

# Functional implication of Netrin expression in malignant melanoma

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**Abstract.** *Background:* Malignant melanoma cells are known to have altered expression of genes supporting proliferation and invasion, however, the expression of molecules of the Netrin family of repellent factors has not been analyzed in melanomas until now.

*Results:* Here, we show that Netrin-1 expression is strongly induced in melanoma cells compared to melanocytes *in vivo* and *in vitro* controlled at the transcriptional level via ETS-1. In addition, the expression of the netrin receptor UNC5B was induced and that of UNC5C was reduced in the tumor cells. In order to determine the functional relevance of Netrin-1 expression in malignant melanoma, Netrin expression in melanoma cells was reduced by siRNA attempts and primary human melanocytes were treated with recombinant Netrin-1. The cells showed no changes in proliferation or apoptosis, however, a strong reduction of migratory properties was observed in the melanoma cells after reduction of Netrin expression whereas melanocyte migration was strongly induced by treatment with Netrin.

*Conclusion:* Our study suggests that Netrin-1 promotes melanoma cell invasion and migration and therefore has an important role in the progression of malignant melanoma.

**Keywords:** Malignant melanoma, migration, repellent factor, Netrin

## 1. Introduction

Cellular repulsion and attraction can be mediated by a group of receptors and ligands known as “repellent factors”. They are best known for their expression and function in the developing central nervous system. Here, they direct the sprouting of nerve fibers as axon guiding cues by repelling or attracting the growth cone from or towards certain structures [1]. Apparently, repellent factors also contribute to the development of a variety of organs such as lung, mammary gland, cardiovascular system and kidney by exerting repellent or attractant activities [6].

Besides their function in embryonic development and normal cellular processes, repellent factors in general are also linked to tumor formation and progression. Several groups have shown, that e.g. Ephs and Ephrins, belonging to the group of repellent factors, influence tumor development, angiogenesis and invasiveness [3,4].

The receptor family of DCC (deleted in colon cancer) and UNC5H (UNC5A-C) mediate the responses to the guidance factor Netrin-1. Netrin-1 was first identified due to its potential to attract axon outgrowth [7,20]. Today it is known that Netrin-1 is a crucial regulator of embryonic development also mediating cell migration, angiogenesis and morphogenesis [2]. In the physiological situation in axons DCC is the receptor which induced attraction after binding Netrin-1 whereas Netrin-1 binding to the UNC5Hs leads to repulsion. However, next to DCC there seem to be other, yet unidentified receptors, which lead to chemoattraction towards Netrin-1 as shown for endothelial cells [16]. DCC action is mediated via small GTPases linking receptor activation to cytoskeletal dynamics. Netrin-1 binding to UNC5Hs was revealed to activate RhoA thus promoting cytoskeletal changes (reviewed in [22]). In other studies Netrin-1 was also linked to apoptosis presenting DCC as dependence receptor [13].

Until today Netrin-1 function in tumorigenesis was only rarely described. Proliferation of schwannoma cells was induced by Netrin-1 [11]. In addition a role in colon carcinoma invasion and metastasis was claimed [17]. We aimed to analyze the role of Netrin-1 in malignant melanoma.

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## 2. Materials and methods

### 2.1. Cell culture

The melanoma cell lines Mel Im, Mel Ei, Mel Wei, Mel Juso and SK Mel 3, were described previously [19]. The cell lines Mel Ei, Mel Wei and Mel Juso were derived from a primary cutaneous melanoma, Mel Im and SK Mel 3, were derived from metastases of malignant melanomas. Cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 µg/ml), L-glutamine (300 µg/ml) and 10% fetal calf serum (FCS; Pan, Aidenbach, Germany) and split at a 1:5 ratio every three days. The human microvascular endothelial cell line CDC/EU.HMEC-1 (HMEC) was kindly provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA). HMEC were cultured in MCDB131 (Sigma) medium, supplemented with 15% fetal calf serum, 1 mg/ml hydrocortisone (Sigma), 10 ng/ml epidermal growth factor (Collaborative Biochemical Products, Bedford, MA, USA), penicillin (100 U/ml) and streptomycin (10 µg/ml). Cells were split 1:5 at confluence.

