNA#460 treated ones. The images were taken using a Nikon microscope; bar = 100 μ m.

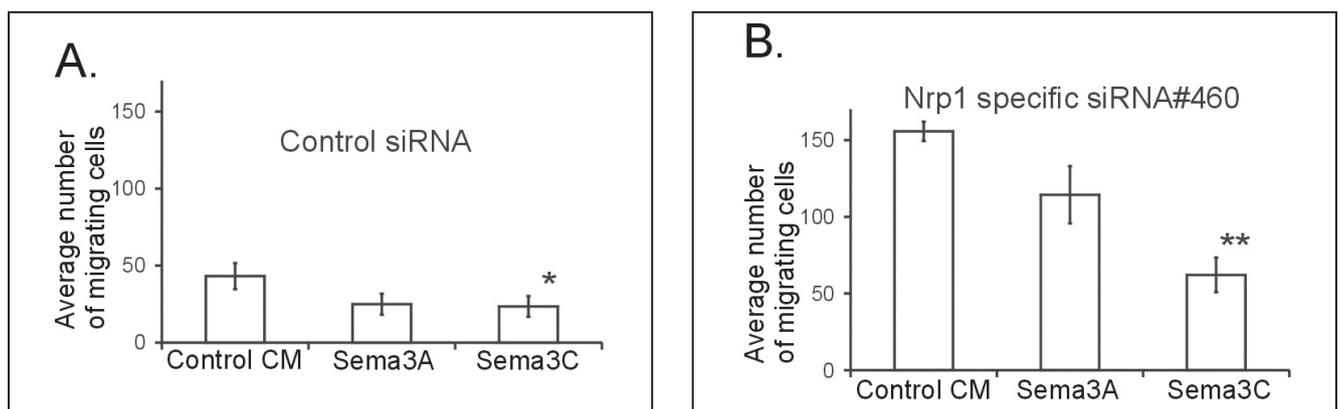


Fig. 2. Influence of Nrp1 downregulation in melanoma B16(F10) cells on chemotactic activity of semaphorin 3A and semaphorin 3C. The migration ability of the cells towards Sema3A-AP or Sema3C-AP conditioned medium was evaluated using the Boyden chamber system. The bottom chamber contained either control CM or medium with Sema3A-AP or Sema3C-AP. The migration assay was performed for 4 hours, using cells transfected with either control siRNA (Figure A) or Nrp1-specific siRNA#460 (Figure B). The data represents the mean values of 4 separate experiments \pm SE. Error bars represent the standard error of the mean. Statistical significance was evaluated using an unpaired t-test, * - $p = 0.02$ vs. control CM; ** - $p = 0.009$ vs. control CM; and $p = 0.04$ vs. Sema3A.

