Analiza antiproliferativnog učinka Schwannovih stanica na stanice neuroblastoma

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Analysis of the anti-proliferative effects of the Schwann cell on neuroblastoma cells

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Graduation Thesis

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This graduation thesis was conducted in the Children´s Cancer Research Institute, Vienna, Austria, under the supervision of Assoc. Prof. Peter F. Ambros, PhD, and co-supervision of Asst. Prof. Petra Korać, PhD. The thesis was submitted for evaluation to the Department of Biology at the Faculty of Science, University of Zagreb with the aim of obtaining the title Master in Molecular Biology.

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Graduation Thesis

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Schwann cells are essential for the maturation process of benign forms of neuroblastoma, ganglioneuroma, acting via neuritogenic, anti-proliferative and pro-apoptotic signals. The goal of this master thesis was to identify Schwann cell-secreted proteins involved in the cross-talk with neuroblastoma cells, and define the concentration and combination needed to induce a growth-impairing effect in neuroblastoma cells. Eight factors were chosen based on protein array results, RNA-sequencing data and literature research: IGFBP6, FGF7, CNTF, PTN, NGF, BDNF, GDNF and EGFL8. They were functionally validated *in vitro* by cultivating two neuroblastoma cell lines derived from aggressive tumors, STA-NB-6 and STA-NB-10, in presence of corresponding recombinant proteins. The effects were measured by flow cytometry. PTN, IGFBP6 and EGFL8 were identified as Schwann cell-secreted proteins that cause reduction of cell proliferation and induction of neuronal-like differentiation of STA-NB-6 cells, when individually added to culture media for 17 days. NGF was confirmed as a neuritogenic factor of STA-NB-6 cells. The combination of all factors had no significant effect on STA-NB-6 cells. The factors had no significant effect on STA-NB-10 cells. This work forms basis for further *in vitro* and *in vivo* experiments to address the effect of these factors on neuroblastoma growth in xenograft models.

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Analiza antiproliferativnog učinka Schwannovih stanica na stanice neuroblastoma

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Schwannove su stanice neophodne za proces sazrijevanja benignih tipova neuroblastoma, ganglioneuroma, djelujući kroz neuritogenične, anti-proliferativne i pro-apoptostske signale. Cilj ovog diplomskog rada bio je identificirati proteine koje izlučuju Schwanove stanice, a koji sudjeluju u njihovoj interakciji sa stanicama neuroblastoma, te definirati koncentraciju i kombinaciju potrebnu za inhibiciju rasta stanica neuroblastoma. Usporedbom rezultata dobivenih pomoću analize proteina i podataka dobivenih sekvenciranjem transkriptoma s podacima iz literature, izabrano je osam faktora: IGFBP6, FGF7, CNTF, PTN, NGF, BDNF, GDNF i EGFL8. Za funkcionalnu potvrdu njihova djelovanja *in vitro*, odgovarajući su rekombinantni proteini dodani u medij korišten za kultiviranje dviju staničnih linija neuroblastoma porijeklom iz agresivnih tumora, STA-NB-6 i STA-NB-10, te je učinak izmjeren pomoću protočne citometrije. Ovo je istraživanje identificiralo PTN, IGFBP6 i EGFL8 kao proteine koje izlučuju Schwannove stanice, koji, kada su individualno dodani staničnom mediju tijekom 17 dana, smanjuju proliferaciju i potiču diferencijaciju STA-NB-6 stanica. NGF je potvrđen kao neuritogenični faktor STA-NB-6 stanica. Kombinacija svih navedenih faktora nije imala značajni učinak na STA-NB-6 stanice. Faktori nisu imali značajan učinak na STA-NB-10 stanice. Ovaj rad daje osnovu za daljnja *in vitro* i buduća istraživanja *in vivo,* za ispitivanje učinka navedenih faktora na rast neuroblastoma metodom staničnog eksplantata.

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List of Abbreviations

CAA: cytokine antibody array

CCRI: Children’s Cancer Research Institute

CNS: central nervous system

CoCu: co-culture

CR: complete remission

CTRL: control

DAPI: 4',6-diamidino-2-phenylindole

dmin: double minute chromosomes

DMSO: dimethyl sulfoxide

DOD: dead of disease

D-PBS: Dulbecco's phosphate-buffered saline

FACS: fluorescence activated cell sorting

FBS: fetal bovine serum

FI: fluorescence intensity

HSR: homogenously stained region

INRG: International Neuroblastoma Risk Group

INSS: International Neuroblastoma Staging System

MFI: mean fluorescence intensity

n.a.: not available

NB: neuroblastoma

NCA: numerical chromosomal abnormality

PCA: principle component analysis

PLL: poly-L-lysine hydrobromide

PNS: peripheral nervous system

PS: penicillin Streptomycin

Resp.: respectively

RT: room temperature

SC: Schwann cell

s.c.: subcutaneously

SCA: segmental chromosomal abnormality

SCEM: Schwann cell expansion media

TW: transwell

vs: versus, against

wcUPD: whole chromosome uniparental disomy

1. INTRODUCTION

1.1 Schwann cells and their role in the peripheral nervous system

The peripheral nervous system (PNS) includes the cranial nerves, spinal nerves, peripheral nerves and neuromuscular junctions. Nerve fibers (axons) of these nerves extend far from the clusters of neuron cell bodies (ganglions) to conduct information towards and away from the central nervous system (CNS). They are bound together by sheaths of connective tissue, with the endoneurium surrounding individual fibers, perineurium binding fibers into fascicles and epineurium binding fascicles into nerves [1].

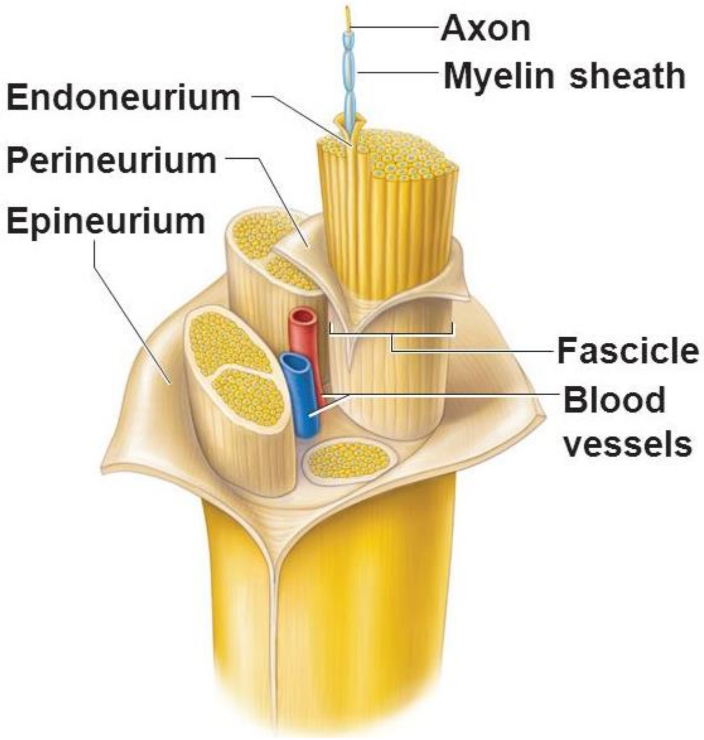


Figure 1 Structure of an adult peripheral nerve.
Nerve fibers, or axons, are surrounded with a myelin sheath and are bound together with a layer of connective tissue, called the endoneurium. Fibers are further bound into fascicles and surrounded by the perineurium. Many fascicles brought together and surrounded by the epineurium make up an adult peripheral nerve. Figure adapted from [1].

The sheath adjacent to the neuron membrane (neurilemma) is the endoneurium (Figure 1). It contains blood capillaries that provide nutrients and oxygen, as well as transport macrophages for nerve protection. The endoneurium also contains cells that are unique for the PNS, Schwann cells (SCs). The SC membrane forms a multilayered insulating lipid sheath (myelin sheath) enwrapping the axon in segments (Figure 2). Each segment belongs to a single SC and is separated by non-myelinated spots (nodes of Ranvier, Figure 2). These spots make the border between adjacent SCs and allow the signal to be propagated in a series of jumps from node to node. The myelin sheath prevents signal loss and reduces any additional metabolic requirements for neural activity [1].

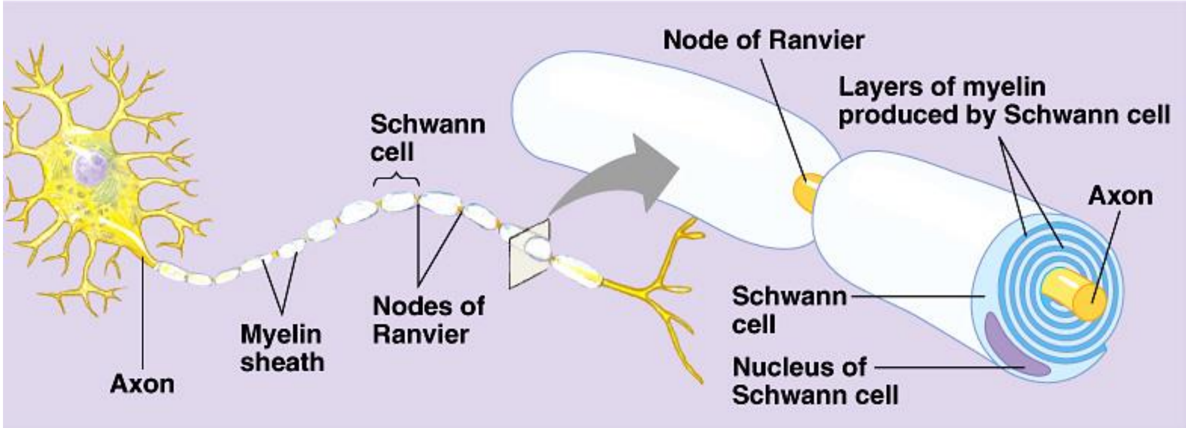


Figure 2 Schwann cells surround the axon and form the myelin sheath.
The Schwann cell membrane forms a myelin sheath which enwraps the axon in segments. Each segment belongs to a single Schwann cell and is separated by non-myelinated spots (nodes of Ranvier). These spots make the border between adjacent Schwann cells and allow a continuous propagation of the nerve signal. Figure adapted from https://shwannomawebpaper.wordpress.com/ on 11.08.2016.

First described by physiologist Theodor Schwann [2], the Schwann cells, glia of the PNS, are derived from the neural crest and migrate together with nerve cells to form peripheral nerves [3]. They have various roles in the PNS, such as being involved in nerve development and regeneration, or cleaning up of neuronal debris [4]. Furthermore, they provide trophic factors for neurons [5], modulate neuromuscular synaptic activity and can recruit antigen-presenting cells to the site of injury [6].

