No functional and transductional significance of specific neuropilin 1 siRNA inhibition in colon carcinoma cell lines lacking VEGF receptor 2

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Abstract. Neuropilins are membrane proteins that mediate effects on tumor cells directly and indirectly by affecting angiogenesis. Recent findings indicate that neuropilin 1 (NRP1) and the associated tyrosine kinase vascular endothelial growth factor receptor 2 (VEGFR2) play a regulatory role in developmental angiogenesis as well as in tumor angiogenesis. NRP1 and VEGFR2 might play a role in colon carcinogenesis and development of metastases. The significance of NRP1 expression in colon cancer seems to be controversial. Therefore, we aimed to distinguish between different expression patterns of signalling cascades in human colon carcinoma cell lines in order to analyze the role of NRP1 in tumorigenesis. We analyzed the biological significance of NRP1 in respect to VEGFR, EGFR, neuropilin and their ligands by RT-PCR and Western blot with functional knock-out of NRP1 in different colon adenocarcinoma cell lines. There was no expression of VEGFR2 in tumor cell lines. There were cells that expressed: i) only NRP1 (HT-29, LS174T), ii) NRP2 (Colo320) or iii) both (SW480, LoVo). Cells without NRP1 expression strongly expressed EGFR but only when NRP2 was co-expressed. Inhibition of NRP1 expression by RNA interference did not alter growth characteristics in soft agar experiments. Furthermore, there were no differences in intracellular signalling pathways (ERK1/2 or AKT) in NRP1 inhibited cells. In *ex vivo* transfer experiments animals with tumors from siRNA-NRP1 transfected cells showed no significant inhibition of tumor growth compared to siRNA control. In conclusion, our results question the role of NRP1 function in VEGFR2 negative colon adenocarcinoma cells. NRP1 seems to have no detectable effect on proliferation or migration nor does it induce any changes in intracellular signalling pathways without the expression of VEGFR2. According to our data, further studies are needed to analyze the therapeutic relevance of NRP1 inhibition *in vivo*.

Introduction

Colorectal carcinoma is one of the most common malignant diseases in the Western countries. Even after successful resection of the primary tumor, about one-third of the patients develop metastases in the liver and lungs (1,2). Metastatic colorectal cancer is associated with a poor prognosis. Although in many clinical studies chemotherapy is administered in combination with different tyrosine kinase inhibitors (3,4), new approaches for targeted therapies are needed. The antitumoral efficiency of vascular endothelial growth factor (VEGF) antagonism has already been described (2,5,6). VEGF expression correlates with tumor vascularization and therefore tumor growth in colorectal cancer (5,7,8). Treatment with tyrosine kinase inhibitors leads to apoptosis and tumor shrinking. Generally it is accepted that VEGF binds to its cell surface receptors [Flt-1 (VEGFR1), Flk-1 (VEGFR2) or Flt-4 (VEGFR3)] and regulates angiogenesis by either controlling intercellular endothelial interactions or intracellular tumor cell pathways (8,9). In the same way neuropilin (NRP) may indirectly mediate effects on tumor progression by affecting angiogenesis or directly through effects on tumor cells (10).

Neuropilin 1 (NRP1) is a transmembrane glycoprotein with a molecular weight of 130 to 140 kDa that was originally described to be expressed in the developing nervous system (11). Subsequently it was identified as a coreceptor for VEGF suggesting a role in vascularisation and angiogenesis (12). Transgene NRP1 knock-out mice suffer from insufficient and delayed vascularisation leading to embryonic death (13) whereas overexpression is lethal because of hemorrhage and excess blood vessel formation (14). Unlike VEGF receptors, NRP1 does not contain a tyrosine kinase domain and therefore mainly functions as a coreceptor for VEGFR2 for the binding of VEGF165 to induce VEGFR2 related migration and angiogenesis in endothelial cells (15). Several

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Table I. RT-PCR primer pairs.

Target	Forward primer	Reverse primer	Fragment
VEGFA189	5'-GTATAAGTCCTGGAGCGT-3'	5'-AAACCCTGAGGGAGGCTC-3'	257 bp
VEGFA165/121	5'-GTGAATGCAGACCAAAGAAAG-3'	5'-AAACCCTGAGGGAGGCTC-3'	100/240 bp
VEGFB186/167	5'-TGTCCCTGGAAGAACACAGCC-3'	5'-GCCATGTGTCACCTTCGCA-3'	260/363 bp
VEGFD	5'-GTATGGACTCTCGCTCAGCAT-3'	5'-AGGCTCTCTTCATTGCAACAG-3'	226 bp
VEGFR1	5'-ATCAGAGATCAGGAAGCACC-3'	5'-GGAACTTCATCTGGGTCCAT-3'	452 bp
VEGFR2	5'-GACTTCAACTGGGAATACCC-3'	5'-CATGGCCCTGACAAATGTG-3'	614 bp
NRP1	5'-AGCCCCTCCTCCTGTTGTGTCCTTC-3'	5'-GCTATCGCGCTGTCGGTGTAAAAA-3'	441 bp
NRP2	5'-CCCCGAACCCAACCAGAAGA-3'	5'-GAATGCCATCCCAGATGTCCA-3'	476 bp
EGFR	5'-GGAAGTATGCAGATGC-3'	5'-CGTAGTGTACGCTTTCG-3'	239 bp

Table II. Real-time RT PCR primer sets.