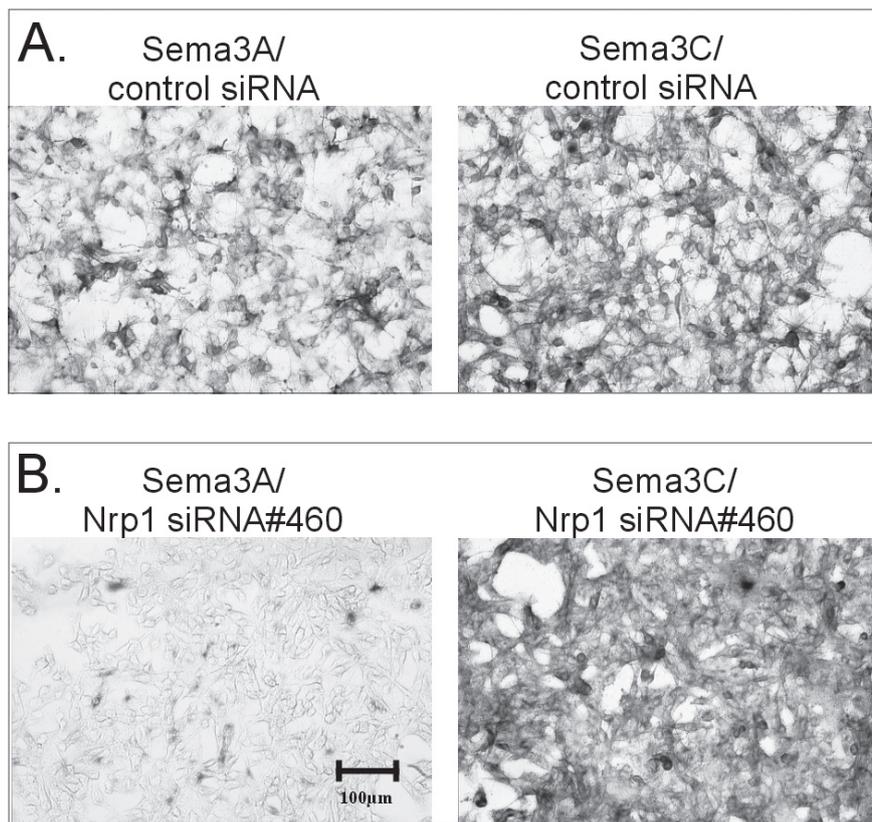


Fig. 3. Binding of Sema3A-AP and Sema3C-AP to B16(F10) melanoma cells. The binding assays of Sema3A-AP or Sema3C-AP to cells transfected with control siRNA (panel A) or to cells transfected with Nrp1-specific siRNA#460 (panel B). Binding assay was performed for 90 minutes. Binding of Sema3A-AP or Sema3C-AP to cell membrane was detected using alkaline phosphatase substrates. The images were taken using a Nikon microscope; bar = 100 µm.

in the bottom chamber. As shown in Fig. 4, B16(F10) cells did not change their migratory ability toward the VEGF₁₆₅-containing medium. After downregulation of Nrp1, it was difficult to assess the influence of VEGF₁₆₅ on B16(F10) cells due to inconsistency between the results obtained in different experiments.

Discussion

Cell movement from one area to another in response to chemical signals is fundamental to embryonic development and wound repair, as well as tumor metastasis and progression. Neuropilins (Nrp1, Nrp2) are proteins that regulate these processes and are generally regarded as tumor promoting factors [37]. Neuropilins may influence cancer growth by affecting not only tumor angiogenesis and migration of cancer cells, but also by regulating apoptosis and immune processes. The molecular mechanisms by which neuropilins modulate cancer progression are rather poorly understood. The complexity of the signalling pathways and large number of neuropilins ligands, as well as dependence of neuropilin-mediated effects on cancer type and cellular context, may be a source of the

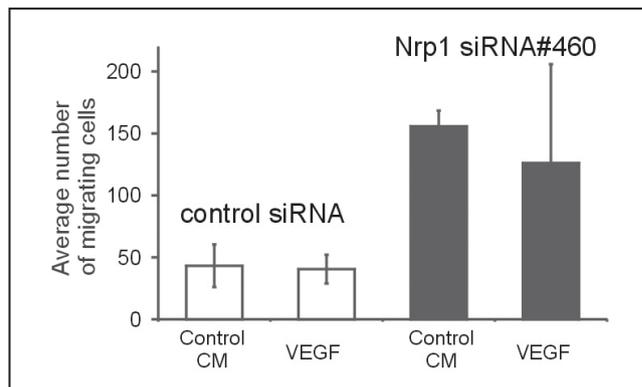


Fig. 4. Influence of Nrp1 downregulation in B16(F10) cells on chemotactic activity of VEGF₁₆₅. The migration ability of cells towards VEGF₁₆₅-conditioned medium was evaluated using the Boyden chamber system. Control CM or medium with VEGF₁₆₅ were placed in the bottom chamber of the Boyden chamber system. The migration assay was performed for 4 hours, using cells transfected with either control siRNA (white columns) or Nrp1-specific siRNA#460 (black columns) after silencing procedure. The mean number of migrated cells from 4 non-overlapping microscope fields was calculated. Results obtained in 4 separate experiments (mean ± SE) were not statistically significant. Error bars represent the standard error of the mean. Statistical significance is presented when p<0.05.

divergent results of studies concerning the role of neuropilins in tumorigenesis [18, 40]. It has been reported that Nrp1 can mediate both promigratory and antimigratory activities of tumor cells [8-13] while Nrp2 has been shown to stimulate angiogenesis in multiple tumors [38]. However it was also reported that a decreasing of its expression in melanoma cells can activate endothelial cells [39].