Cell proliferation was determined using the XTT assay (Roche, Mannheim, Germany). Apoptosis was measured by Annexin V staining as described in [14, 21].

For adenoviral transduction either Adeno-lacZ or Adeno-IκB were used as described previously [9].

Staining of actin cytoskeleton was performed using Phalloidin-Rhodamin (Cytoskeleton Inc., Denver, CO, USA).

### 2.2. Immunohistochemistry

Paraffin-embedded preparations of normal skin, *nevi*, primary melanoma and metastases of malignant melanomas were screened for Netrin-1 protein expression by the avidin-biotin complex (ABC) method (DAKO-LSAB2-Kit, DAKO, Hamburg, Germany, [23]). The tissues were deparaffinated, rehydrated and incubated with primary polyclonal Netrin-1 antibody (1:200, Abcam) over night at 4°C. The secondary antibody supplied with the kit was incubated for 30 min at room temperature. Antibody binding was visualized using AEC-solution (for LSAB2-Kit). Finally, the tissues were counterstained by hemalaun.

### 2.3. RNA isolation and reverse transcription

Total cellular RNA was isolated from cultured cells using the RNeasy kit (QIAGEN, Hilden, Germany) and cDNAs were generated by reverse transcriptase reaction performed in 20 µl reaction volume containing 2 µg of total cellular RNA, 4 µl of 5× first strand buffer (Gibco), 2 µl of 0.1 M DTT, 1 µl of dN<sub>6</sub>-primer (10 mM), 1 µl of dNTPs (10 mM) and DEPC-water. The reaction mixture was incubated for 10 min at 70°C, 200 units of Superscript II reverse transcriptase (Gibco) were added and RNAs were transcribed for 1 h at 37°C. Reverse transcriptase was inactivated at 70°C for 10 min and the RNA was degraded by digestion with 1 µl RNase A (10 mg/ml) at 37°C for 30 min.

### 2.4. Analysis of mRNA expression by quantitative PCR

Quantitative real time-PCR was performed on a Lightcycler (Roche, Mannheim, Germany). cDNA template (2 µl), 0.5 µl (20 mM) of forward and reverse primers (see Table 1) and 10 µl of SybrPremix ExTaq (Takara (Lonza), Switzerland) in a total of 20 µl were applied to the following PCR program: 30 s 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 15 s, 3 s – 60°C, 5 s – 72°C, 81°C acquisition mode single, repeated for 40 times (amplification). The PCR reaction was evaluated by melting

Table 1  
Primer sequences for primers used in RT-PCR

Gene	Primer
β-actin forward	5'-CTA CGT CGC CCT GGA CTT CGA GC
β-actin reverse	5'-GAT GGA GCC GCC GAT CCA CAC GG
Netrin-1 forward	5'-AAC CCG CAC AAC CTG ACG
Netrin-1 reverse	5'-TAG AAC TGG AAG GGC ACC CA
Netrin-3 forward	5'-CAC TGT GAC CTG GAC TAT GGC
Netrin-3 reverse	5'-TGC GTA GGA GTA AGG GAC GA
Netrin-4 forward	5'-CGT GTT CCT GCC ATC CAG TA
Netrin-4 reverse	5'-ACA GCC ATA GTC TCC GAA GC
DCC forward	5'-GAC TCC AAT CCC AGG TGA CT
DCC reverse	5'-TGA CTT CCT CGC CTC GTA AC
UNC5A forward	5'-GCC CAT CTG TGT GTG TGT ATG
UNC5A reverse	5'-TGG TCG TGT GCC TGA ATC CA
UNC5B forward	5'-TTC ACG GGC GAG TCC TAT TC
UNC5B reverse	5'-TCG GCT CCT CCA CCA AGT AT
UNC5C forward	5'-ACA TCA AGG CAG CAA GAC AAG
UNC5C reverse	5'-TCT CCA ACA ACG ACT GGG TC
UNC5D forward	5'-ACA CAA AGT AGC CCA TCT GCT
UNC5D reverse	5'-GCC TGC TGT AGT TGA AGT CG

curve analysis and checking the PCR products on 1.8% agarose gels.