Target	Forward primer	Reverse primer	Probe no.
Alas	agg cat ggt tcc cag aat c	ccc tct tca ccc tgg cta	64
VEGFA	cac cac ttc gtg atg att ctg	cta cct cca cca tgc caa gt	63
VEGFB	gct tca cag cac tgt cct	ttt ggg gac atg tcc ctg gaa	76
VEGFD	tgg tat gaa agg ggc atc tg	tga gtg caa aga aag tct gga g	74
VEGFR1	cgc ctt acg gaa gct ctc t	cca ctc cct tga aca cga g	85
NRP1	cat caa ttt taa ttt ctg ggt tct tt	cac att tca caa gaa gat tgt gc	85
NRP2	tgt tgg agc agg gag cat '	gac ctg gca ctc agg tat cg	19
EGFR	ggc aca gat gat ttt ggt ca	gat cca agc tgt ccc aat g	55

studies have further focused on the role of NRP1 in tumor progression and carcinogenesis (11). NRP1 has been associated with increased angiogenesis and tumor growth *in vitro* and *in vivo* (16) and is expressed by several non-gastrointestinal tumors but also by pancreas, bile duct, gastric and colorectal cancer. In terms of colorectal cancer, it has been reported that NRP1 is expressed in colon adeno-carcinoma but not in the adjacent non-malignant colon mucosa (10) and contributes to angiogenesis and suppression of tumor cell apoptosis. Therefore, it was concluded that NRP1 expression may be involved in tumor development with metastasis in colon cancer and may be used as a prognostic factor or therapeutic target (10,17).

Nevertheless, the significance of NRP1 expression in colon cancer has not been clearly characterized to date. Here, we analyze different expression patterns and post-receptor signalling following NRP1 inhibition via RNA interference in different colon cancer cell lines.

Materials and methods

Animals and cell lines. The tumor cell lines SW480 (ATCC Number: CL228), LoVo(ATCC Number: CCL229), HT-29 (ATCC Number: HTB38), LS174T (ATCC Number: CL188), Colo320 (ATCC Number: CCL220), and Caco2 (ATCC Number: HTB37) were maintained in DMEM supplemented with 10% FBS, 200 mM glutamine, 100 μg streptomycin, and 100 μg penicillin. The human umbilical vein endothelial cell line HUVEC was obtained from Cascade Biologies Inc. (Portland, USA) and cultured in Medium 200 with LSGS. Primary colon epithelial cells were taken from human specimens after routine biopsy.

Eight-week-old male severe combined immunodeficient (SCID) mice were supplied by Charles River (Sulzfeld, Germany) and kept in the local central animal facility of the University Hospital Bonn. The mice were housed under standard conditions and had free access to water and food. Animal procedures were performed in accordance with approved protocols and followed recommendations for proper care and use of laboratory animals.

RT-PCR analysis. All tumor cells, endothelial cells, and primary epithelial cells were analyzed by PCR for expression of VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167 and VEGFD, and the receptors VEGFR1, VEGFR2, NRP1, NRP2 and EGFR. Human ß-actin was used as positive control. For this, RNA was isolated and reverse transcribed as described previously (18). Sequences are given in Table I. PCR products were separated with agarose gel electrophoresis.

Table III. Western blot antibodies and conditions.

Primary antibody	Protein size (kDa)	Dilution	Secondary antibody
EGF (Z-12): sc-275	6	1/200	1/2000 goat anti-rabbit IgG-HRP: sc-2004
EGFR (1005): sc-03	170	1/200	1/2000 goat anti-rabbit IgG-HRP: sc-2004
ERK 1/2	44/42	1/500	1/2000 goat anti-rabbit IgG-HRP: sc-2004
p-ERK1/2 (Thr 177)-R: sc-16981-R	44/42	1/500	1/1000 goat anti-mouse IgG-HRP: sc-2005
p-AKT1/2/3 (Ser 473)-R: sc-7985-R	60/56/60	1/200	1/2000 goat anti-rabbit IgG-HRP: sc-2004
p-p38 (Thr 180/Tyr 182)-R: sc-17852	38	1/200	1/2000 goat anti-rabbit IgG-HRP: sc-2004
PI 3-kinase p85a (B-9): sc-1637	85	1/200	1/2000 goat anti-mouse IgG-HRP: sc-2005
neuropilin (A-12): sc-5307	130	1/200	1/2000 goat anti-mouse IgG-HRP: sc-2005
neuropilin-2 (C-9): sc-13117	116	1/200	1/2000 goat anti-mouse IgG-HRP: sc-2005
Flt-1 (H-225): sc-9029	180	1/200	1/2000 goat anti-rabbit IgG-HRP: sc-2004
Flk-1 (C-20): sc-315	150/200	1/200	1/2000 goat anti-mouse IgG-HRP: sc-2005
ß-actin (C4): sc-47778	43	-	1/5000 goat anti-mouse IgG-HRP: sc-2005