In the case of melanoma it was shown that Nrp2 could be a potential mediator of melanoma-endothelial interactions [41] and a therapeutic target [42]. On the other hand, Nrp2 was found to be functionally involved in chemorepulsive [43] and antimigratory properties of semaphorin 3F in melanoma [39]. Whether and how the Nrp1 can influence melanoma cell migration is not known. Here, using siRNA technology we investigated the role of Nrp1 in the migratory activity of metastatic mouse melanoma cancer cell line B16(F10), widely used as a model system for studying various aspects of melanoma biology.

We have shown that a reduction of Nrp1 level resulted in a significant enhancement of migration rate of these cells. A similar effect of Nrp1 depletion had already been observed in the case of pancreatic cancer cells (Panc-1) [11] and breast cancer cells (MDA-435, MCF7, MDA-231) [10]. The authors of the latter report concluded that the effect was caused by the reduction of the chemorepellent activity of Sema3A, for which Nrp1 is the main functional receptor. This explanation could also be true for B16(F10) cells since we have observed decreased Sema3A binding to melanoma cells deprived of Nrp1 protein, while no similar effect was observed for Sema3C.

The role of secreted class-3 semaphorins in tumor progression has emerged during the last decade. It has been documented that semaphorins modulate the adhesive and migratory properties of responsive malignant cells influencing tumor development. Some of them have protumorigenic properties (Sema3C, Sema3E) while other (Sema3A, Sema3F, Sema3B) exert a rather opposite effect [18]. So far knowledge about the role of semaphorins in melanoma biology is limited to mainly semaphorin 3F, an overexpression of which was found to inhibit lymph node and lung metastasis in human melanoma tumor cells (A375SM) [43].

Our data presented here suggests for the first time that two other semaphorins, namely Sema3A and Sema3C may also be chemorepulsive for melanoma cells. Both semaphorins reduced chemotaxis of B16(F10) cells at a similar rate, although the results obtained for the Sema3A were not statistically significant. Chemorepellent activity of semaphorin 3A was earlier shown to be responsible for Sema3A inhibitory effect on migration of prostate PC3 [17] and breast cancer cells (MDA-435, MCF7, MDA-231) [10]. In the latter case the effect was mediated by a Nrp1 receptor. In our study, even if the depletion of Nrp1 almost completely reduced the binding capability of Sema3A, its antimigratory effect on B16(F10) cells was nevertheless observable. These results suggest that Nrp1 could be the main receptor for Sema3A in B16(F10) cells, but presumably not the only one. Serini et al. [44] has shown that

Sema3A inhibited the adhesion of endothelial cells via integrin $\alpha_v\beta_3$, independently of Nrp1. Since this integrin is highly expressed in B16(F10) melanoma cells [45], one may speculate that it could also be an alternative receptor for Sema3A.

In contrast to Sema3A, Sema3C expression seems to be correlated with the chemoresistance and metastatic potential of tumor cells. The expression of Sema3C was detected in numerous glioma cell lines (U138MG, U87MG, LN-428, D247MG, T98G, LN-319, LN-229, A172, U251MG, U373MG, LN-308) [46]. A significantly higher expression level of Sema3C was found in the CDDP-resistant ovarian cell lines (TYKnuR) and lung cancer cell lines (Lu65/CDDP, MS-1/CDDP) as compared to their CDDP-sensitive parental cells. [47]. A much higher level of Sema3C was also reported in metastatic human lung adenocarcinoma cells (HAL-8Luc) than in a non-metastatic cell line (HAL-24Luc) [19]. Sema3C acts as a chemoattractant, *via* Nrp1 or Nrp2, upon EC cells (MGEC cell line) [48], as well as upon prostate cancer PC3 cells [17].