SC precursor cells originate from the neural crest and, along with generation of immature SCs, can develop into melanoblasts [7], parasympathetic neurons [8] and endoneurial fibroblasts, which form the connective tissue that appears in nerves [9]. Survival of SC precursor cells is essentially dependent on neuronal signals [10, 11], while immature SCs are less neuron-dependent and can rely on the secretion of their own autocrine signals [10, 11]. Further immature SC proliferation and differentiation, though, remains neuron-dependent to a great extent [12].

Immature SCs develop into myelinating or non-myelinating cells (Figure 3) that retain their phenotypic plasticity and can switch back to former developmental stages in response to nerve injury [13], which is a characteristic that contributes to the striking regenerative properties of the PNS.

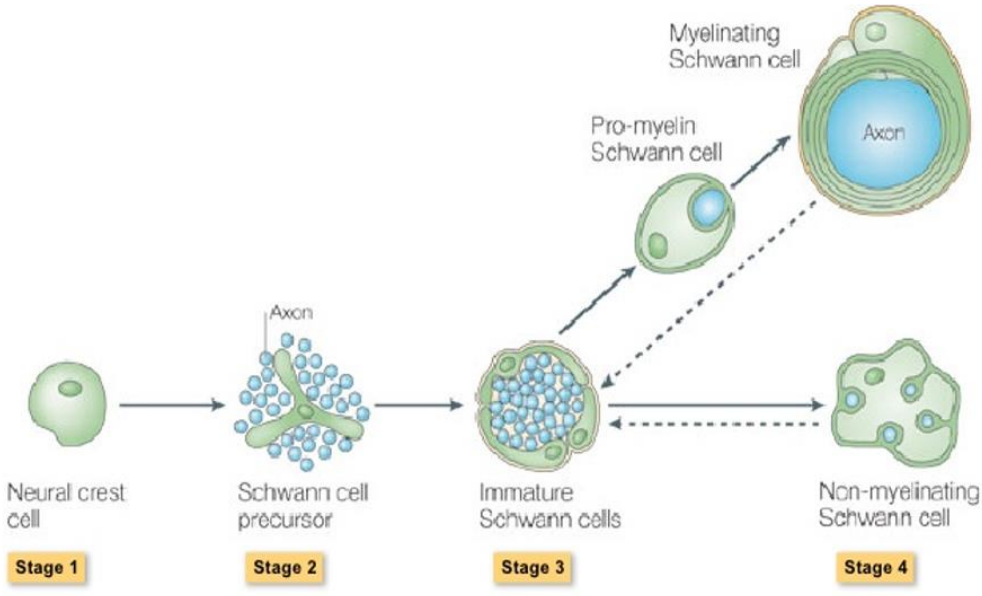


Figure 3 Four stages of Schwann cell development.
Schwann cell precursor cells originate from the neural crest and, while developing towards the Schwann cell lineage, can form myelinating or non-myelinating Schwann cells, which retain their phenotypic plasticity and can switch back to former developmental stages in response to nerve injury. Figure adapted from [14].

Myelinating SCs remain consistently dependent on neurons and, even though they have the ability to survive without axonal contact in response to nerve damage, after longer periods of no axonal contact, most of them eventually die [15]. Similarly, neurons need the presence of SCs for normal branching and target interactions in early development [12, 16], as well as their neurotrophic support for cell differentiation [17] and survival in later stages of development. The role of SCs in nerve cell differentiation makes the pronounced interdependence between these two cell types especially interesting in regards to deciphering the biology of tumors originating from the sympatho-adrenal lineage of the PNS, neuroblastomas.

1.2 Neuroblastoma

Neuroblastoma (NB) is a solid tumor originating from trunk neural crest-derived neuroblasts that most commonly affects infants and children. Tumors most frequently arise in the adrenal glands or ganglia but, in some cases, also in other sites like the abdomen or chest. The biology of NB differs greatly among different types of tumors and the characteristics are highly dependent on the age of the patient, as well as stage and genetic profile of the tumor [18]. Manifestations can range from spontaneously regressing to very aggressive metastasizing forms and several genomic alterations, which can be linked to the different patterns of clinical behavior, have so far been identified.

Tumors can be divided into 2 major types [18]:

1. Type 1 NBs are characteristic for very young patients and show numerical chromosome abnormalities (NCAs) (near triploidy) (Figure 4), without additional segmental chromosome abnormalities (SCAs) [19, 20]. SCAs in general go together with diploidy or tetraploidy [21], although can sometimes occur in near-triploid NBs, thereby affecting the prognostic impact of these tumors [20]. Type 1 NBs show high expression of the TrkA (tropomyosin receptor kinase) neurotrophin receptor and have the ability to undergo differentiation in the presence of normal SCs and its ligand NGF (nerve growth factor) in the microenvironment, or spontaneous regression (apoptosis) in the absence of NGF [22], frequently leading to a favorable clinical outcome for patients (in both cases).

2. Type 2 tumors (Figure 4) are associated with patients of older age and an unfavorable advanced tumor stage that is linked to frequent SCAs - like an unbalanced gain of chromosome arm 17q, which is a frequent event in both subtypes (2A, 2B). Subtype 2A often has additional segmental losses of chromosome arms 3p, 4p and/or 11q and gain of chromosome 7. Subtype 2B is characterized by *MYCN* amplification, frequently associated with 1p deletion [23]. Type 2 tumors also show high expression of the TrkB neurotrophin receptor and its ligand BDNF (brain-derived neurotrophic factor). Activation of the TrkB-BDNF autocrine signaling pathway leads to invasion, metastasis, angiogenesis and drug resistance [24].

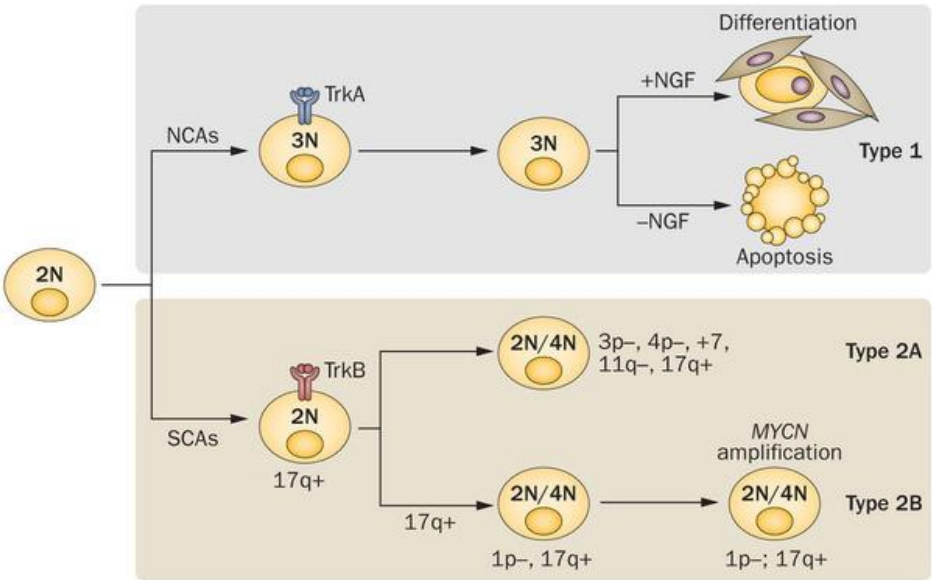


Figure 4 The major genomic alterations involved in neuroblastoma development.
Type 1 neuroblastomas are tumors with a favorable clinical outcome, which have the ability to undergo differentiation in the presence of NGF in the microenvironment, or apoptosis in its absence. Type 2 neuroblastomas are clinically unfavorable and linked to frequent SCAs, with the subtype 2B tumors being the most rapidly progressive ones. NCAs: numerical chromosomal abnormalities; SCAs: segmental chromosomal abnormalities; NGF: nerve growth factor; TrkA/B: tropomyosin receptor kinase A/B. Figure adapted from [18].

Although aggressive forms of neuroblastoma frequently relapse and lead to death of patients, NB is considered as one of the cancers with the highest probability to undergo spontaneous regression [25]. This phenomenon contributed to classification of a subset of infants currently designated as L1, L2 and MS stages according to the International Neuroblastoma Risk Group (INRG) staging system [26]. MS stage patients present small primary tumors, less than 10% of bone marrow involvement, are less than 18 months of age at diagnosis and the tumors lack *MYCN* amplification. Additionally, the tumors of these patients were shown to be mostly near triploid with whole chromosome gains [27].

Fully mature types of tumors, so-called ganglioneuroma, are composed of few ganglionic cells (differentiated neuroblasts) that are surrounded by a dense SC stroma. There are also intermediate types called ganglioneuroblastomas, in which the extent of the SC stroma varies, which is directly correlated with the level of tumor maturation, as well as to a favorable prognosis [28, 29]. The finding that the SCs in these tumors do not originate from NB cells was first described by Ambros et al. [22].

1.3 Previous research on the Schwann cell – neuroblastoma interaction in fully mature neuroblastomas

SCs originate from a pluripotent neural crest cell (Figure 5) and thus share a common progenitor with neuroblasts, which is why they were for long considered to be of neoplastic origin. This was based on two observations: first, in ganglioneuroma and ganglioneuroblastoma, SCs frequently make up the majority of the tumor mass and second, in neuroblastoma *in vitro* cell cultures, besides the neuronal-type cells, an additional fibroblast-like cell type, was found and considered to represent SCs [30, 31]. However, Ambros et al. showed that, in *MYCN* amplified NB cell lines, these fibroblast cells with flat morphology are revertants (senescent cells), and not SCs [32, 33], proving that these previous assumptions were misconceptions.

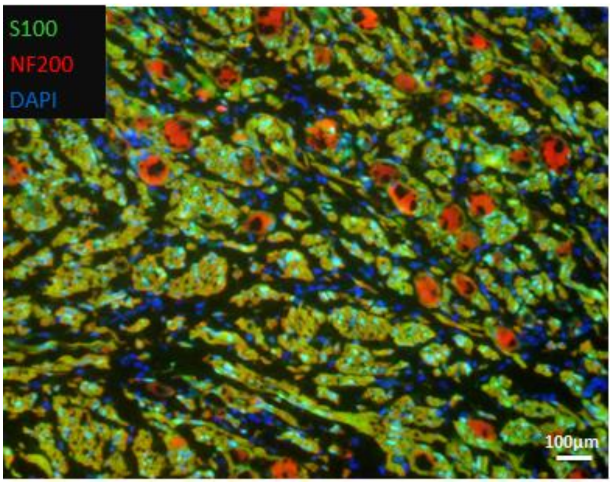


Figure 5 Immunofluoresence-stained cryosection of a fully mature neuroblastoma (ganglioneuroma). Cells positive for the differentiation marker NF200 are shown in red and cells positive for the Schwann cell marker S100 in green. Cell nuclei labeled with DAPI-stain are shown as blue. Nuclei negative for either of the

markers belong to fibroblasts or blood vessels. The predominance of stromal Schwann cells in the tumor is clearly visible. Image kindly provided by Tamara Weiss, CCRI, Vienna. DAPI: 4', 6-diamidino-2-phenylindole; CCRI: Children's Cancer Research Institute.