For semiquantitative PCR, RNA was isolated from tumor tissue samples using the HighPure RNA Isolation Tissue Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. RNA concentrations were determined and RNA was used in the following RT-reaction with random primers (Transcriptor cDNA Synthesis Kit, Roche Diagnostics). Transcript levels of VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167, and VEGFD and the receptors VEGFR1, VEGFR2, NRP1, NRP2, and EGFR were determined in relation to ALAS mRNA levels by semiquantitative real-time PCR (LightCycler, Roche Diagnostics). Primers were obtained from Invitrogen GmbH and probes from Roche Diagnostics. Sequences are given in Table II.

Western blot analysis. For analysis of intracellular signal transduction molecules Western blot was performed. Lysates from tumor cells were harvested in a buffer (50 mM Hepes, pH 7.4, 0.1% Nonidet P-40, 250 mM NaCl) with 1 mM protease and 10 mM phosphatase inhibitors. Whole protein concentrations were determined using the DC protein assay (Bio-Rad, Munich, Germany). Protein was separated by 10% SDS-PAGE and transferred on nitrocellulose membrane via semi-dry blot. Blocking was performed in 5% milk and 10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.2% Tween-20 overnight, followed by incubation with primary and secondary antibodies. Detection was done by enhanced chemiluminescence on X-ray film. Primary and secondary antibodies are given in Table III.

Transfection of siRNA. siRNA-NRP1 and a non-sense siRNA (siRNA-CONT) were purchased from Dharmacon (Munich, Germany). The sequences have been selected according to a recent publication (17). A second set of siRNA-NRP1 purchased from Dharmacon as a so-called On-Target plus SMARTpool for NRP1 with defined sequences was used as a control siRNA. Transfection was performed in 6-well plates using 100 pmol siRNA-CONT or siRNA-NRP1 and 5 μl Lipofectamine 2000 (Invitrogen) in OptiMEM (Invitrogen) according to the manufacturer's instructions. Cells were assayed for alterations in expression levels 2 days post transfection after they were ruptured with PBS and three freeze-thaw cycles. Protein concentration was determined using the DC protein assay (Bio-Rad) and 100 μg whole protein was analyzed by Western blot. Transfection efficiency was analyzed by transfection of siRNA-GFP (Block-It Fluorescent Oigo, Invitrogen) followed by fluorescence microscopy. Gene knock-down was demonstrated by RT-PCR and Western blot of NRP1 as described above.

Analysis of proliferation after siRNA-treatment. After demonstrating gene knock-down, we tested whether treatment with siRNA-NRP alters functional properties of tumor cells. Selected colon cancer cell lines were transfected with siRNA-NRP or siRNA-CONT as described above and analyzed for changes in proliferation. Proliferation was examined with tetrazolium (MTT); cells were washed with PBS and incubated with MTT at 37°C for 45 min. MTT was solubilised with DMSO for 15 min and optical density was determined at 550 nm (19).

Ex vivo gene transfer of siRNA transfected cells. Orthotopic tumors were established as described previously (19): after transfecting siRNA-NRP and siRNA-CONT in HT-29 and LoVo cells (each n=5) as described above, 10^6 cells were implanted in the liver of SCID mice 24 h after transfection (20). Fourteen days after tumor cell inoculation, animals were sacrificed and livers explanted. Tumor growth was determined by measurement of the visible tumor in length and width.