In contrast to the aforementioned results we observed the chemorepellent activity of Sema3C on B16(F10) cells. Silencing of Nrp1 in these cells did not influence Sema3C chemorepellent activity and, contrarily to Sema3A, did not change its binding to B16(F10) cells. This observation may suggest that Nrp1 is not a receptor for Sema3C in B16(F10) cells, or at least not the main one. The candidate for functional receptor mediating Sema3C antimigratory activity in melanoma cells B16(F10) could be Nrp2, which was shown to transfer chemorepulsive signal of previously mentioned semaphorin Sema3F in breast cancer cell line C100 [49] as well as melanoma A375SM cell line [43].

On the basis of the previously performed microarray-based experiments (50) (see also Table 1) we suggest that potential candidate Nrp1 co-receptors expressed highly in B16(F10) cells could be plexin A3 and plexin D1. It has been reported previously that only Nrp1/PlexinD1 or Nrp2/PlexinD1 complexes are responsible for mediating semaphorin signalling in endothelial cells [27]. The strong expression of plexin D1 in B16(F10) cells suggests that it could be a candidate for functional co-receptor required for Sema3C activity also in melanoma cells. On the other hand, insignificant expression of Nrp1 co-receptors such as Kdr, Flt1 and Flt4, may explain our observation that VEGF does not affect migration of B16(F10) melanoma cells.

In summary, our results indicate that Sema3A and Sema3C exhibit chemorepellent activity in B16(F10) melanoma cells and presumably act through different or overlapping receptors. While Sema3A seems to exert its antimigratory effect mainly through Nrp1, Sema3C most likely acts through other signal transducers, possibly Plexin D1 and Nrp2. However further studies are needed to verify this supposition. The molecular mechanisms by which neuropilins modulate cancer progression are very poorly understood, mostly due to the large number of their ligands and co-receptors. The great complexity of these molecular processes results in a high diversity of observed effects between cell lines. It would be of great interest

Table 1. Neuropilin-1 co-receptors gene expression profile in B16(F10) murine melanoma cell line. Data presented in the table was retrieved from our earlier microarray study of gene expression in B16(F10) [50]. The pre-processing of data was carried out by Robust MultiArray Analysis (RMA) using the Bioconductor Affy package. The microarray data was analyzed using Statistical Algorithms implemented in Affymetrix Microarray Suite Version 5.0. The real signal for a single probe pair is estimated by taking the log of the perfect match intensity after subtracting the stray signal estimate. The Signal value is the mean of the weighted intensity values for a probe set corrected back to a linear scale. The Signal value represents a quantitative measure of the relative abundance of a transcript in 3 independent experiments and was calculated using the One-Step Tuckey's Biweight Estimate. Expression levels were assessed as: P – presence ($p < 0.05$); A – absence ($p > 0.05$).

Probe Set ID	Gene Symbol	Gene Name	Signal \pm SD Expression
95016_at	Nrp1	neuropilin 1	73,65 \pm 18,36 (P)
102933_at	Plxna3	plexin A3	71,20 \pm 16,33 (P)
97967_at	Plxnd1	plexin D1	410,20 \pm 86,78 (P)
97699_at	Plxna1	plexin A1	41,72 \pm 7,92 (A)
95368_at	Plxna2	plexin A2	3,37 \pm 2,84 (A)
104265_at	Kdr	kinase insert domain protein receptor (counterpart of human Vascular endothelial growth factor receptor 2; VEGFR2)	7,85 \pm 3,09 (A)
98452_at	Flt1	FMS-like tyrosine kinase 1 (counterpart of human Vascular endothelial growth factor receptor 1; VEGFR1)	8,90 \pm 4,98 (A)
104416_at	Flt4	FMS-like tyrosine kinase 4 (counterpart of human Vascular endothelial growth factor receptor 3; VEGFR3)	9,08 \pm 7,5 (A)
99800_at	L1cam	L1 cell adhesion molecule	6,12 \pm 0,51 (A)

and importance to validate the obtained results in a broad panel of cell lines derived from human melanoma, a tumor heterogeneity of which is well established. Elucidating the role of neuropilins in melanoma biology is especially warranted, since metastatic melanoma is still an incurable disease. It is worth noting that neuropilins have emerged recently as a highly attractive target for cancer therapy (51), due to their participation in binding of tumor penetrating peptide iRGD which has a potential to selectively deliver various therapeutics to tumor tissue (reviewed in: 52).