As the physiologic properties and functions of SCs became clearer in the early 1990s, the interest for their role in tumor maturation arose, and several groups aimed to decipher this matter. Nonetheless, this was challenging at that time, since cells in ganglioneuromas do not proliferate and conventional cytogenetic methods could not be used. Ambros et al. used *in situ* hybridization and immunohistochemical analysis on paraffin sections, together with flow cytometry, to measure DNA content and detect numerical and segmental chromosomal aberrations [22]. They demonstrated that SCs in ganglioneuromas have a diploid DNA content and a disomic *in situ* hybridization pattern, whereas the ganglionic cells showed clonal aneuploidy (e.g. trisomy) [22], suggesting that SCs in ganglioneuromas and ganglioneuroblastomas are cells of non-neoplastic origin that invade the tumor and form a specialized tumor stroma.

Based on these data, they proposed a model (Figure 6) in which undifferentiated aneuploid NB cells secrete chemotactic factors that recruit SCs. The neuron-associated mitogens provoke SC proliferation and migration, and the SCs migrate into the tumor using neuritic processes for their guidance. Further stimulated by the NB cells, once in tumor proximity, the SCs secrete neurotrophins, such as NGF, BDNF and CNTF (ciliary neurotrophic factor), that act on NB cells and inhibit NB cell proliferation, induce cell differentiation and/or apoptosis, leading to a fully mature tumor [22].

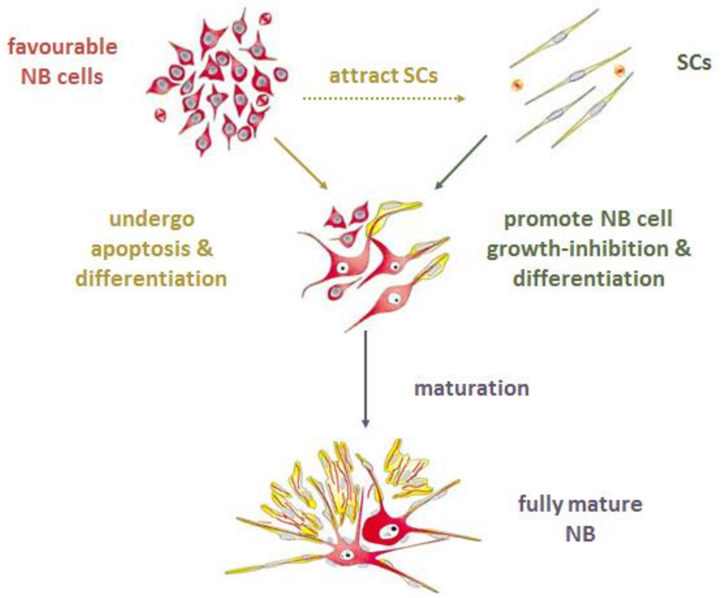


Figure 6 Proposed model of neuroblastoma maturation.
Favourable neuroblastoma cells have the ability to attract Schwann cells by secretion of Schwann cell-recruiting chemotactic factors. Once in tumor proximity, the Schwann cells secrete neuritogens that inhibit neuroblastoma cell proliferation, induce cell differentiation and/or apoptosis, in that way stimulating the formation of a fully mature neuroblastoma. Figure adapted from [22].

The proposed model is supported by the fact that aggressive *MYCN* non-amplified types of tumors can present with a SC stroma after chemotherapy [34], indicating the proposed ability of tumor cells to recruit SCs. Secondly, the SCs present in differentiating tumors can frequently be found at the outer portion of the tumor and are not intermingled with NB cells in a way that would be expected if they arose from the same cells as tumor cells [22]. Moreover, SCs occurring in fully mature NBs, ganglioneuromas, cannot be distinguished from SCs present in the PNS in their spatial organization [22]. These SCs have also been shown to have only limited morphological similarities to neoplastic SC versions that form schwannomas [35]. Another important fact is the high expression of high and low affinity NGF receptors, $p140^{trkA}$ and $p75^{NGFR}$, respectively, in aneuploid forms of NB [36, 37]. The ligand for these receptors, NGF, is essential for inducing differentiation of sympathetic neuronal cells and, since SCs are a valuable source of NGF, they could, according to the proposed model [22], trigger the maturation process in NB.

Soluble SC-secreted factors that mediate the SC-NB cross-talk and that could influence neuroblastoma maturation, have so far been investigated by several groups. SPARC (secreted protein acidic and cysteine rich) is an example of a factor that was identified as one of the SC-derived inhibitors controlling neuroblastoma tumor angiogenesis [38]. Another example is PEDF (pigment epithelium-derived factor), that was shown to induce tumor cell differentiation *in vitro*. Its recombinant form was shown to have the same effects *in vitro* and *in vivo* [39]. However, the analysis of the SC secretory profile has so far been restricted to single candidate factors and a comprehensive analysis has not yet been performed.

1.4 Preliminary data on Schwann cell - neuroblastoma *in vitro* co-cultivation

The published [35] and new preliminary data of the Tumor Biology group at the Children's Cancer Research Institute (CCRI), Vienna, on *in vitro* co-cultivation experiments of primary human peripheral nerve-derived SCs and NB cell lines with different genetic backgrounds, derived from high-risk NB patients (representing type 2 tumors, Figure 4), have confirmed a tumor-inhibiting and differentiation-inducing effect of SCs on NB cells, as examined by flow cytometry and immunocytology (manuscript in preparation). These experiments also demonstrated induction of apoptosis according to TUNEL assays, all together proving that the SC-NB interactions in the tumor can be replicated *in vitro*. Furthermore, SC-NB transwell cultures that prohibit direct cell-cell contact, while media and proteins diffuse freely through the microporous membrane of the insert, demonstrated that secreted factors are involved, since the effects were similar as with direct co-cultivations (manuscript in preparation).

1.5 Hypothesis and aim of study

Based on the hypothesis that SCs secrete factors that have an anti-proliferative and differentiation-inducing effect on NB cells, the aim of this master thesis was to investigate the mechanism of this process by identifying the factors involved, and defining the concentration and combination needed to provoke inhibition of cell proliferation and induction of cell differentiation of neuroblastoma cells.

2 MATERIALS AND METHODS

2.1 Cell lines and cell culture

Cultivation of NB cell lines was performed using αMEM complete medium (αMEM+): αMEM (Gibco, Austria) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, Austria), 25 mM HEPES buffer solution (Pan Biotech, Germany), 1 mM sodium pyruvate (Na-pyruvate, Pan Biotech, Germany) and 1% Penicillin/Streptomycin (P/S, Pan Biotech, Germany). The cell lines used and their genetic characteristics are listed in Table 1.

Table 1 Characteristics of neuroblastoma cell lines used in *in vitro* cultivation experiments.

	STA–NB–10	**STA-NB-6**	**CLB-Ma**	**IMR5**	**SHSY5Y**
INSS stage	3	3	4	n.a.	4
Patient outcome	DOD	CR	n.a.	n.a.	DOD
***MYCN* amplification**	Yes dmin	No	Yes dmin	Yes HSR	No
Ploidy	di-/tetra-ploid	aneuploid	diploid	n.a.	diploid
17q gain	Yes	Yes	Yes	n.a.	Yes
1p loss	Yes	Yes	Yes	Yes	No
Reference	[33, 40]	[33, 40]	[41]	[42]	[43]

INSS: International neuroblastoma staging system; n. a.: not available; DOD: dead of disease; CR: complete remission; dmin: double minute chromosomes; HSR homogenously staining region; wcUPD: whole chromosome uniparental disomy.

All cell lines were previously derived from tumor tissue or bone marrow samples from INSS stage 3 or 4, i.e. high-risk NB patients. Tumor tissue and bone marrow samples were provided by the St. Anna Children’s Hospital, Vienna. Informed consent was obtained for the collection and research use of human tumor tissue and bone marrow samples according to the guidelines of the Council for International Organizations of Medical Sciences and the World Health Organization and was approved by the local ethics committees of the Medical University of Vienna and the St. Anna Children’s Hospital.

The cells were stored in liquid nitrogen, or at -80 °C in an FBS solution with 10% Dimethylsulfoxid (DMSO, Roth, Germany). When needed, cells were thawed, αMEM+ was added and they were kept in a CO_2-incubator (5% CO_2) at 37 °C. Cells were sub-cultured after reaching 80% confluency. Media was removed, cells were rinsed with 1x Dulbecco's Phosphate-Buffered Saline (D-PBS, PAN Biotech, Germany) and detached by adding 1.5 mL of Accutasse (cell detachment solution, PAN Biotech, Germany) for a 75 cm^2 culture flask (T75), or 700 µL for a 25 cm^2 sized culture flask (T25), respectively, for 2-3 minutes. αMEM+ media was added in a volume of 8 mL, the cell suspension was transferred to a 15 mL tube and centrifuged at 1100 rpm for 5 minutes at 4 °C (all cell detachment, harvesting and splitting procedures mentioned throughout the text have been performed in the same manner, if not otherwise stated). The pellet was resuspended in αMEM+ medium, in a volume depending on the splitting ratio (*i.e.* for a 1:2 splitting, 1 mL of αMEM+ medium was used) and the cells were further cultured in a total volume of 12 mL media for a T75 culture flask or 7 mL media for a T25 flask, including 1/3 of conditioned media (media conditioned by the NB cell line from the previous passage) (e.g.. for a T75 flask, 8 mL of fresh and 4 mL of conditioned media was combined).

2.2 Isolation of human Schwann cells from peripheral nerve tissue and their co-cultivation with the STA-NB-6 cell line

2.2.1 Isolation of nerve fascicles

Peripheral nerve tissue samples were provided by the Department of Orthopedic Surgery or the Department of Plastic and Reconstructive Surgery at the Vienna General Hospital. Informed consent was obtained for the collection and research use of human peripheral nerve tissues according to the guidelines of the Council for International Organizations of Medical Sciences and the World Health Organization and was approved by the local ethics committees of the Medical University of Vienna and the St. Anna Children's Hospital. Human peripheral nerves were collected during reconstructive surgery, amputations or organ donations of male and female patients between 16 and 70 years of age. Nerves were stored in sterile saline for 1–3 h until further processing.

The peripheral nerve was washed one time with cold 1x D-PBS, transferred into an autoclaved glass dish and covered with αMEM+ medium (Figure 7a). The nerve was cut with the use of a lancet into pieces of around 3 cm in length and any extra connective or adipose tissue was thereby removed. Nerve fascicles were pulled out of the epineurium using forceps and separated from the remaining tissue into a different autoclaved glass dish containing αMEM+ medium (Figure 7b). The fascicles were further cut into 5 mm pieces. The isolated cut fascicles were equally distributed into wells of a 6-well plate (Figure 7c) and incubated overnight in 2 mL of digestion solution (αMEM+, 0.125% collagenase Type IV (GIBCO), 1.25 U/ml Dispase II (SIGMA) and 3 mM CaCl) at 37 °C [44].