Statistical analysis. Tumor volumes were calculated with the formula length x width^2 x 0.52. Data are expressed as mean ± SEM. Differences of different experimental groups were analyzed for statistical significance by a non-parametric, two-tailed test (Mann-Whitney test) for unpaired samples. Significance was calculated by the log-rank test. $p < 0.05$ was considered to be significant.
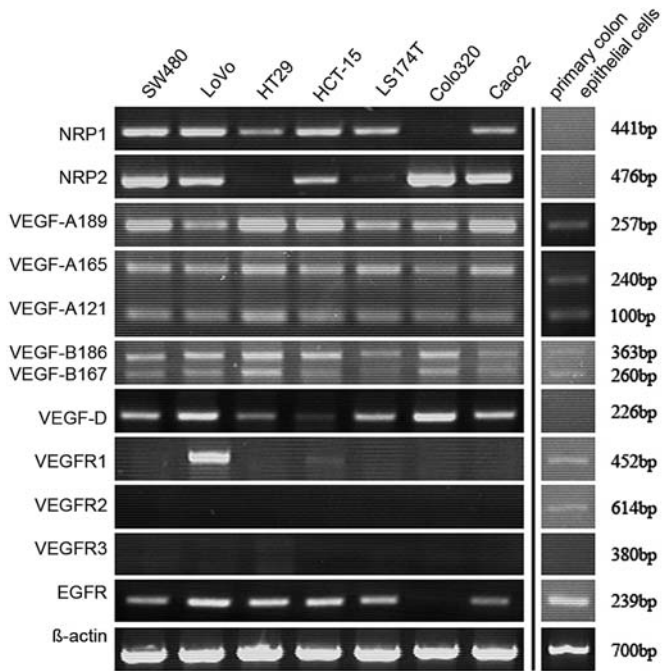
Figure 1. RNA expression profile in different colon cancer cell lines (RT-PCR). RNA from SW480, LoVo, HT-29, HCT-15, LS174T, Colo320, Caco2 and primary epithelial cells was analyzed by RT-PCR regarding the expression of VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167 and VEGFD and the receptors VEGFR1, VEGFR2, NRP1, NRP2 and EGFR.

Results

Receptor expression profile in different colon cancer cell lines. RNA from untreated tumor cells (SW480, LoVo, HT-29, HCT-15, LS174T, Colo320, Caco2) and primary epithelial cells was analyzed by RT-PCR regarding the expression of VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167, VEGFD and the receptors VEGFR1, VEGFR2, NRP1, NRP2, and EGFR, before starting siRNA-treatment (Fig. 1). All tumor cells and primary epithelial cells expressed VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167 and VEGFD with slightly different intensity. NRP1 and NRP2 were expressed in all tumor cells except in Colo320, HT-29 and LS174T cells. Both, NRP1 and

NRP2 expression was not detectable in primary epithelial cells. Only in LoVo cells and primary epithelial cells VEGFR1 was expressed. There was no expression of VEGFR2 and VEGFR3 in all analyzed tumor cell lines. Slight expression of VEGFR2 in biopsy samples may possibly be attributable to capillaries and not to epithelium. EGFR was expressed in all, but in Colo320 cells. These results correlated with those of additionally performed real-time RT-PCR experiments (data not shown).

RNA and protein expression profile after inhibition of NRP1. Small interfering RNA transfection in selected tumor cells (SW480, HT-29, LoVo, Colo320) was performed with two different siRNA-NRP1 sequences - one of a recent publication (17) and the other from Dharmacon as a so called On-Target plus SMARTpool for NRP1 - after optimizing the transfection efficiency up to 90% (Fig. 2). In tumor cells inhibition of NRP1 expression peaked 48 h after transfection compared to the control. An additional endothelial cell line (HUVEC) served as a transfection control. Differences in RNA and protein expression pattern (VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167, VEGFD, VEGFR1, VEGFR2, NRP1, NRP2, EGFR) in tumor cells after treatment with siRNA-NRP1 or siRNA-CONT were analyzed by RT-PCR (Fig. 3) and Western blot (Fig. 4). RNA expression of NRP1 was reduced in SW480, LoVo, HT-29 and in the control cell line (HUVEC) 48 h after siRNA-NRP1 inhibition. In Colo320 cells NRP1 expression remained un-detected. Quantitative analysis of NRP2 expression showed an upregulation of NRP2 in LoVo cells and a downregulation in SW480 and HT-29 cells after siRNA-NRP1 transfection compared to the control. Further quantitative analysis of the associated receptors showed downregulation of EGFR and VEGFR1 in LoVo cells but upregulation of EGFR in HT-29 cells after siRNA-NRP1 transfection compared to the control (Fig. 5). The expression profile of VEGFA189, VEGFA165, VEGFA121, VEGFB186, VEGFB167 and VEGFD remained unchanged after SiRNA-NRP1 transfection compared to the siRNA control.

Protein expression in untreated tumor cells (SW480, LoVo, HT-29, HCT-15, LS174T, Colo320) showed different expression patterns of VEGFR1, VEGFR2, NRP1, NRP2 and EGFR. NRP1 was strongly expressed in HT-29 and LS174T
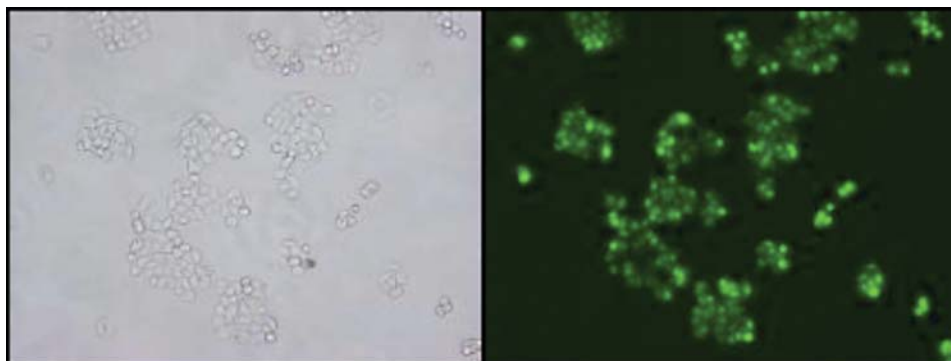