Acknowledgements: We wish to express our gratitude to Joanna Solowska-Baird for providing pAG-3/AP, pAG-3/AP/Sema3A pAG-3/AP/Sema3C plasmid vectors. We would also like to thank Maciej Malecki for providing the pSec/VEGF₁₆₅ plasmid vector. This study was supported by a grant KBN 2PO5A02227 (to ZK) from the State Committee for Scientific Research in Poland.

References

- [1] KOLODKIN AL, MATTHES DJ, GOODMAN CS The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell*;1993 75: 1389-1399. [http://dx.doi.org/10.1016/0092-8674\(93\)90625-Z](http://dx.doi.org/10.1016/0092-8674(93)90625-Z)
- [2] LUO Y, RAIBLE D, RAPER JA Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* 1993; 75: 217-227. [http://dx.doi.org/10.1016/0092-8674\(93\)80064-L](http://dx.doi.org/10.1016/0092-8674(93)80064-L)
- [3] BAGRI A, TESSIER-LAVIGNE M, WATTS RJ Neuropilins in tumor biology. *Clin Cancer Res*. 2009; 15:1860-4. <http://dx.doi.org/10.1158/1078-0432.CCR-08-0563>
- [4] LATIL A, BIECHE I, PESCHE S, VALERI A, FOURNIER G et al. VEGF overexpression in clinically localized prostate tumours and neuropilin-1 overexpression in metastatic forms. *Int J Cancer* 2000; 89: 167-171. [http://dx.doi.org/10.1002/\(SICI\)1097-0215\(20000320\)89:2<167::AID-IJC11>3.0.CO;2-9](http://dx.doi.org/10.1002/(SICI)1097-0215(20000320)89:2<167::AID-IJC11>3.0.CO;2-9)
- [5] VANVELDHUIZEN PJ, ZULFIQAR M, BANERJEE S, CHERIAN R, SAXENA NK et al. Differential expression of neuropilin-1 in malignant and benign prostatic stromal tissue. *Oncol Rep* 2003; 10: 1067-1071.
- [6] KAWAKAMI T, TOKUNAGA T, HATANAKA H, KIJIMA H, YAMAZAKI H et al. Neuropilin 1 and neuropilin 2 co-expression is significantly correlated with increased vascularity and poor prognosis in nonsmall cell lung carcinoma. *Cancer* 2002; 95: 2196-2201. <http://dx.doi.org/10.1002/cncr.10936>
- [7] STRAUME O, AKSLEN LA Increased expression of VEGF-receptors (FLT-1, KDR, NRP-1) and thrombospondin-1 is associated with glomeruloid microvascular proliferation, an aggressive angiogenic phenotype, in malignant melanoma. *Angiogenesis* 2003; 6: 295-301. <http://dx.doi.org/10.1023/B:AGEN.0000029408.08638.aa>
- [8] MIAO HQ, LEE P, LIN H, SOKER S, KLAGSBRUN M Neuropilin-1 expression by tumour cells promotes tumour angiogenesis and progression. *FASEB J* 2000; 14: 2532-2539. <http://dx.doi.org/10.1096/fj.00-0250com>
- [9] PARIKH AA, FAN F, LIU WB, AHMAD SA, STOELTZING O et al. Neuropilin-1 in human colon cancer: expression, regulation, and role in induction of angiogenesis. *Am J Pathol* 2004; 164: 2139-2151. [http://dx.doi.org/10.1016/S0002-9440\(10\)63772-8](http://dx.doi.org/10.1016/S0002-9440(10)63772-8)
- [10] BACHELDER RE, LIPSCOMB EA, LIN X, WENDT MA, CHADBORN NH et al. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. *Cancer Res* 2003; 63: 5230-5233.
- [11] GRAY MJ, WEY JS, BELCHEVA A, MCCARTY MF, TREVINO JG et al. Neuropilin-1 suppresses tumourigenic properties in a human pancreatic adenocarcinoma cell line