### 2.5. Transfection experiments

Cells ( $2 \times 10^5$  per well) were seeded into 6-well plates and transfected with 0.5  $\mu\text{g}$  of plasmid per well using lipofectamine plus (Invitrogen). The following plasmids were used: Ets-1 construct in pcDNA3 (15) and p50 and p65 expression plasmids [10]. Twenty-four hours after transfection the cells were harvested and RNA was isolated. SiRNA transfection was performed using Lipofectamin RNAiMAX (Invitrogen) and the siRNAs against Netrin-1 (Hs\_NTN1; Qiagen). All transfection experiments were repeated three times.

### 2.6. Migration assay

Migration assays were performed using Boyden chambers containing polycarbonate filters with 8  $\mu\text{m}$  pore size (Neuroprobe Inc., Gaithersburg, MD, USA), essentially as described previously [19]. Filters were coated with gelatine (Becton Dickinson, Heidelberg, Germany). The lower compartment was filled with fibroblast-conditioned medium, used as a chemo-attractant. Melanoma cells were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of  $2 \times 10^5$  cells/ml and placed in the upper compartment of the chamber. After incubation at 37°C for 4 h, the filters were collected and the cells adhering to the lower surface fixed, stained and counted. Netrin-1 was used in a concentration of 100 ng/ml.

### 2.7. Statistical analysis

Results are expressed as mean  $\pm$  SD (range) or percent. Comparison between groups was made using the Student's paired *t*-test. A *p*-value  $< 0.05$  was considered statistically significant. All calculations were performed using the GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

### 3.1. Analysis of expression of the Netrin family in malignant melanoma

We first analyzed the expression of Netrins (Netrin-1, -3 and -4) and their receptors (UNC5A, B, C, D

and DCC) in melanoma cell lines and normal human epidermal melanocytes (NHEM) by using RT-PCR. Expression of DCC was not detectable in the analyzed cell lines (Fig. 1A), whereas all other family members were expressed in all cells. Interestingly, a strong induction of Netrin-1 and UNC5B expression was observed comparing melanoma cells to primary melanocytes whereas UNC5C expression was strongly reduced. Expression of each molecule in comparison to actin as housekeeper to assess expression level is displayed in Fig. 1B. For further analysis we concentrated on the role of the ligand Netrin-1 in malignant melanoma. Expression of Netrin-1 mRNA was analysed by quantitative RT-PCR in melanoma tissue (Fig. 1C). In accordance to cell lines, in tissue samples of malignant melanoma increased expression of Netrin-1 mRNA was determined in primary tumors (PT1-4) and metastases (MM1, 2). Immunohistochemistry supported the mRNA data and revealed moderate to strong expression of Netrin-1 in samples of primary melanoma ( $n = 5$ ) and melanoma metastasis ( $n = 5$ ) whereas only weak expression was observed in nevi (Fig. 1D). These results confirmed that Netrin-1 is up-regulated during transformation.

### 3.2. Ets-1 as possible regulators of Netrin-1 expression

In order to analyze the mechanisms of regulation of Netrin-1 expression in melanoma cells, we focused on the promoter regions of the human *netrin-1* gene. Sequence analysis of the *netrin-1* promoter region revealed the presence of putative binding sites for several transcription factors, including Ets-1. In addition, recent publications suggested a regulation of Netrin-1 expression by NF $\kappa$ B in colorectal cancer cells [15]. Since we have shown previously that Ets-1 was up-regulated in melanomas [19], we assessed the importance of both.

We chose to down-regulate the expression of Ets-1 by using an anti-sense approach in order to assess Ets-1 function on the *netrin-1* gene. Transfection of the melanoma cell line Mel Im with Ets-1 anti-sense expression plasmids resulted in a significant down-regulation of the levels of expression of the endogenous transcript (Fig. 2A). On the other hand, modulation of the strong NF $\kappa$ B activity in malignant melanoma [10] by either stable I $\kappa$ B or by transfection of NF $\kappa$ B subunits p50 and p65 did not result in changes in Netrin-1 expression (Fig. 2B). Taken together, these data demonstrate that Ets-1 is an endogenous activator of the *netrin-1* gene in melanoma cells.