Figure 2. Control of transfection efficiency. siRNA transfection was performed in 6-well plates using Lipofectamine 2000. Transfection efficiency was analyzed by transfection of siRNA-GFP followed by fluorescence microscopy (left, microscopy of *in vitro* cultured cells; right, fluorescence microscopy of *in vitro* cultured cells after transfection).
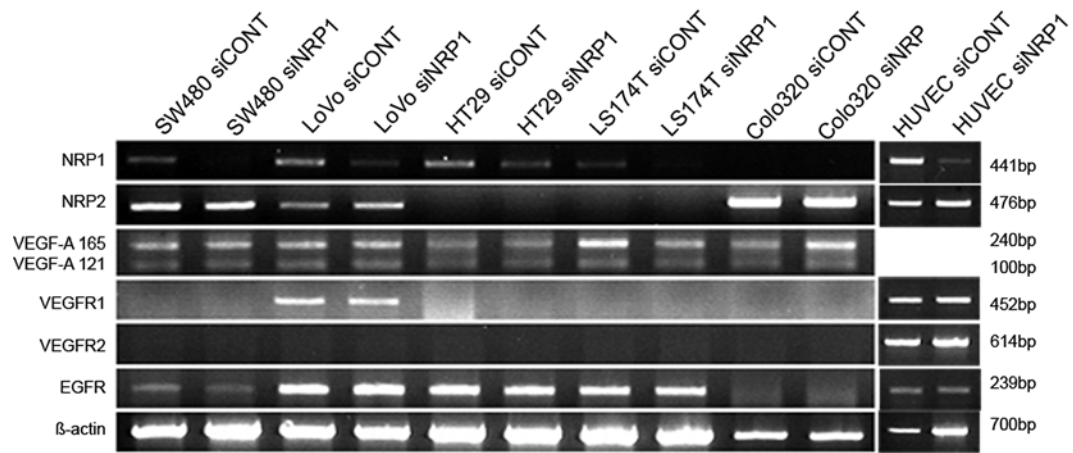
Figure 3. RNA Expression after siRNA transfection. Differences in RNA expression patterns in tumor cells after treatment with siRNA-NRP1 (siNRP1) or control siRNA (siCONT) were analyzed by RT-PCR. Small interfering RNA transfection in selected tumor cells (SW480, HT-29, LoVo, Colo320) was performed with two different siRNA-NRP1 sequences. An additional endothelial cell line (HUVEC) served as a transfection control. RNA expression patterns of VEGFA165, VEGFA121, VEGFR1, VEGFR2, NRP1, NRP2, EGFR correlated with real-time RT PCR.
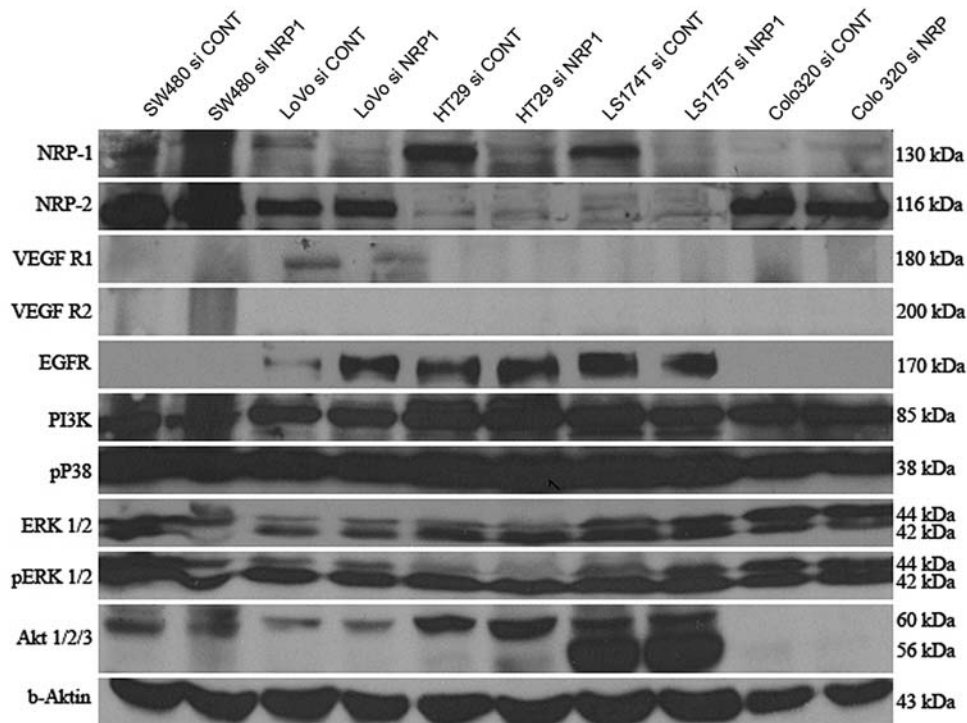