- lacking neuropilin-1 coreceptors. *Cancer Res* 2005; 65: 3664-3670. <http://dx.doi.org/10.1158/0008-5472.CAN-04-2229>
- [12] BACHELDER RE, CRAGO A, CHUNG J, WENDT MA, SHAW LM et al. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* 2001; 61: 5736-5740.
- [13] OCHIUMI T, KITADAI Y, TANAKA S, AKAGI M, YOSHIIHARA M et al. Neuropilin-1 is involved in regulation of apoptosis and migration of human colon cancer. *Int J Oncol* 2006; 29:105-116.
- [14] MIAO HQ, SOKER S, FEINER L, ALONSO JL, RAPER JA et al. Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. *J Cell Biol* 1999; 146: 233-242.
- [15] BAGNARD D, VAILLANT C, KHUTH ST, DUFAY N, LOHRUM M et al. Semaphorin 3A-vascular endothelial growth factor-165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. *J Neurosci* 2001; 21: 3332-3341.
- [16] OSADA R, HORIUCHI A, KIKUCHI N, OHIRA S, OTA M et al. Expression of semaphorins, vascular endothelial growth factor, and their common receptor neuropilins and allelic loss of semaphorin locus in epithelial ovarian neoplasms: increased ratio of vascular endothelial growth factor to semaphorin is a poor prognostic factor in ovarian carcinomas. *Hum Pathol* 2006; 37: 1414-1425. <http://dx.doi.org/10.1016/j.humpath.2006.04.031>
- [17] HERMAN JG, MEADOWS GG Increased class 3 semaphorin expression modulates the invasive and adhesive properties of prostate cancer cells. *Int J Oncol* 2007; 30: 1231-8.
- [18] GAUR P, BIELENBERG DR, SAMUELS, BOSE D, ZHOU Y et al. Role of class 3 semaphorins and their receptors in tumor growth and angiogenesis. *Clin Cancer Res.* 2009; 15: 6763-70. <http://dx.doi.org/10.1158/1078-0432.CCR-09-1810>
- [19] MARTIN-SATUE M, BLANCO J Identification of semaphorin E gene expression in metastatic human lung adenocarcinoma cells by mRNA differential display. *J Surg Oncol* 1999; 72: 18-23. [http://dx.doi.org/10.1002/\(SICI\)1096-9098\(199909\)72:1<18::AID-JSO5>3.0.CO;2-P](http://dx.doi.org/10.1002/(SICI)1096-9098(199909)72:1<18::AID-JSO5>3.0.CO;2-P)
- [20] GALANI E, SGOUROS J, PETROPOULOU C, JANINIS J, ARAVANTINOS G et al. Correlation of MDR-1, nm23-H1 and H Sema E gene expression with histopathological findings and clinical outcome in ovarian and breast cancer patients. *Anticancer Res* 2002; 22: 2275-80
- [21] KOLODKIN AL, LEVENGOOD DV, ROWE EG, TAI YT, GIGER RJ et al. Neuropilin is a semaphorin III receptor. *Cell* 1997; 90: 753-762. [http://dx.doi.org/10.1016/S0092-8674\(00\)80535-8](http://dx.doi.org/10.1016/S0092-8674(00)80535-8)
- [22] HE Z, TESSIER-LAVIGNE M Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. *Cell* 1997; 90: 739-751. [http://dx.doi.org/10.1016/S0092-8674\(00\)80534-6](http://dx.doi.org/10.1016/S0092-8674(00)80534-6)
- [23] TAKAHASHI T, FOURNIER A, NAKAMURA F, WANG LH, MURAKAMI Y et al. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* 1999; 99: 59-69. [http://dx.doi.org/10.1016/S0092-8674\(00\)80062-8](http://dx.doi.org/10.1016/S0092-8674(00)80062-8)