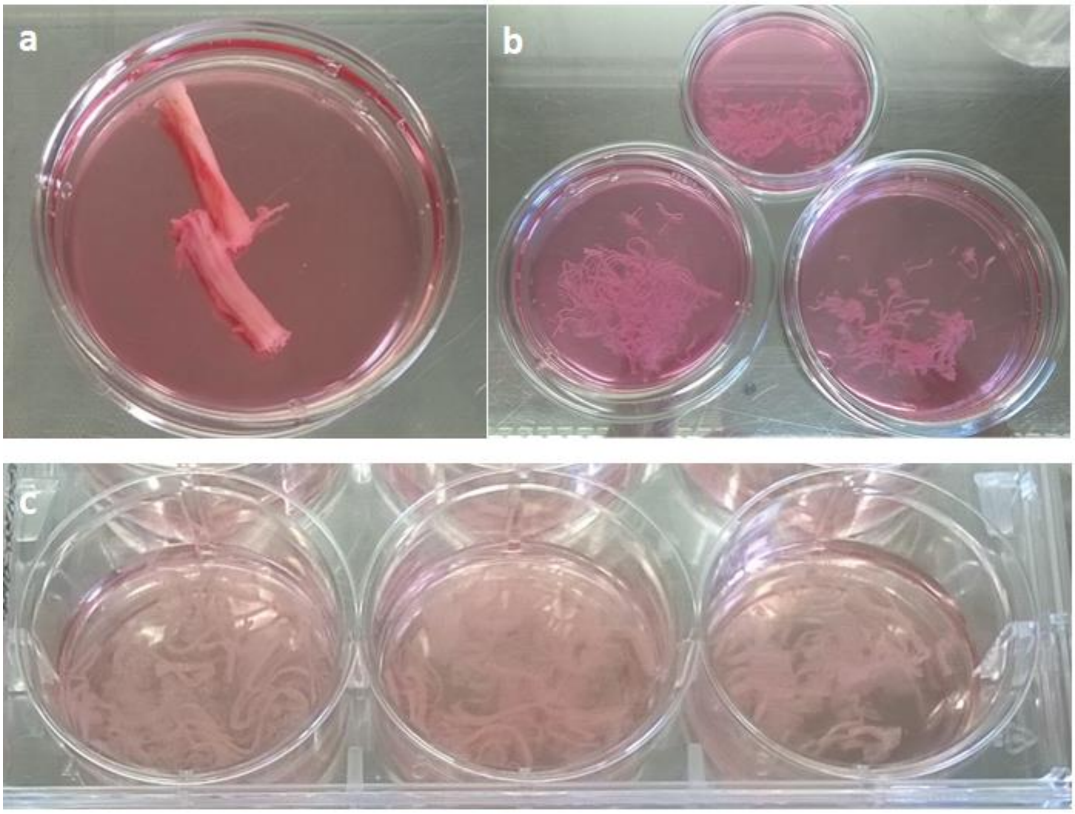


Figure 7 Schwann cell isolation procedure.
a) Peripheral nerve tissue covered with αMEM+; b) pulled nerve fascicles covered with αMEM+; c) nerve fascicles in 2 mL digestion solution.

2.2.2 Coating of culture dishes

Surfaces of culture dishes were coated with 0.01% Poly-L-lysine Hydrobromide solution (PLL, Sigma-Aldrich, Austria), which was added in a volume of 1 mL to each well of a 6-well plate and distributed equally. The plate was shaken every minute to maintain the equal distribution of the solution. After 10 min, the wells were gently washed two times with ddH_20 and the plate was left to dry for 2 h at room temperature (RT) [44]. The plate was then incubated at 37 °C with 1 mL of Laminin solution (6 µg/mL in 1x D-PBS, Engelbreth-Holm-Swarm murine sarcoma, Sigma-Aldrich, Austria) and washed the following day two times with 1x D-PBS, after which 1 mL of Schwann cell expansion media (SCEM; MEMα, 1% P/S, 2% FBS, 1 mM sodium pyruvate, 25 mM HEPES, 10 ng/ml human FGF basic, 10 ng/ml human Heregulinβ1, 5 ng/ml human PDGF-AA (all PeproTech), 0.5% N2 supplement (GIBCO) and 2 µM forskolin (SIGMA) [44]) was added to each well. The same procedure was used for coating of transwell inserts.

2.2.3 Schwann cell seeding

After overnight incubation, residual tissue was mechanically resuspended with a pipette to ensure homogeneity of the solution. After the addition of αMEM+, digested tissue was transferred into

15 mL tubes and centrifuged at 1000 rpm, RT. The pellet was resuspended in 1 mL SCEM and transferred to coated wells that already contained 1 mL of SCEM. Electrostatic charge was reduced by tapping the 6-well plate on a paper tissue soaked with Mikrozid (Mikrozid Liquid, Schülke & Mayr, UK). The cells were cultured for 5-7 days (Figure 8) and half of the media was changed every 2-3 days [44].

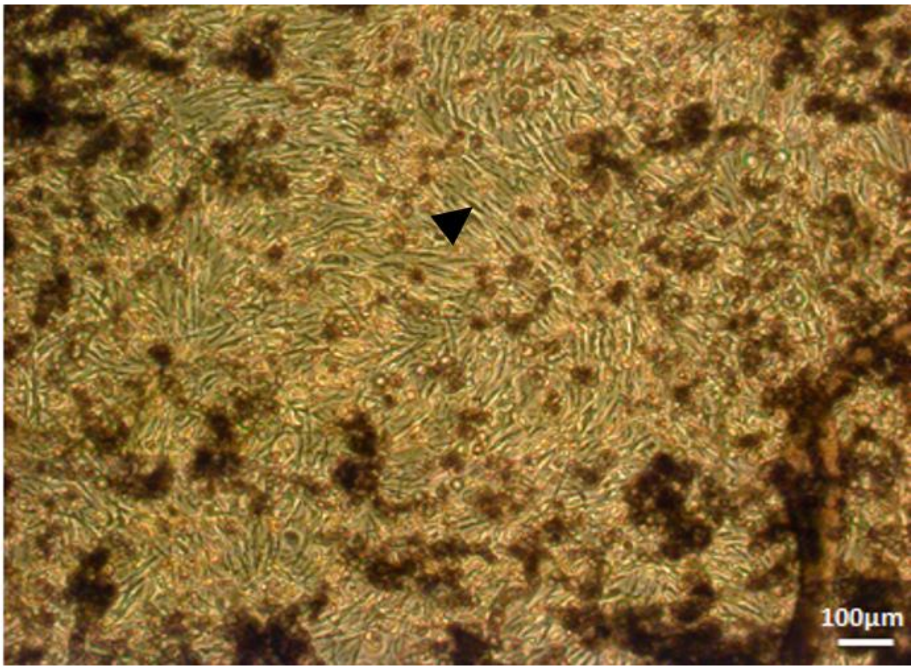


Figure 8 Schwann cells growing on a coated well 5 days after seeding.
A Schwann cell is indicated with the black arrow.

2.2.4 Schwann cell purification procedure

After 7 days in culture, SCs were purified from fibroblasts by addition of ice cold Accutasse for 2-3 minutes [44]. Cells were gently washed off with a pipette, collected into 15 mL tubes and centrifuged at 900 rpm for 5 minutes at 4 °C. The pellet was resuspended in 1 mL αMEM+ and 500 µL of the suspension was transferred to 2 wells of an uncoated 6-well plate that already contained 2 mL of αMEM+ media each. Electrostatic charge was reduced as mentioned previously. The plate was incubated for 30 minutes at 37 °C [44], allowing the fibroblasts to attach. The supernatant that mostly contained SCs in suspension was further centrifuged at 900 rpm for 5 min [44], cells were counted and 100 000 cells/well were seeded in duplicates in a fresh plate coated as described previously, serving either as Schwann cell control (Figure 9) or for co-cultivation with the STA-NB-6 cell line.

The SCs were seeded in media consisting of conditioned SCEM and freshly prepared SCEM at a ratio of 1:1 [44]. After 1.5 days, half of the media was changed to αMEM+. On day 3 after seeding, the entire media was changed to αMEM+ and 140 000 cells of STA-NB-6 cells were added for co-cultivation experiments.

Fibroblasts that remained in the uncoated plate after the purification procedure were kept and cells from one well were transferred to a coated well. They were cultivated in αMEM+ and half of the media was changed every 3-4 days.

SCs and fibroblasts, as well as all cells from co-cultivation and transwell cultures, and all STA-NB-6 cells mentioned in the following paragraphs, were cultivated for a maximum of 17 days.

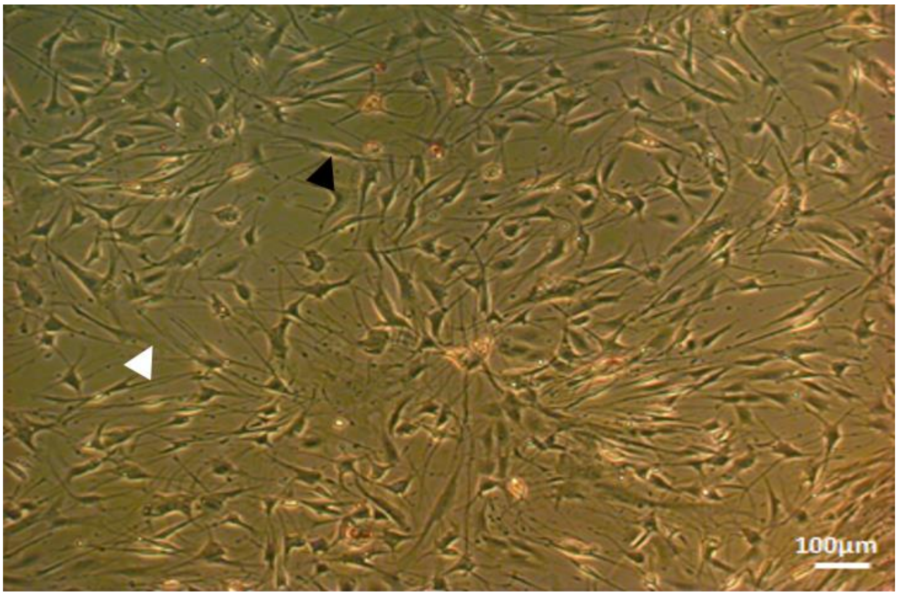


Figure 9 Purified Schwann cells serving as control in αMEM+, 7 days after the purification procedure. Fibroblasts are visible as flat, substrate adherent cells, indicated with the white arrow, between mostly di- or tri-polar Schwann cells, indicated with the black arrow.

2.2.5 Direct Schwann cell/STA-NB-6 co-cultivation

STA-NB-6 cells, used for co-cultivation experiments, were not sub-cultured for at least 3-4 days before the start of the experiment to ensure that they reach a high cell number. The media was changed 2 days before. Cells were maximally 80% confluent when harvested, and 140 000 cells were added to wells (Figure 10) already containing SCs in a drop-wise manner, thereby not disturbing cells growing at the bottom of the well. Cells were carefully distributed throughout the well by gentle shaking and electrostatic charge was reduced as described above. Half of the media was changed every 3-4 days. Furthermore, 300 000 STA-NB-6 cells from the same flask were seeded on a coated well as control cells. Cells were sub-cultured at a ratio of 1:2 when necessary, and half of the media was changed every 3-4 days.