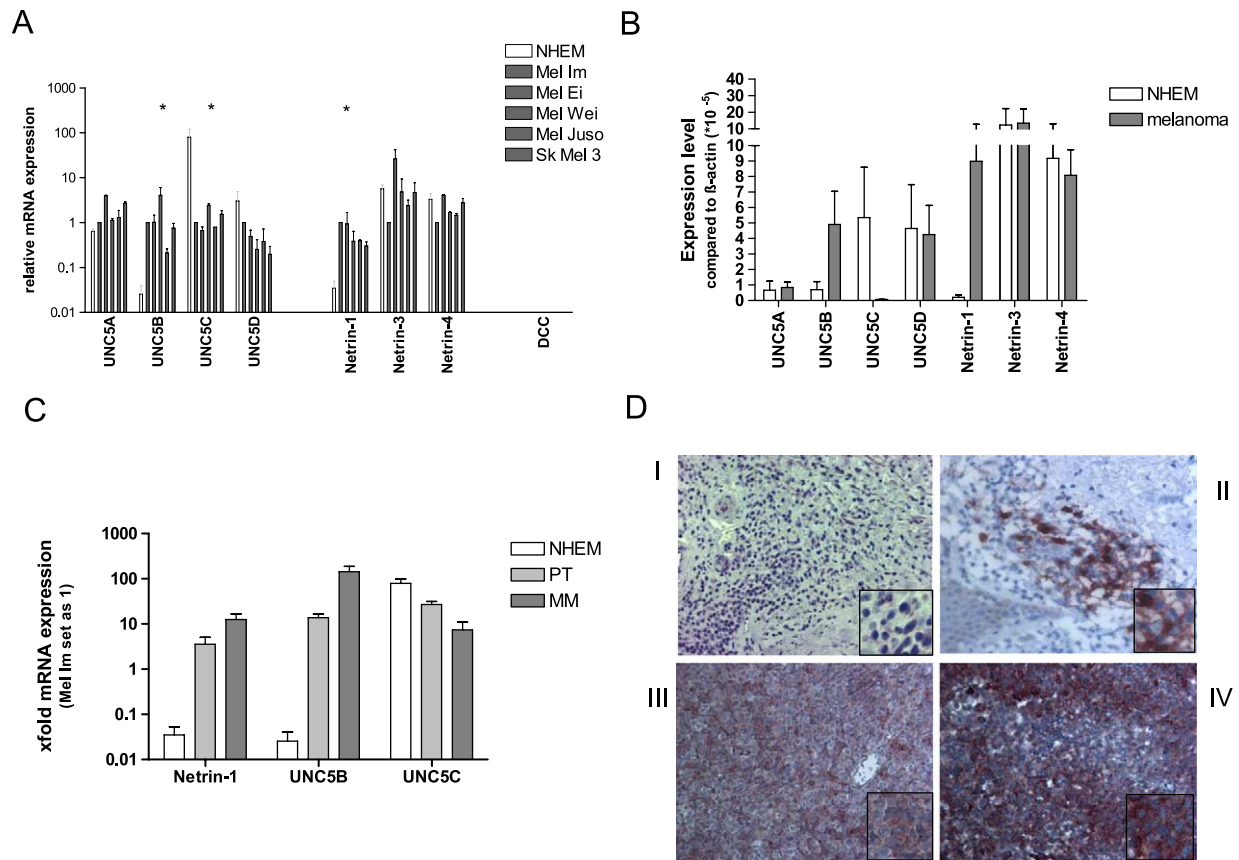


Fig. 1. Expression pattern of Netrin family repellent factors in melanoma. (A, B, C) expression of Netrins (Netrin-1, -3 and -4) and their receptors (UNC5A, B, C and D) and DCC was determined (A) in melanoma cell lines and normal human epidermal melanocytes (NHEM) and in (C) RNAs extracted from primary melanomas (PT,  $n = 7$ ) and melanoma metastasis (MM,  $n = 6$ ) by using quantitative RTPCR. In (B) expression of the molecules compared to actin are displayed to assess expression level. All experiments were performed in triplicate and repeated three times, bars display mean + SD, statistical analysis was performed using a Student's unpaired  $t$ -test. ( $*p < 0.05$ .) (D) Immunohistochemical staining of Netrin-1 in melanoma samples revealed expression in the tumor cells. (I: nevus; II: primary melanoma; III-IV: melanoma metastasis.)