- [24] ROHM B, OTTEMEYER A, LOHRUM M, PUSCHEL AW Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. *Mech Dev* 2000; 93: 95-104. [http://dx.doi.org/10.1016/S0925-4773\(00\)00269-0](http://dx.doi.org/10.1016/S0925-4773(00)00269-0)
- [25] CHENG HJ, BAGRI A, YARON A, STEIN E, PLEASURE SJ et al. Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. *Neuron* 2001; 32: 249-63. [http://dx.doi.org/10.1016/S0896-6273\(01\)00478-0](http://dx.doi.org/10.1016/S0896-6273(01)00478-0)
- [26] SUTO F, ITO K, UEMURA M, SHIMIZU M, SHINKAWA Y et al. Plexin-a4 mediates axon-repulsive activities of both secreted and transmembrane semaphorins and plays roles in nerve fiber guidance. *J Neurosci* 2005; 25: 3628-3637. <http://dx.doi.org/10.1523/JNEUROSCI.4480-04.2005>
- [27] GITLER AD, LU MM, EPSTEIN JA PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. *Dev Cell* 2004; 7: 107-116. <http://dx.doi.org/10.1016/j.devcel.2004.06.002>
- [28] CHEN H, CHEDOTAL A, HE Z, GOODMAN CS, TESSIER-LAVIGNE M Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* 1997; 19: 547-559. [http://dx.doi.org/10.1016/S0896-6273\(00\)80371-2](http://dx.doi.org/10.1016/S0896-6273(00)80371-2)
- [29] CASTELLANI V, CHEDOTAL A, SCHACHNER M, FAIVRE-SARRAILH C, ROUGON G Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* 2000; 27: 237-249. [http://dx.doi.org/10.1016/S0896-6273\(00\)00033-7](http://dx.doi.org/10.1016/S0896-6273(00)00033-7)
- [30] CASTELLANI V, FALK J, ROUGON G Semaphorin3A-induced receptor endocytosis during axon guidance responses is mediated by L1 CAM. *Mol Cell Neurosci* 2004; 26: 89-100. <http://dx.doi.org/10.1016/j.mcn.2004.01.010>
- [31] SOLOWSKA JM, MAZUREK A, WEINBERGER L, BAIRD DH Pontocerebellar axon guidance: neuropilin-1- and semaphorin 3A-sensitivity gradients across basilar pontine nuclei and semaphorin 3A variation across cerebellum. *Mol Cell Neurosci* 2002; 21: 266-284. <http://dx.doi.org/10.1006/mcne.2002.1187>
- [32] MALECKI M, PRZYBYSZEWSKA M, JANIK P In vitro and in vivo study of the expression vector encoding vascular endothelial growth factor. *Arch Immunol Ther Exp* 2001; 49: 243-6.
- [33] FLANAGAN JG, LEDER P The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell.* 1990; 63: 185-94. [http://dx.doi.org/10.1016/0092-8674\(90\)90299-T](http://dx.doi.org/10.1016/0092-8674(90)90299-T)
- [34] NASARRE P, CONSTANTIN B, ROUHAUD L, HARNOIS T, RAYMOND G et al. Semaphorin SEMA3F and VEGF have opposing effects on cell attachment and spreading. *Neoplasia* 2003; 5: 83-92.
- [35] GUO P, FANG Q, TAO HQ, SCHAFFER CA, FENTON BM et al. Overexpression of vascular endothelial growth factor by MCF-7 breast cancer cells promotes estrogen-independent tumor growth in vivo. *Cancer Res* 2003; 63: 4684-91.
- [36] KURENOVA EV, HUNT DL, HE D, FU AD, MASSOLL NA et al. Vascular endothelial growth factor receptor-3 promotes breast cancer cell proliferation, motility and survival in vitro and tumor formation in vivo. *Cell Cycle* 2009; 8: 2266-80. <http://dx.doi.org/10.4161/cc.8.14.9101>