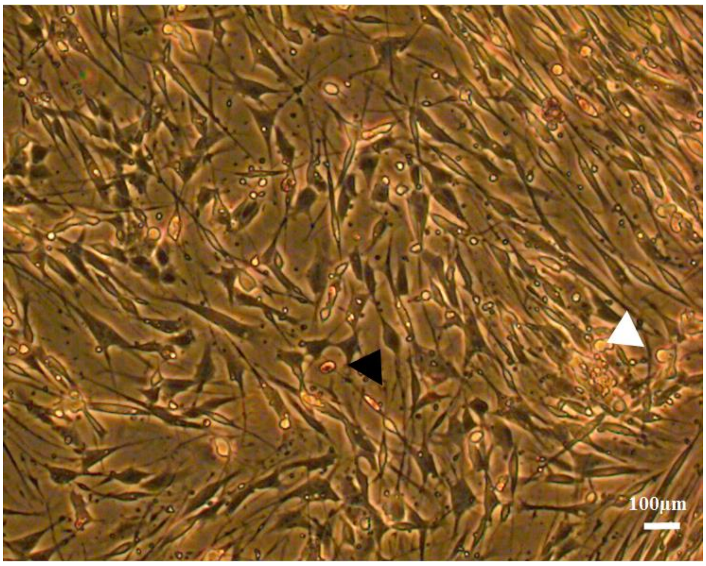


Figure 10 Schwann cells and STA NB 6 cells in αMEM+, 5 days after co-cultivation.
The black arrow indicates Schwann cells, the white arrow indicates neuroblastoma cells.

2.2.6 Transwell Schwann cell/STA-NB-6 culture

SCs were seeded in duplicates, 100 000 cells/well. STA-NB-6 cells were seeded in duplicates on the coated surfaces of inserts (Transwell Permeable Supports, 24 mm Inserts/0.4 µm Polyester Membrane, Corning Inc.), in the same number as for the co-cultivation experiments (i.e. 140 000 cells/well). The transwell (TW) inserts were then transferred on top of the wells, while ensuring there is enough media underneath (around 3 mL) and inside the insert (around 800 µl), so that constant exchange of media is allowed (Figure 11). Additionally, 300 000 STA-NB-6 cells were seeded in duplicates to serve as transwell controls. Half of the media was changed every 3-4 days, very carefully in order to avoid the STA-NB-6 cells geting transferred to the bottom well containing SCs. For this reason, it was important to change pipette tips between changing medium of the well and the insert, and to gently transfer the insert to a separate plate with the use of forceps while sub-culturing STA-NB-6 cells.

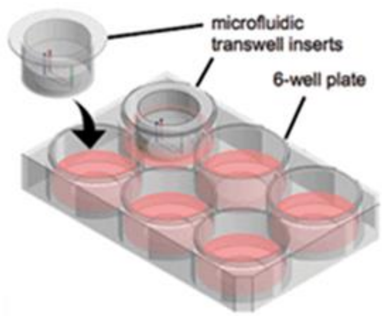


Figure 11 A scheme of a 6-well plate and a transwell insert.
The microfluidic transwell inserts contain micropores on the bottom for ensuring constant media flow between the insert and the well, while prohibiting direct cell-cell contact. Figure adapted from [45].

2.3 Collection of culture supernatants

Culture supernatants from co-cultivation and transwell experiments, as well as from corresponding SCs and STA-NB-6 controls and fibroblast cultures, were collected and centrifuged at 1200 rpm for 10 minutes at 4 °C. The supernatants were collected into labelled protein low-bind tubes (Protein LoBind Microcentrifuge Tubes, 1.5 mL, Eppendorf), snap-frozen and stored at -80 °C.

2.4 Identification of Schwann cell-secreted factors in co-cultivation and transwell supernatants

2.4.1 Cytokine Antibody Arrays

Supernatants from 2 previous independent co-cultivation and transwell experiments with 5 different NB cell lines (Figure 12, Sample), as well as from corresponding controls, were pooled, respectively, and analyzed for the presence of various cytokines, chemokines, growth factors and proteases (which will from now on be referred to as ´factors´ for simplicity) with cytokine antibody arrays (CAA or protein arrays, RayBio Human Cytokine Antibody Array G-Series 4000, RayBiotech Inc., USA). Each of the 5 arrays used, contained antibodies for 55 different factors printed on their glass surface, so, in total, 275 factors could be detected.

It is important to note that all reagents and samples were added only to wells that contain printed antibodies on their glass surface (Figure 12) and that all steps that include aspiration were carried out with extreme care touching only the corners of the well with a pipette tip. After the protein arrays were equilibrated to RT, 100 µL of 1x blocking buffer was added into each well and incubated for 30 minutes at RT. The blocking buffer was decanted and the remaining liquid was aspirated, after which 100 µL/well of undiluted sample (culture supernatant) was added. The chambers were covered with adhesive film and incubated for 2 h at RT. The adhesive film was removed and the samples were aspirated. The wells were washed 3 times for 2 min with 150 µL of 1x wash buffer I. After the last washing step, glass slides were placed into a container, submerged in wash buffer I and washed with gentle rocking two times for 10 min. This step was repeated with 1x wash buffer II. After the buffer was decanted and aspirated, 70 µL of streptavidin fluor was added to each well, the chambers were covered with adhesive film and aluminum foil on top and incubated for 2 h at RT while being gently shaken. The streptavidin fluor was removed and the two washing steps with wash buffers I and II, respectively, were repeated in the same manner as described above. The glass slides were then removed from the frame assemblies, placed in 30 mL centrifuge tubes and washed while being gently shaken, two times with wash buffer I for 10 minutes, afterwards one time with wash buffer II for 3 minutes. The glass chips were then removed from tubes, vigorously rinsed with de-ionized water and dried for 20 minutes in a laminar flow hood protected from light. A simplified version of the protocol is shown on Figure 13.

Sample Nr	Sample
1	SC CTRL
2	SC / NB6 CoCu
3	SC / NB6 TW
4	α-MEM+ CTRL
5	NB6 TW CTRL
6	NB6 CTRL
7	SC CTRL
8	SC / CLBMa CoCu
9	SC / CLBMa TW
10	CLBMa TW CTRL
11	CLBMa CTRL
12	SC CTRL
13	SC / IMR5 CoCu
14	SC / IMR5 TW
15	IMR5 TW CTRL
16	IMR5 CTRL
17	SC CTRL
18	SC / SHSY5Y CoCu
19	SC / SHSY5Y TW
20	SHSY5Y TW CTRL
21	SHSY5Y CTRL
22	SC CTRL
23	SC / NB10 CoCu
24	NB10 CTRL

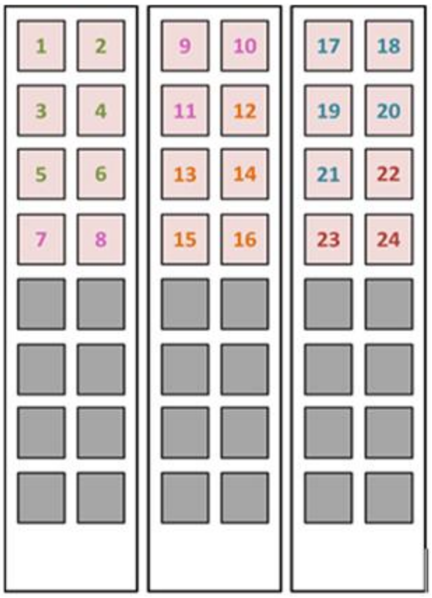


Figure 12 Scheme of samples and their distribution on protein arrays.
The 3 subarrays, shown right, contain the same antibodies for 55 factors printed on their glass surface. Supernatants of the 24 samples, shown left, were added to wells of the arrays and were tested for the presence of these 55 factors. This procedure was performed 5 times, with 5 different arrays containing antibodies for different factors, so, in total, 275 different factors could be detected. The cell lines used and their characteristics are listed in Table 1. CTRL: control; CoCu: co-cultivation; TW: transwell; NB6: STA-NB-6; NB10: STA-NB-10.

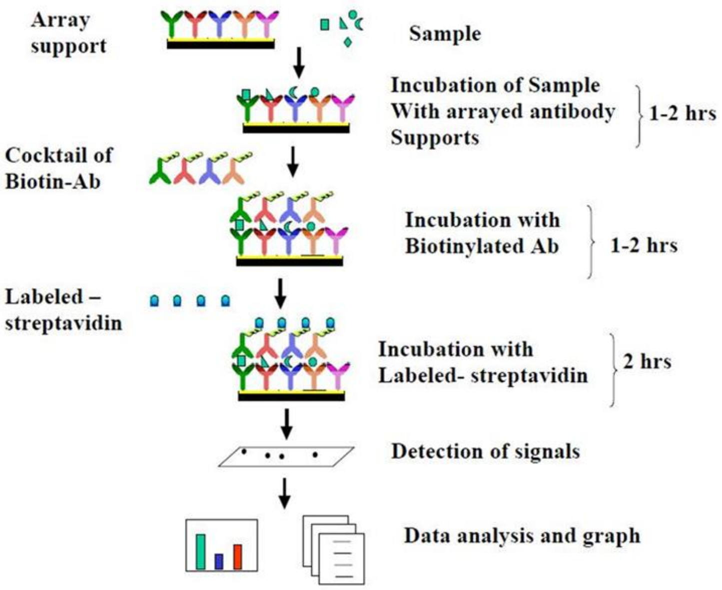


Figure 13 Simplified scheme of the protein array protocol.
After the addition of samples, the arrays are incubated with a cocktail of biotinylated antibodies, followed by fluorescently labeled-streptavidin. The signals are detected with a laser scanner. Figure adapted from RayBiotech CAA User Manual.

2.4.2 Measurement and data analysis

Fluorescence signals were obtained with the GenePix 4000 array scanner (Molecular Devices) using the green channel (Cy3) at an excitation frequency of 532 nm and 700 PMT. The image files generated in this way were aligned to respective .gal files (RayBiotech) and Gene Pix Pro 7 (Molecular Devices) was used to create .gpr files. Each spot was manually inspected on the .gpr file images to ensure accuracy. After background correction and normalization to the internal control, averages of logarithmic (log2) fluorescence intensity (FI) values of direct (CoCu) and indirect (TW) experiments were calculated for each cell line. The mean fluorescence intensity (MFI) ratio of these experiments when compared to CTRLs was calculated. The MFI values were combined for all cell lines and proteins that had an at least 2-fold increase in secretion in CoCu/TW experiments as compared to controls, together with a $p \leq 0.05$ value, were selected for visualization. Data was uploaded into the Qlucore Omics Explorer V3.1 software to generate Principle Component Analysis (PCA) plots and heatmaps.