Figure 4. Protein expression after siRNA transfection. Differences in protein expression of receptor and signal cascade proteins in tumor cells after treatment with neuropilin siRNA (siNRP1) or control siRNA (siCONT). siRNA transfection in selected tumor cells (SW480, HT-29, LoVo, LS174T, Colo320) was performed with two different siRNA-NRP1 sequences. For Western blot analysis protein of tumor cells were separated by SDS-PAGE and transferred to nitrocellulose membrane via semi-dry blot, followed by incubation with primary and secondary antibodies (Table III). Detection was done by enhanced chemiluminescence on X-ray film.

and only slightly in SW480 cells. NRP2 was expressed in SW480, LoVo and Colo320 cells. VEGFR1 was only expressed in LoVo cells. VEGFR2 was not expressed in any of the cell lines. EGFR was strongly expressed in HT-29 and LS174T cells and only slightly expressed in LoVo cells (Fig. 4). Transfection of tumor cells with siRNA-NRP1 did not influence protein expression of NRP2 and VEGFR2. NRP1 expression was nearly abolished after siRNA-NRP1 transfection. Furthermore, EGFR expression was induced

and VEGFR1 expression reduced after NRP1 transfection in LoVo cells.

Analysis of the effects of NRP inhibition on intracellular signal cascades revealed a strong expression of phosphorylated P38, ERK1/2 and phosphorylated ERK1/2. Phosphorylated AKT1/2/3 was strongly expressed in LS174T followed by HT-29 and SW480 cells and only slightly expressed in LoVo cells. There were no changes in the phosphorylation of AKT1/2/3 or ERK1/2 respectively nor PI3K and p38 MAPK
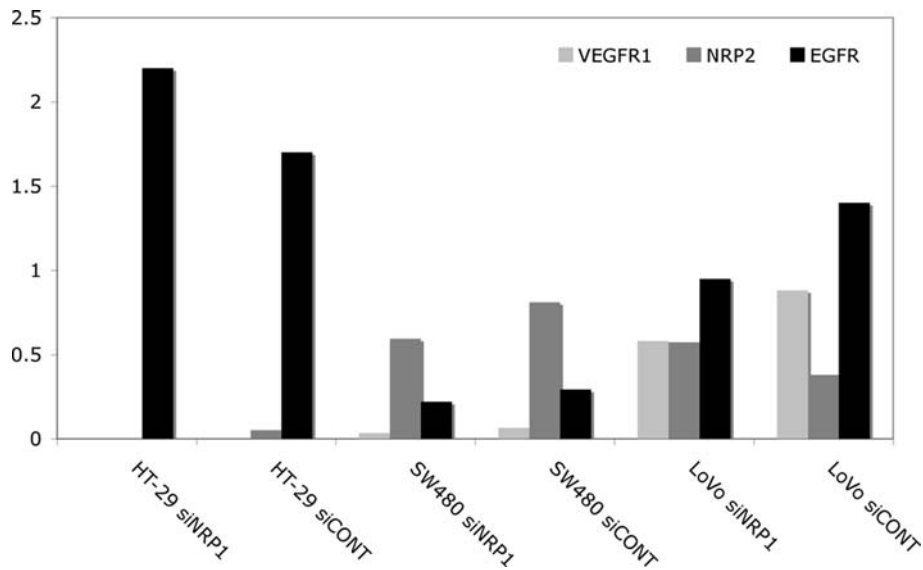
Figure 5. Real-time PCR expression after siRNA transfection. Differences in RNA expression patterns in tumor cells after treatment with siRNA-NRP1 (siNRP1) or control siRNA (siCONT) were analyzed by semiquantitative RT-PCR. Transcript levels of VEGFR1, NRP2, and EGFR were determined in relation to ALAS mRNA levels by semiquantitative real-time PCR (LightCycler). Sequences are given in Table II.
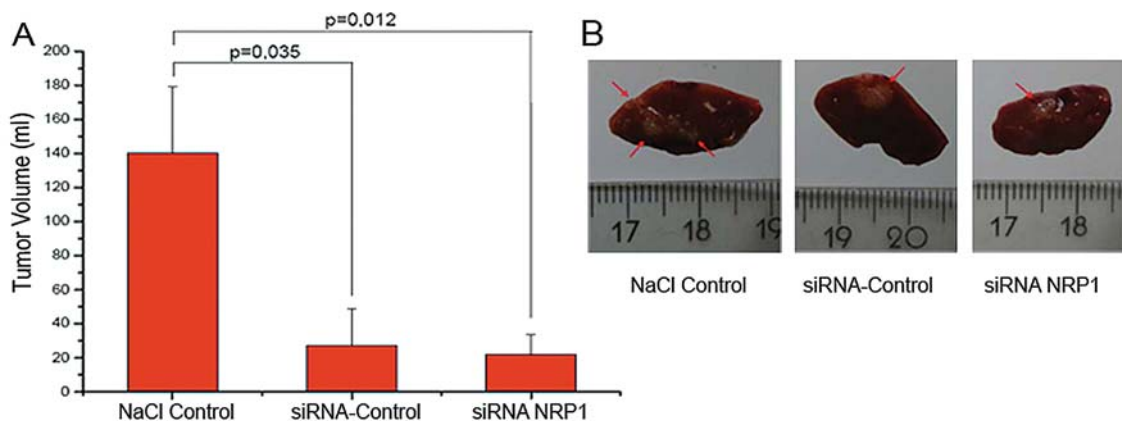