- [37] BAGRI A, TESSIER-LAVIGNE M, WATTS RJ Neuropilins in tumor biology. *Clin Cancer Res.* 2009; 15: 1860-4. <http://dx.doi.org/10.1158/1078-0432.CCR-08-0563>
- [38] GERETTI E, KLAGSBRUN M Neuropilins: novel targets for anti-angiogenesis therapies. *Cell Adh Migr.* 2007; 1: 56-61. <http://dx.doi.org/10.4161/cam.1.2.4490>
- [39] COMA S, SHIMIZU A, KLAGSBRUN M Hypoxia induces tumor and endothelial cell migration in a Semaphorin 3F- and VEGF-dependent manner via transcriptional repression of their common receptor Neuropilin 2. *Cell Adh Migr.* 2011; 5: 266-75. <http://dx.doi.org/10.4161/cam.5.3.16294>
- [40] SHIBUYA M Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol.* 2006; 39: 469-478. <http://dx.doi.org/10.5483/BMBRep.2006.39.5.469>
- [41] STINE MJ, WANG CJ, MORIARTY WF, RYU B, CHEONG R et al. Integration of genotypic and phenotypic screening reveals molecular mediators of melanoma-stromal interaction. *Cancer Res.* 2011;71: 2433-44. <http://dx.doi.org/10.1158/0008-5472.CAN-10-1875>
- [42] GERETTI E, VAN MEETEREN LA, SHIMIZU A, DUDLEY AC, CLAESSESON-WELSH L, KLAGSBRUN M A mutated soluble neuropilin-2 B domain antagonizes vascular endothelial growth factor bioactivity and inhibits tumor progression. *Mol Cancer Res.* 2010; 8: 1063-73. <http://dx.doi.org/10.1158/1541-7786.MCR-10-0157>
- [43] BIELENBERG DR, HIDA Y, SHIMIZU A, KAIPAINEN A, KREUTER M et al. Semaphorin 3F, a chemorepellent for endothelial cells, induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. *J Clin Invest* 2004; 114: 1260-1271.
- [44] SERINI G, VALDEMBRI D, ZANIVAN S, MORTERRA G, BURKHARDT C et al. Class 3 semaphorins control vascular morphogenesis by inhibiting integrin function. *Nature* 2003; 424: 391-397. <http://dx.doi.org/10.1038/nature01784>
- [45] SMOLARCZYK R, CICHON T, GRAJA K, HUCZJ, SOCHAN-
IK A et al. Antitumor effect of RGD-4C-GG-(D)(KLAKLAK)(2) peptide in mouse B16(F10) melanoma model. *Acta Biochim Pol* 2006; 53: 801-805.
- [46] RIEGER J, WICK W, WELLER M Human malignant glioma cells express semaphorins and their receptors, neuropilins and plexins. *Glia* 2003; 42: 379-89. <http://dx.doi.org/10.1002/glia.10210>
- [47] YAMADA T, ENDO R, GOTOH M, HIROHASHI S Identification of semaphorin E as a non-MDR drug resistance gene of human cancers. *Proc Natl Acad Sci USA* 1997; 94: 14713-14718. <http://dx.doi.org/10.1073/pnas.94.26.14713>
- [48] BANU N, TEICHMAN J, DUNLAP-BROWN M, VILLEGAS G, TUFRO A Semaphorin 3C regulates endothelial cell function by increasing integrin activity. *FASEB J* 2006; 20: 2150-2152. <http://dx.doi.org/10.1096/fj.05-5698fje>
- [49] NASARRE P, KUSY S, CONSTANTIN B, CASTELLANI V, DRABKIN HA, BAGNARD D, ROCHE J Semaphorin SEMA3F has a repulsing activity on breast cancer cells and inhibits E-cadherin-mediated cell adhesion. *Neoplasia.* 2005; 7: 180-9. <http://dx.doi.org/10.1593/neo.04481>
- [50] OLBRYT M, JARZAB M, JAZOWIECKA-RAKUS J, SIMEK K, SZALA S et al. Gene expression profile of B 16(F10) murine melanoma cells exposed to hypoxic conditions in vitro. *Gene Expr* 2006; 13: 191-203. <http://dx.doi.org/10.3727/000000006783991818>
- [51] GRANDCLEMENT C, BORG C Neuropilins: a new target for cancer therapy. *Cancers*, 2011; 3: 1899-1928. <http://dx.doi.org/10.3390/cancers3021899>
- [52] FERON O Tumor-penetrating peptides: a shift from magic bullets to magic guns. *Sci. Transl. med.* 2010; 34, 34ps26.