### 3.3. Functional role of Netrin-1 in melanocytic cells

To assess the functional effects of Netrin-1 on melanocytic cells we, first, treated primary melanocytes with Netrin-1 and measured the effects on cell proliferation and migration. Cell proliferation was not different after Netrin-1 treatment (data not shown). On the other hand, the migratory properties of primary melanocytes were clearly induced (Fig. 3).

Melanoma cells were treated with two siRNAs targeting Netrin-1 compared to a control siRNA. Quantitative RT-PCR revealed strong reduction of Netrin-1 expression by both specific siRNAs (Fig. 4A). The rate of cellular apoptosis (Fig. 4B) and proliferation (Fig. 4C) stayed unchanged after treatment; however, cellular migration was strongly reduced (Fig. 4D).

### 3.4. Molecular regulation of Netrin-1 function

We were interested to get an insight into the molecular basis of Netrin-1 function in melanoma. We, therefore, first analyzed whether treatment of the melanoma cells with Netrin-1 (100 ng/ml) for 24 h resulted in changes in gene expression. Genes chosen to be analyzed were all correlated with melanoma invasiveness. No significant changes could be determined in mRNA expression of RhoA, RhoC, MMP2, MMP9, MMP14, E-cadherin and N-cadherin (data not shown). We, therefore, speculate that the effect of Netrin-1 is a direct mechanism modulating signaling pathways that control migration and invasion. This is supported by the fact that the effects on migration determined in the Boyden Chamber model were observed during 4 h of assay. To get insight whether the cytoskeleton is

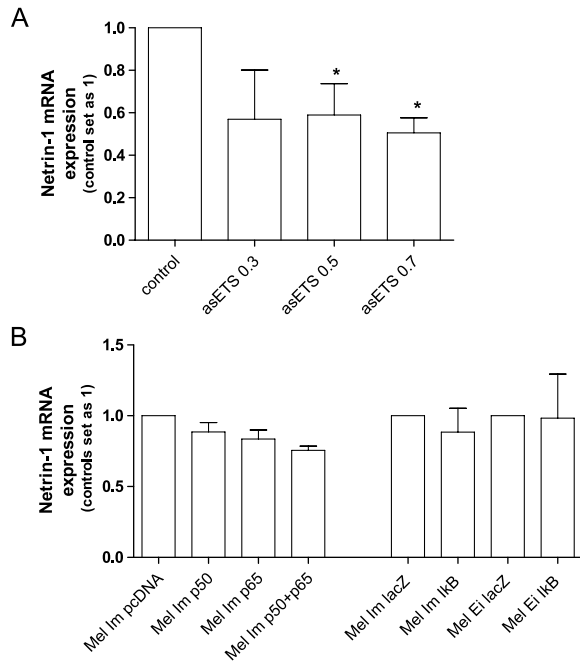


Fig. 2. Regulation of Netrin-1 expression. (A) Mel Im cells were transiently transfected with different amounts of antisense Ets-1 expression construct. Regulation of Netrin-1 mRNA was assessed by quantitative RT-PCR 48 h after transfection compared with the mock transfected control. (B) Mel Im cells were either transfected with expression plasmids for p50, p65 or both, or transduced with an adenoviral vector expressing a stable form of I $\kappa$ B. Regulation of Netrin-1 mRNA was assessed by quantitative RT-PCR 48 h after transfection/transduction compared with the mock control. All experiments were performed in triplicate and repeated three times with similar results, bars display mean + SD, statistical analysis was performed using a Student's unpaired *t*-test. (\**p* < 0.05.)

involved in this regulation, we stained siRNA Netrin treated cells with Phalloidin–Rhodamine and revealed marked changes in the actin cytoskeleton compared with control siRNA treated cells (Fig. 5). As expected by unchanged expression of E- and N-cadherin, cell–cell adhesion was not affected.