Figure 6. *Ex vivo* gene transfer were performed in an intrahepatic tumor model. (A) Animals with tumors from siRNA-NRP1 transfected HT-29 and LoVo cells showed no significant inhibition of tumor growth compared to siRNA control. There was a significantly reduced tumor growth of up to 70% comparing all siRNA treated cells of control and target (NRP1) siRNA to the NaCl treated cells. (B) After transfecting siRNA-NRP and siRNA-CONT in HT-29 and LoVo cells (each n=5) as described above, 10^6 cells were implanted in the liver of SCID mice 24 h after transfection. Fourteen days after tumor cell inoculation, animals were sacrificed and livers explanted.

after siRNA-NRP transfection compared to the control. AKT1/2/3 was not detectable in Colo320 (Fig. 4).

Corresponding to the unchanged effects on signal transduction molecules there was also no effect after siRNA-NRP1 transfection on proliferation in tumor cells and endothelial cells (data not shown).

Influence of ex vivo transfer of siRNA on orthotopic tumor growth. After verifying the inhibitory effects of siRNA-NRP1 on NRP expression in tumor cell lines, *in vivo* experiments after *ex vivo* gene transfer were performed in an intrahepatic tumor model: animals with tumors from siRNA-NRP1 transfected HT-29 and LoVo cells showed no significant inhibition of tumor growth compared to siRNA control (Fig. 6). Interestingly, significantly reduced tumor growth of up to

70% was observed comparing all siRNA treated cells of control and target (NRP1) siRNA to the NaCl treated cells. Both the target and control siRNA could significantly reduce the tumor growth whereas there was no reduction in tumors of NaCl treated cells.

Discussion

The members of the neuropilin family were originally identified as receptors for the repulsive axon guidance factor belonging to the class-3 semaphorin sub-family (11). NRP1 is known to function as a coreceptor of VEGFR2 for the binding of VEGF165 and suggested to play a role in embryonic vasculogenesis and angiogenesis (10,13). NRP1 expression has been described as a VEGF coreceptor

in colon adenocarcinoma and other tumor entities, indicating a role in the process of carcinogenesis by affecting tumor angiogenesis, as well as migration, invasiveness and progression. It was explained that the NRP1 function in tumor cells by tumor-derived NRP1 that acts as a positive modulator of angiogenesis and suggests to target NRP1 as a potential anti-cancer strategy (16). Our results suggest that NRP1 seems to have neither detectable effect on proliferation or migration nor does it activate commonly related intracellular signalling pathways. It does not interact with VEGFR2 in colon cancer cell lines and might interact with VEGFR1 in a metastatic colon cancer cell line (Lovo). Among the analyzed colon cancer cell lines we found cell lines that: i) express NRP1 only (HT-29, LS174T), ii) NRP2 only (Colo320) or iii) both NRP1 and NRP2 (SW480, LoVo). These cell lines represent different stages (Dukes I-III) of colon cancer and further represent a natural knock-out of NRP1 and NRP2.

The fact that there is no VEGFR2 expression in colon adenocarcinoma cell lines corresponds to recent reports from Hansel *et al* (21) and Parikh *et al* (10) who did not find VEGFR2 expression in colon cancer. Assuming any functional or transductional significance of NRP1 in VEGFR2 negative colon cancer cell lines these results are contrary to former data indicating an interaction of NRP1 with VEGFR2 to activate cellular proliferation and angiogenesis (12). Pan *et al* recently reported that blocking of NRP1 and VEGF in tumor models results in an additive effect in reducing tumor growth. But at the same time they present evidence that NRP1 may also be acting through mechanisms other than VEGFR2 signalling. They showed that VEGF121 directly binds to NRP1. However, unlike VEGF165, VEGF121 is not sufficient to bridge the NRP1-VEGFR2 complex. VEGFR2 enhances VEGF165 but not VEGF121 binding to NRP1 (22,23). The lack of downstream effects (except from EGFR upregulation) of VEGF121 or VEGF165 in the absence of VEGFR2 in our study underlines a mandatory role of VEGFR2 for NRP1 signalling. Additionally, this is the first time a correlation of VEGFR1 and NRP1 expression in a metastatic colon cancer cell line has been demonstrated. NRP1 inhibition leads to a downregulation of the tyrosine-kinase VEGFR1 in metastatic colon adenocarcinoma cells via an Erk1/2 or Akt-independent pathway. Hansel *et al* (21) showed that neuropilin is being upregulated during the process of carcinogenesis and correlates to invasive behaviour of gastrointestinal adenocarcinomas. First results from a clinical study from our group (unpublished data) underline the correlation of NRP1 expression and metastases in colon cancer patients with subsequent VEGFR1 up-regulation.