2.5 Fluorescence activated cell-sorting of co-cultivated cells

2.5.1 Fluorescence-activated cell sorting (FACS)

Replicates from co-cultivation and transwell experiments, as well as their corresponding controls, were harvested and pooled, and the pellet was resuspended in 250 µL FACS buffer (0.1% FBS, 0.05% Na-azide in 1x D-PBS), or in 400 µL for samples nr. 2 and 6 (Table 2).

Table 2 Samples used for FACS-sorting and the corresponding antibodies used. Antibody specifications are listed in the text.

SAMPLE	Antibody
1 SC CTRL (αMEM+)	**GD2-A546 / p75NTR A647**
2 SC/STA NB 6 CoCu	**GD2-A546 / p75NTR A647**
3 SC TW	**GD2-A546 / p75NTR A647**
4 STA NB 6 TW	**GD2-A546**
5 STA NB 6 TW CTRL	**GD2-A546**
6 STA NB 6 CTRL	**GD2-A546**
7 Fibroblasts on coated surfaces	**GD2-A546**
8 Fibroblasts without coating	**GD2-A546**

CTRL: control; SC: Schwann cell; CoCu: co-cultivation; TW: transwell.

Cells were transferred into FACS tubes and the following antibodies were added: 4 µL of p75NTR-A647 (rabbit anti human, Cell Signalling, USA; labelled with AlexaFluor647 [A20173], ThermoFisher Scientific, USA; diluted 1:60 in FACS buffer) and 5 µL of GD2-A546 (humanized chinese hamster, POLYMUN GmbH, Austria; labelled with AlexaFluor546 [A10237], ThermoFisher Scientific, USA; diluted 1:10 in FACS buffer).

The samples were incubated in the dark for 30 minutes at 4 °C, washed one time with 1 mL of FACS buffer and centrifuged for 5 minutes at 1200 rpm and 4 °C (all washing steps for FACS procedures were performed in this manner). The pellet was resuspended in 200 µL FACS buffer and 8 µL of 2µg/mL 4', 6-Diamidino-2-Phenylindole (DAPI) solution in 1x D-PBS was added. Cells were filtered through a cell strainer (BD) to remove clumps and sorted at FACS Aria (BD) in tubes containing 1.5 mL FBS. Sorted cells were then centrifuged at 1200 rpm for 5 minutes at 4 °C, the pellet was resuspended in 1 mL 1x D-PBS and transferred to a RNA-free tube (RNAse free, Eppendorfer). After another centrifugation step, the pellet was finally resuspended in 700 µL of Qiazol (Qiazol Lysis Reagent, Qiagen, Germany) and stored at -80 °C.

2.5.2 Gene expression analysis

Analysis of already existing RNA sequencing (RNAseq) data was performed using a custom RNA-sequencing pipeline built with Anduril [46] and with Qlucore Omics Explorer V3.1 software. Differentially expressed genes for the dataset were selected as $|logFC| > 2$ and $q \leq 0.05$.

2.6 Cultivation of STA-NB-6 and STA-NB-10 NB cell lines in the presence of recombinant proteins identified as Schwann cell-secreted factors

Table 3 Initial concentrations of the recombinant proteins analogous to Schwann cell-secreted factors selected for addition to STA-NB-6 and STA-NB-10 cell lines.

FACTOR	SOLVENT	CONCENTRATION (ng/mL)	PROVIDER
IGFBP6	dH2O/0.1 % BSA/PBS	100	Peprotech
BDNF	dH2O/0.1 % BSA/PBS	40	Peprotech
CNTF	5mM Na3PO4, pH 7.5/0.1 % BSA/PBS	10	Peprotech
GDNF	dH2O/0.1 % BSA/PBS	30	Peprotech
β-NGF	dH2O/0.1 % BSA/PBS	20	Peprotech
PTN	dH2O/0.1 % BSA/PBS	50	Peprotech
FGF7	dH2O/0.1 % BSA/PBS	10	Miltenyi Biotec
EGFL8	50 mM Tris HCL, 10 mM reduced glutathione	100	Abnova

STA-NB-10 and STA-NB-6 cell lines were seeded at a density of 250 000 cells/well and in 2 mL of α-MEM+. Recombinant proteins were added to the media the following day, at concentrations (Table 3) that were chosen according to literature data. Each protein was added as a single factor, and a combination of all proteins was also tested with all the same concentrations as for single factors. The cells were sub-cultured when required, and/or 2/3 of media supplemented with recombinant factors was replenished every 3-4 days. All cell lines were cultivated in this manner until day 8 or day 17 after the start of the experiment, with the two time-points according to the procedure used for SC-NB co-cultivation and TW experiments (manuscript in preparation).

2.6.1 Cell proliferation rate measurement of STA-NB-6 and STA-NB-10 cell lines by flow cytometry

Cells were incubated with 1 μM EdU (Life Technologies, USA) for 14 h and were harvested, washed 1x in αMEM+ media, then resuspended in 100 μL of FACS buffer. The cell solution was split and 50 μL was used for the proliferation assay, while the remaining 50 μL for the differentiation assay. After centrifugation at 1200 rpm for 5 min at 4 °C (all centrifugation and washing steps for the FACS procedure were performed in this manner), 200 μL Roti-Histofix (acid free, pH 7, phosphate-buffered formaldehyde solution 4 %, Roth, Germany) was added and cells were fixed for 20 minutes at RT, protected from light. They were afterwards washed in 1 mL 1x D-PBS / 1 % FBS and the pellet was resuspended in 100 μL of 1x EdU Perm/Wash Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific, USA) for permeabilization. Cells were washed with 1 mL EdU Perm/Wash and resuspended in 100 μL of Click-it reaction cocktail (Click-it, Life Technologies, USA) and incubated for 30 minutes at RT.

After a washing step with 1 mL of the EdU Perm/Wash, cells were again resuspended in 50 μL of the same buffer and 3 μL of the GD2-A546 antibody (humanized chinese hamster, POLYMUN GmbH, Austria; labelled with AlexaFluor546 [A10237], ThermoFisher Scientific, USA; diluted 1:100 in FACS buffer) was added and incubated for 30 min at 4 °C. Samples were washed with 1 mL of the EdU Perm/Wash, cells were resuspended in 100 μL of the EdU Perm/Wash and 1 μL of FxCycle Violet stain (Thermo Fisher Scientific, USA) was added and incubated for 20 minutes at 4 °C, protected from light. Samples were analyzed immediately without washing with the flow cytometer (Fortessa, Beckton Dickinson, BD Biosciences, Austria).

2.6.2 Cell diferentiation measurement of STA-NB-6 and STA-NB-10 cell lines by flow cytometry

For measurement of cell differentiation, 5 µL of the GD2-A546 antibody (specifications as described previously) was added to 50 µL of cell suspension and incubated for 20 minutes at 4 °C, protected from light. Cells were then washed with 1 mL of FACS buffer, the pellet was resuspended in 100 µL of Cytofix/Cytoperm solution (Fixation/Permeabilization kit, BD Biosciences,Austria) and incubated for 20 minutes at 4 °C, protected from light. Samples were then washed with BD Perm/Wash buffer (diluted 1:10 with ddH_2O), resuspended in 50 µL of the BD Perm/Wash and 5 µL of the NF200-A647 antibody (mouse anti human, Millipore, USA; labelled with AlexaFluor647 [A20173], ThermoFisher Scientific, USA; diluted 1:40 in FACS buffer) was added and incubated for 20 minutes at 4 °C, in the dark. Cells were finally washed one time with 1 mL of the BD Perm/Wash, resuspended in 100 µL Perm/Wash and analyzed with the flow cytometer (Fortessa, BD, Austria).

2.7 Isolation of mouse Schwann cells from peripheral nerve tissue (*Nervus ishiadicus*)

Animal studies have been approved by the Medical University of Vienna institutional review board for animal ethics (GZ 66.009/0274-II/3b/2010). Mice peripheral nerve tissue samples (*Nervus ishiadicus*) were provided by the Anna Spiegel Scientific Institution of the Medical University of Vienna. The SC isolation procedure was performed in the same manner as described for human peripheral nerve tissue. Since mice nerves are significantly smaller than human ones (Figure 14a), there was no need for cutting with a lancet, so the fascicles were pulled out directly from the fibers with the use of forceps, and cleaned from the remaining tissue, as much as it was possible considering the size. The isolated fascicles (Figure 14b) were equally distributed into 3 wells and incubated for 3h in 2 mL of digestion solution (αMEM+, 0.0625% collagenase Type IV (GIBCO), 0.625 U/ml Dispase II (SIGMA) and 3 mM CaCl) at 37 °C.

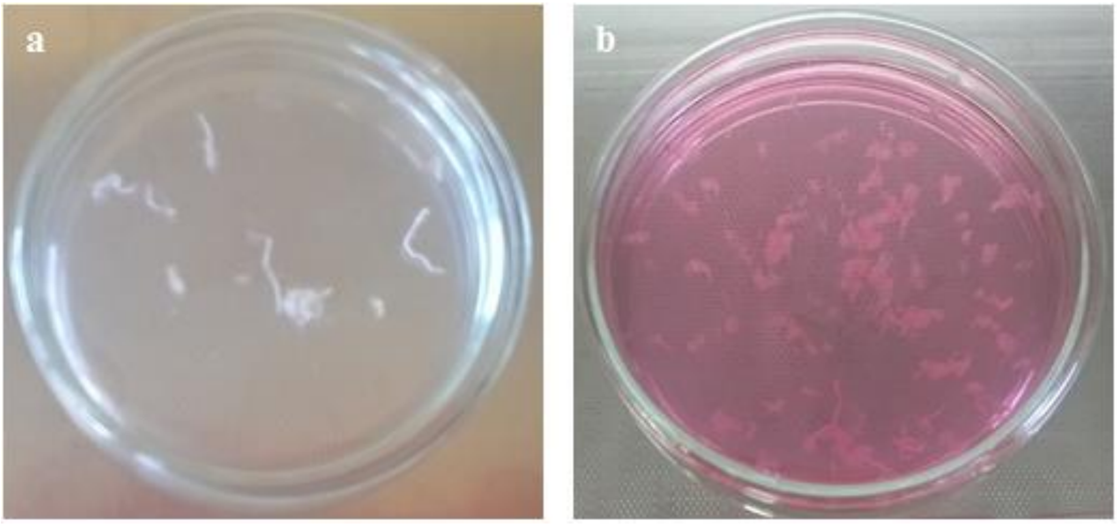


Figure 14 Mouse Schwann cell isolation.
a) isolated nerves in D-PBS; b) nerve fascicles covered with αMEM.

After 3 h, the tissue was mechanically resuspended with a pipette tip and the solution was transferred into 15 mL tubes. The wells were rinsed with αMEM+ which was added to tubes, together with 8 mL of fresh media. The tissue was centrifuged as stated for human SCs, and seeded on coated wells, in 2 mL SCEM. The cells were cultured for 3 weeks in SCEM. Due to the large amount of fibroblasts present, the purification procedure needed to be performed once a week and half of the media was changed every 3-4 days (Figure 15).