#### 4. Discussion

Netrin-1 has been shown to play a major role in the control of axon guidance of neurons during the development of the nervous system by interacting with its main receptors of the families DCC and UNC5H. DCC and UNC5H were shown to belong to the family of “dependence receptors” that share the ability to induce apoptosis in the absence of their ligands [12]. Expression of this type of receptors at the surface of cancer

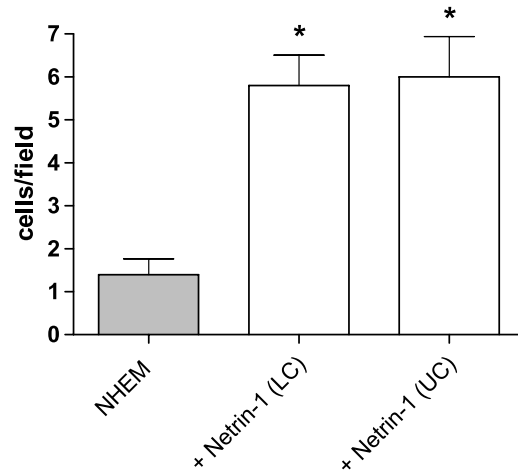


Fig. 3. Influence of Netrin-1 on migration of melanocytes. Human normal epidermal melanocytes were treated with Netrin-1 and migration was assessed in the Boyden Chamber model. Netrin (100 ng/ml) was either given into the lower (LC) or the upper chamber (UC). All experiments were performed in duplicate and repeated three times with consistent results. Bars display mean + SD, statistical analysis was performed using a Student's unpaired *t*-test. (\**p* < 0.05.)

cells is indeed speculated to create a dependency of tumor cells on ligand availability for its survival.

In this study we could show changes in expression of UNC5H receptors and strong induction of Netrin-1 expression in melanoma cells in comparison to normal primary melanocytes *in vitro* and *in vivo*. In addition, UNC5B mRNA expression was induced in the tumor cells. The fact that Netrin-1 expression confers a selective advantage for tumor cell survival was also shown for metastatic breast cancer [5]. However, for malignant melanoma we could not reveal an effect on cell proliferation and cell death after repression of Netrin-1 expression.

Recently, it was shown that in colon carcinoma Netrin-1 induction was modulated by active NF $\kappa$ B signaling [15]. Interestingly, for melanoma we could rule out a role of NF $\kappa$ B in Netrin-1 regulation but revealed ETS-1 as an important regulator of Netrin-1 expression. ETS-1 is known to be strongly active in malignant melanoma [19].

To unravel the potential functional role of Netrin-1 in melanoma in more detail we performed functional tests. Surprisingly, we did not observe an effect on proliferation or apoptosis what several groups did show for other kind of tumors [11,18]. This is interesting as Lee et al. could demonstrate that induction of proliferation is mediated via UNC5B, the receptor which we also found upregulated in melanoma. This could hint