The different combination of NRP1 and NRP2 expression in the analyzed cell lines further correlated with a different expression of EGFR. Cells without NRP1 expression did strongly express EGFR and cells with NRP2 expression did not. In metastatic LoVo cells both NRP1 and NRP2 were expressed which resulted in a reduced EGFR expression while VEGFR1 was upregulated. These results suggest an interaction of NRP1 and EGFR although no direct interaction has been proven so far. Recently Wang *et al* (24) demonstrated that NRP1 induced survival in endothelial cells,

which is mediated by a connection of NRP1 with the NRP1 interacting protein. NRP1 activation signals via PI3K/AKT, which subsequently results in an inactivation of p53 pathways. EGFR acts via the PI3K/AKT pathway. We saw EGFR upregulation when NRP1 was silenced (Fig. 4). This implies an interaction of EGFR and NRP1 independent of PI3K/AKT pathway, which was supported by our data. There is almost no phosphorylated AKT in EGFR-negative cell lines. The expression of NRP2 seems to go along with the lack of EGFR and AKT expression.

To analyze the functional significance of siRNA-NRP1 transfection in selected tumor cells (SW480, HT-29, LoVo, Colo320) was performed. Apart for the described changes of EGFR and VEGFR1 expression in metastatic colon cancer cells, the expected inhibition of NRP1 was observed only. Effects on membrane receptors or signalling pathway proteins were missing. The effect of reduced levels of NRP1 after siRNA-NRP1 transfection on proliferation and apoptosis in tumor cells and endothelial cells did not show any significant differences. Since we used two different siRNA sequences, we can conclude that NRP1 inhibition by siRNA seems to have no effect on tumor cells and therefore is not a sufficient therapy strategy. These findings are controversial to the results of Ochiumi *et al* (17). Although they did not find any changes in migration ability they demonstrated increased levels of proliferation and angiogenesis with decreased apoptosis in NRP1 knock down cells. The precise mechanism for this inconsistency remains unknown. One explanation might be the different expression of VEGFR2. In the cell lines used in our study, VEGFR2 expression was not detected. Therefore, interaction of NRP1 and VEGFR2 was not found and could not be analyzed.

Although our *in vitro* data were not in favour of *in vivo* experiments, previous studies described antitumor efficiency. An intrahepatic tumor model for colon cancer metastasis was chosen and *ex vivo* gene transfer was performed. Consistent with our *in vitro* data, siRNA-NRP1 transfected cells showed no significant retardation of tumor growth. Significant reduction of tumor growth of 70% was observed in all siRNA treated cells (control and NRP1) compared to the NaCl treated cells. Targeted RNA interference was performed with two different poly U siRNA sequences. Consistently the siRNA itself seems to have an antitumorigenic or apoptotic effect on injected tumor cells. These observations are supported by different reports on non-specific effects of small interfering RNA. These non-specific effects accompanied by proinflammatory responses would be detrimental to a desired therapeutic effect of siRNA. They might be due to an activation of the interferon response and the presence of a 5'-triphosphate on the RNA (25,26). Judge *et al* reported that poly U rich siRNA complexes activate the innate and adapted immune system via IFN type I upregulation (27). The NRP1 siRNA sequences employed in our study also contain poly U rich motif. A current review favors the merging of gene-silencing and immune stimulation in one siRNA molecule (28). Nevertheless, inhibition of NRP1 expression with the sequences presented in this study seems to be a controversial experimental tool to analyze the functional effects.

In summary, this report questions the role of NRP1 on carcinogenesis in VEGFR2-negative colon adenocarcinoma

cells. NRP1 does not seem to have a detectable effect on proliferation or migration nor does it induce any changes in intracellular signalling pathways without the expression of VEGFR2 when analyzing its function by interfering RNA technique. However, in the absence of VEGFR2 there might be an interaction of NRP1 and EGFR. VEGFR1 is up-regulated in metastatic colon cancer cells. The relevance of NRP1 inhibition is questionable in this context, despite the fact that the used siRNA itself retarded tumor growth. For any therapeutic approach with NRP1 inhibition by siRNA, further experiments are required to fully understand the interaction of NRP1 and its several coreceptors.

Acknowledgements

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