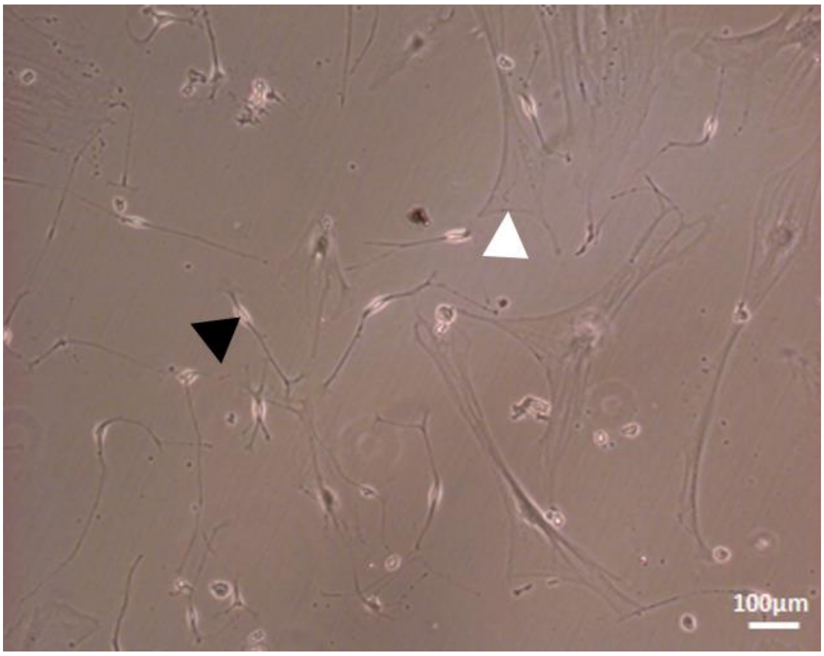


Figure 15 Mouse Schwann cells growing in Schwann cell expansion media after two purification procedures. A lot of fibroblasts are still visible, as bigger flat cells, even after two purification rounds, due to the challenging isolation procedure. Fibroblasts are indicated with the white arrow, while the Schwann cells with a black one.

Considering the low amount of mouse SCs that could be obtained by this procedure, all of the mouse SCs were used for co-cultivation with the STA-NB-6 cell line. They were seeded on one coated well. Half of the media was changed to αMEM+ 1.5 days after seeding and 50 000 STA-NB-6 cells were added 3 days after seeding, with complete change of media to αMEM+. For the STA-NB-6 control, 200 000 cells were seeded on a coated well in αMEM+. Half of the media was changed every 3-4 days for both the co-cultivation culture and the control, and the cells were cultivated for 17 days.

2.7.1 Cell proliferation rate measurement of the mouse Schwann cell/STA-NB-6 co-cultivation by flow cytometry

Cell proliferation of the mouse SC/STA-NB-6 co-cultivation was measured on day 17 after the start of the experiment, with the time-point chosen according to previous human SC co-cultivation experiments with NB cell lines (manuscript in preparation). The entire procedure was performed in the same manner as described for the STA-NB-6 and STA-NB-10 cell lines, up to the point after the addition of the Click-it reaction cocktail, after which samples were resuspended in 50 µL of the EdU Perm/Wash buffer with the addition of the following antibodies: 1µL S100-FITC (rabbit anti-mouse; DAKO, Austria; labelled with FITC [Z25342], ThermoFisher Scientific, USA; diluted 1:50 in FACS buffer); 3 µL GD2-A546 antibody (hamster anti-mouse, POLYMUN GmbH, Austria; labelled with AlexaFluor546 [A10237], ThermoFisher Scientific, USA; diluted 1:100 in FACS buffer); 2.5 µL Vimentin (rabbit anti-mouse, DAKO, Austria; diluted 1:10 in FACS buffer).

Samples were incubated for 30 minutes at 4 °C, after which they were washed with 1 mL of EdU Perm/Wash and resuspended in 50 µL of the same buffer with the addition of 5 µL of Gt-anti-Ms-A594 antibody (goat anti mouse, Li-Cor Biosciences, USA; labelled with AlexaFluor594 [A10239], ThermoFisher Scientific, USA; diluted 1:1000 in FACS buffer). After 20 minutes of incubation at 4 °C, samples were washed with EdU Perm/Wash and resuspended in 100 µL of buffer. The FxCycle Violet stain was added in a volume of 1 µL and the samples were incubated for 20 minutes at 4 °C before measurement with the flow cytometer (Fortessa, BD, Austria).

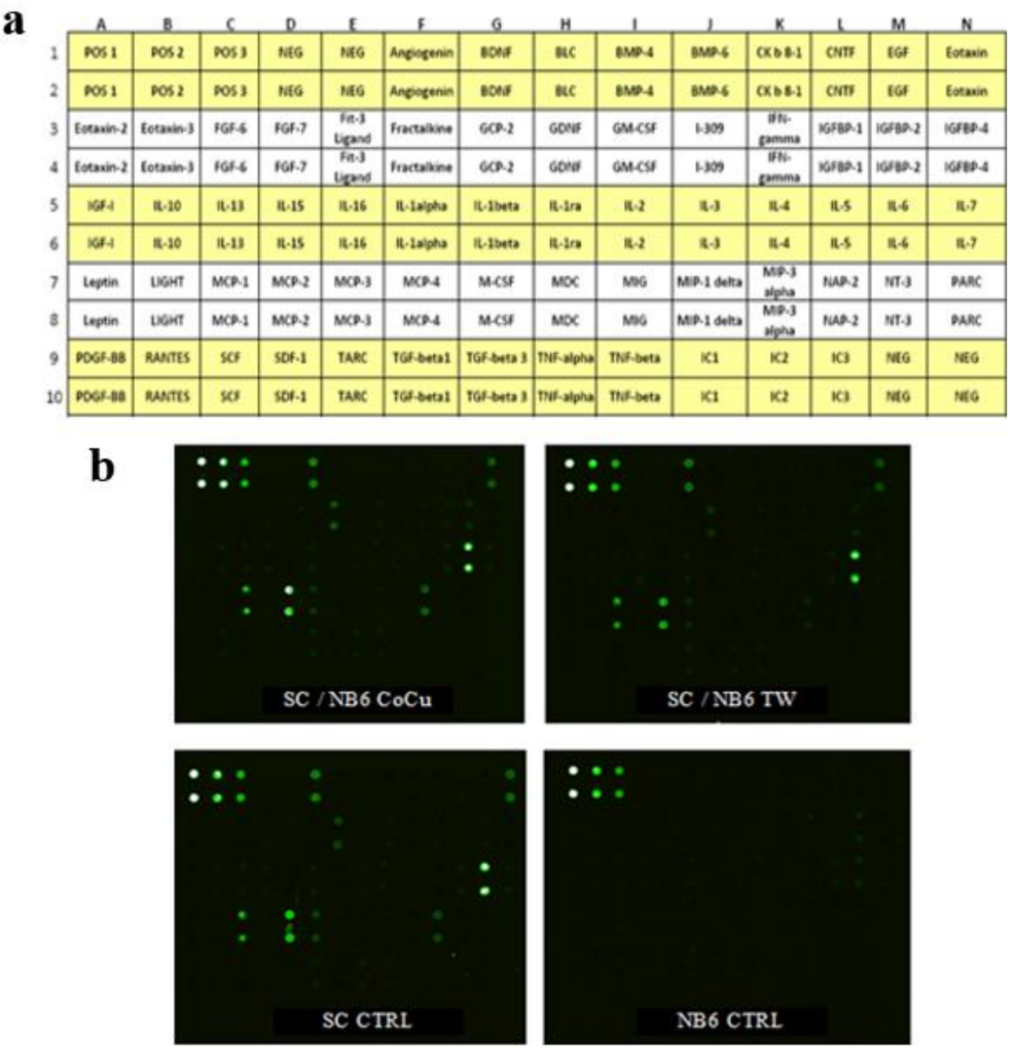
3 RESULTS

3.1 Identification of Schwann cell-secreted factors in control, co-cultivation and transwell supernatants

The identification of factors that are involved in the SC-NB crosstalk was done by a comprehensive analysis of the SC secretome, starting with the identification of proteins present in cell culture supernatants by protein arrays.

3.1.1 Protein arrays identified 49 factors with an increase of secretion in co-cultivation/transwell experiments when compared to controls

For the analysis of the secretome of SCs in direct (CoCu) or indirect (TW) contact with NB cells, when compared to the secretome of SCs and NB cells alone (controls), supernatants from 2 independent co-cultivation and transwell experiments, respectively, with 5 different NB cell lines, as well as from corresponding controls, were pooled and analyzed by protein arrays. After fluorescence signal visualization (Figure 16b), background subtraction and data normalization, 49 factors were identified (Figure 17) with an at least 2-fold increased secretion in CoCu/TW experiments as compared to NB controls.



	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	POS 1	POS 2	POS 3	NEG	NEG	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CK b 8-1	CNTF	EGF	Eotaxin
2	POS 1	POS 2	POS 3	NEG	NEG	Angiogenin	BDNF	BLC	BMP-4	BMP-6	CK b 8-1	CNTF	EGF	Eotaxin
3	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-gamma	IGFBP-1	IGFBP-2	IGFBP-4
4	Eotaxin-2	Eotaxin-3	FGF-6	FGF-7	Flt-3 Ligand	Fractalkine	GCP-2	GDNF	GM-CSF	I-309	IFN-gamma	IGFBP-1	IGFBP-2	IGFBP-4
5	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1alpha	IL-1beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
6	IGF-I	IL-10	IL-13	IL-15	IL-16	IL-1alpha	IL-1beta	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7
7	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
8	Leptin	LIGHT	MCP-1	MCP-2	MCP-3	MCP-4	M-CSF	MDC	MIG	MIP-1 delta	MIP-3 alpha	NAP-2	NT-3	PARC
9	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-beta1	TGF-beta 3	TNF-alpha	TNF-beta	IC1	IC2	IC3	NEG	NEG
10	PDGF-BB	RANTES	SCF	SDF-1	TARC	TGF-beta1	TGF-beta 3	TNF-alpha	TNF-beta	IC1	IC2	IC3	NEG	NEG

Figure 16 Protein array fluorescence signal visualization.

a) Scheme of antibodies spotted on one of the sub-arrays. Each antibody is spotted in duplicates on the array to enable calculation of the average of intensities. The first three spots (upper left corner) are positive controls representing a dilution series of biotinylated immunoglobulins G that were used for data normalization. The last two spots (lower left) are negative controls, printed with a buffer already containing proteins, which are identical to background signals on arrays b) examples of signals obtained with the same sub-array in supernatants from the co-cultivation and transwell experiments of Schwann cells with the STA-NB-6 cell line in the upper panel, and the Schwann cells alone and STA-NB-6 cells alone, as controls, in the lower panel. SC: Schwann cell; NB6: STA-NB-6 cells; CoCu: co-culture; TW: transwell culture, CTRL: control.