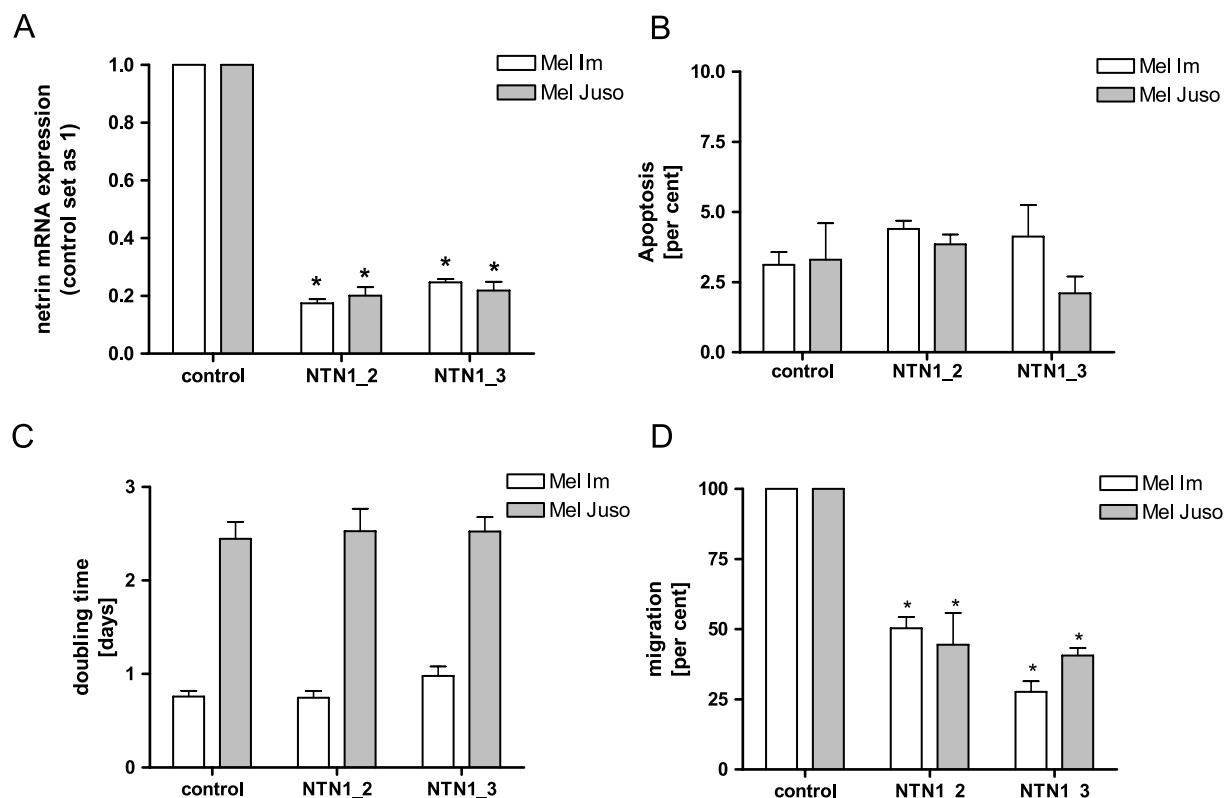


Fig. 4. Functional impact of Netrin-1 on melanoma cells. Mel Im and Mel Juso cells were transfected with two independent siRNAs against Netrin-1 mRNA (NTN1 2, NTN1 3) or control siRNA. (A) Quantitative real time RT-PCR analysis of Netrin-1 mRNA expression revealed strong suppression by both Netrin siRNAs. (B, C) Apoptosis (B) and proliferation (C) assays did not reveal significant differences comparing Netrin siRNA treated cells with control treated Mel Im or Mel Juso cells. (D) Influence of repression of Netrin-1 expression on migration of Mel Im or *Mel Juso cells* was determined in the Boyden chamber model. Here, significant reduction was observed. All experiments were repeated three times, bars display mean + SD, statistical analysis was performed using a Student's unpaired *t*-test. (\* $p < 0.05$ .)

to the fact that cell types react differently to the same signal. However, the analyzed schwannoma cells express only UNC5B so it can also be speculated that interaction of UNC5H receptors modulate the signal. In melanoma we see a strong effect on cell migration. Downregulation of Netrin-1 expression results in a drastic inhibition, whereas treatment of primary melanocytes strongly induces cell migration. It is interesting to see that also an effect on the normal counterpart of the melanoma cells can be seen. This finding suggests that receptor deregulation is not important for induction of motility and only upregulation of Netrin-1 expression is needed.

In addition, one could speculate that Netrin-1 is not only important in tumor migration but also involved in physiological processes where melanocytes need to migrate like wound healing or even in migration of melanoblasts from the neural-crest to their final destination. For the tumor, additional aspects of Netrin-1

function like induction of angiogenesis could also be supportive [16].

Still, the way migration is induced needs to be analyzed in detail as for axon guidance only DCC, which is not expressed in melanoma, was shown to have attractive function. In this study we did not see regulation of gene expression of several genes known to play a role in melanoma migration. In addition, the quick response to Netrin-1 in our functional assay hints to a direct regulatory mechanism.

Rodrigues et al. described the importance of Rho-Rho kinases axis for Netrin-1 to trigger invasion in colon carcinoma cell lines [17]. Other studies describe an effect of Netrin-1 via integrin alpha3 beta1, which is also known to be involved in melanoma progression [8]. Preliminary results present marked changes in the cytoskeletal organization after reduction of Netrin expression in melanoma cells which could be due to one or both of the described mechanisms. In addition,

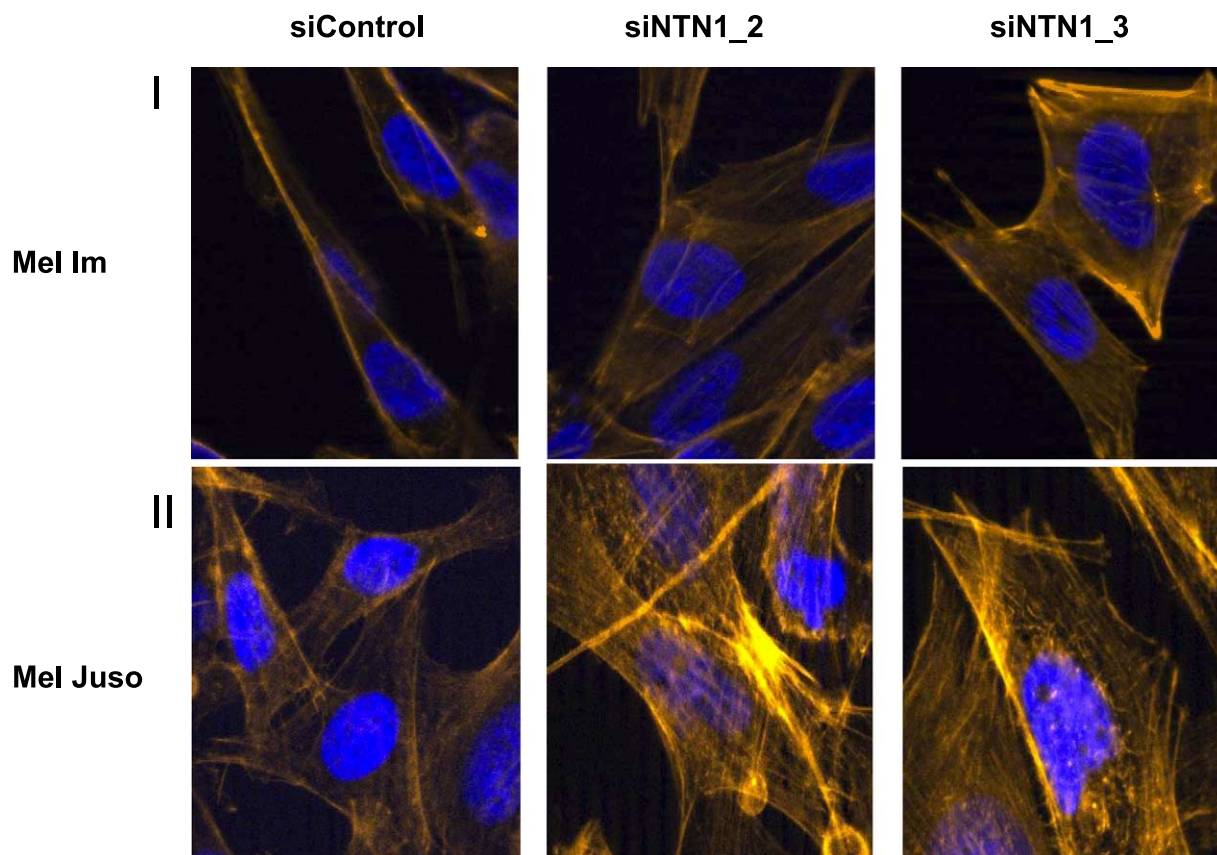


Fig. 5. Cytoskeletal changes after downregulation of Netrin-1 expression. Cells (I) Mel Im and (II) Mel Juso) were treated with siRNAs against Netrin-1 (siNTN1\_2, siNTN1\_3) or control siRNA (siControl) and the cytoskeleton was stained with Phalloidin-Rhodamine. Strong changes were observed comparing siNetrin with siControl treated cells.

our data suggest a role of Netrin-1 in regulation migration and adhesion; however, detailed studies have to prove which pathway or whether both regulatory mechanisms are involved in producing the effect of Netrin-1 on migration. As differential regulation of two receptors is found (UNC5B is upregulated and UNC5C downregulated) additionally a complex interplay between the UNC5 receptors can be expected.

In summary, we present Netrin-1 as a supportive factor of melanoma migration and invasion.

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