49 factors were increased in co-cultures/TW culture as compared to control NB cultures, while 10 of them were differentially secreted in co-cultures when compared to SC control (Figure 17). The increase in secretion ranged in log2 fold changes from 1 (CFD) to 8 (CCL7), with the value of 1 presenting a 2-fold increase (Figure 17).

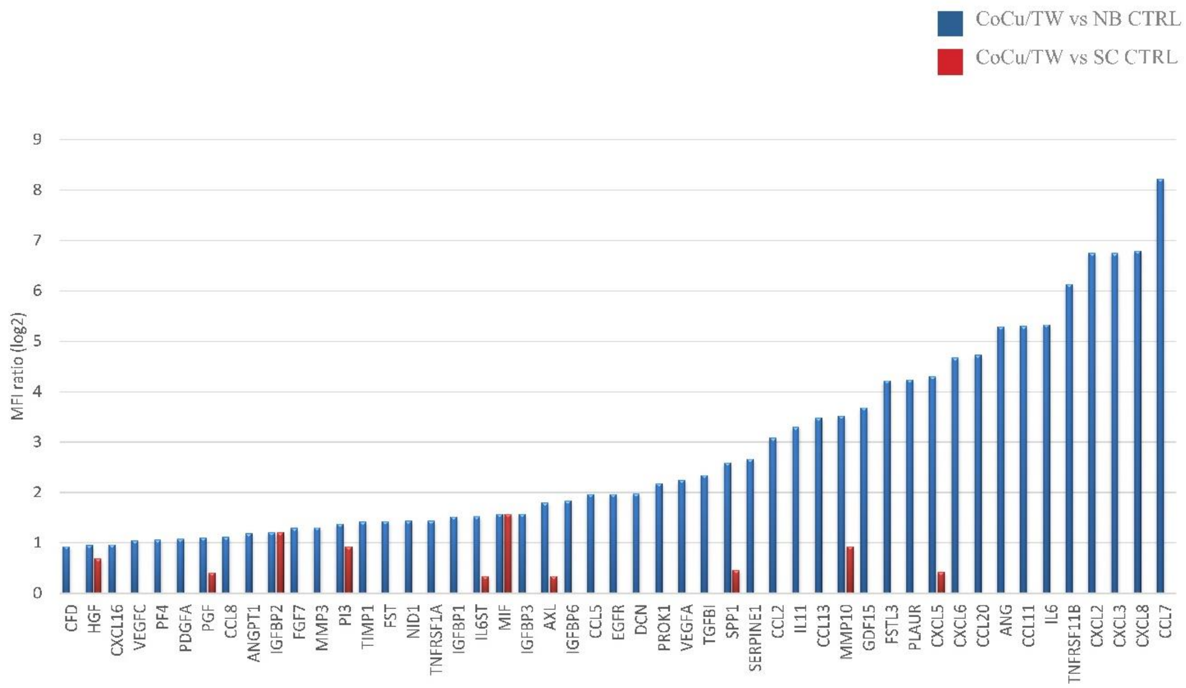


Figure 17 49 factors identified by protein arrays.
Cell culture supernatants of control or Schwann cell/neuroblastoma co-cultivation and transwell experiments were analyzed with protein arrays for the presence of 275 different cytokines and growth factors. An average fluorescence intensity value for the co-cultivation and transwell experiments was calculated and compared to values of neuroblastoma controls or Schwann cell controls. 49 proteins had an increase of secretion in CoCu/TW when compared to NB CTRL (shown in blue), while 10 proteins had an increase of secretion when compared to SC CTRL (shown in red). CoCu: co-culture; TW: transwell culture; CTRL: control; MFI: mean fluorescence intensity.

The normalized data from the protein arrays was further uploaded into the Qlucore Omics Explorer to visualize the data-set and evaluate similarities and/or differences between the samples by generating heatmaps and PCA plots. In unsupervised hierarchical clustering, two main clusters were visible: direct SC-NB co-cultivation, transwell and SC control samples clustered together (Figure 18), while the second cluster contained all NB control samples, even though NB cell lines with various genetic backgrounds were used (Figure 18).

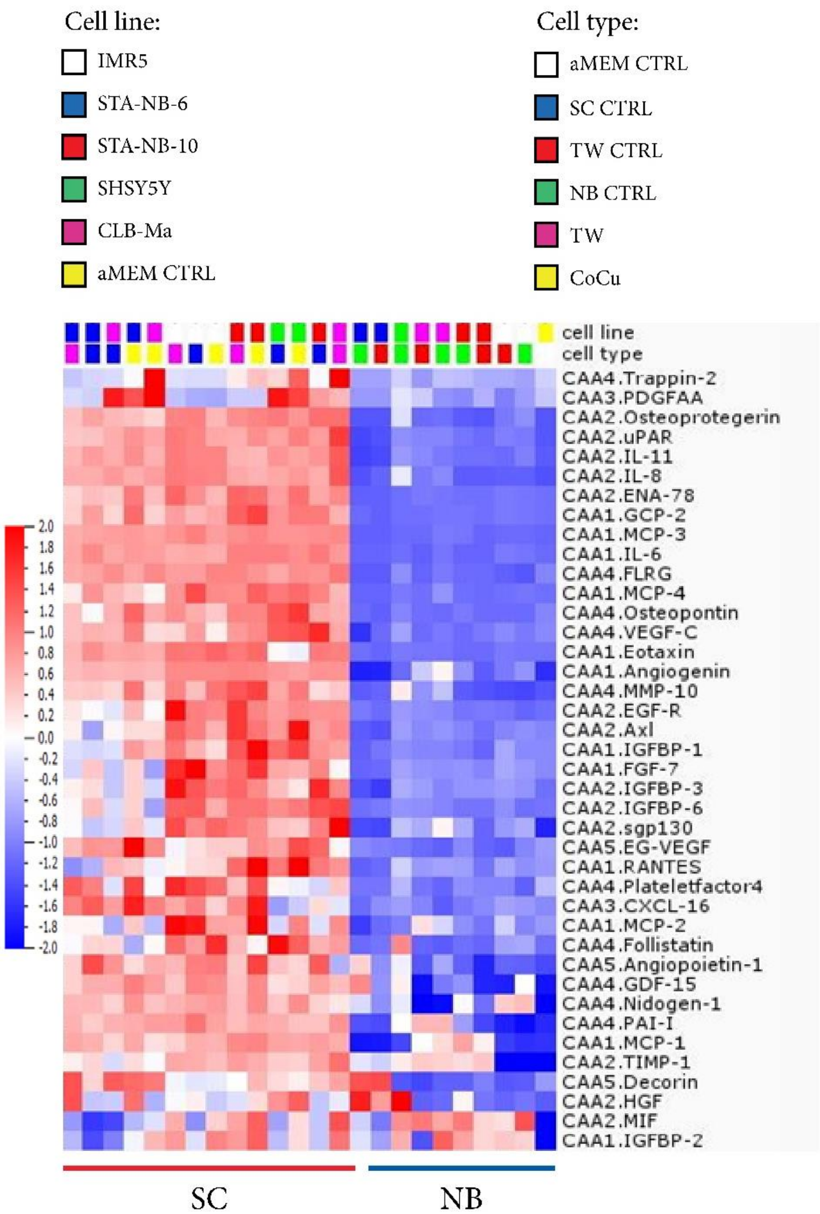


Figure 18 A heatmap showing unsupervised hierarchical clustering of samples based on the top 49 differentially secreted factors derived from the normalized protein array data.

Up-regulated factors are shown in red, downregulated in blue. All cultures containing Schwann cells, including co-cultures, transwell cultures and Schwann cell controls clustered together and are abbreviated by SC, for simplicity. Cultures containing only NB cells are abbreviated by NB. CTRL: control, CoCu: co-culture; TW: transwell.

Also, PCA plots (Figure 19) clearly visualized the NB-containing samples clustering distantly from all samples that contained SCs.

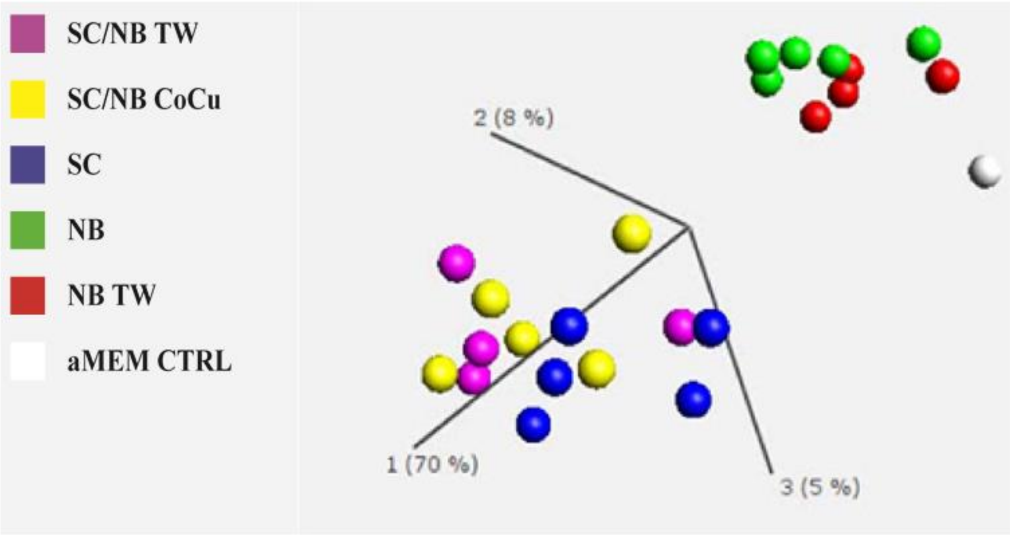


Figure 19 PCA plot showing clustering of all samples analyzed by CAA.
Samples containing only neuroblastoma cells cluster away from all samples containing Schwann cells. SC/NB TW: Schwann cell/neuroblastoma transwell culture; SC/NB CoCu: Schwann cell/neuroblastoma co-culture; SC: Schwann cell control; NB: neuroblastoma control; NB TW: neuroblastoma transwell controls; aMEM CTRL: aMEM media control.

3.2 Prioritization of candidate neurotrophic/neuritogenic factors

Literature research was performed for each of the 49 identified factors to investigate whether they suit the criteria for further functional validation on NB cell lines. As sources for literature research UniProtKB (www.uniprot.org/), the NCBI gene database (www.ncbi.nlm.nih.gov/gene) and PubMed (www.ncbi.nlm.nih.gov/pubmed; with search terms: [factor name] and [Schwann cells]; [factor name] and [neuroblastoma]) were used.

Twelve, out of 49 proteins, were shown to have functions in immune cell attraction (Figure 20; Table 4), most of them being cytokines and chemokines, such as CCL7, CXCL8, CXCL3, CXCL2, CCL11, CCL20, CXCL6, CCL13, CCL2, CCL8, PF4, or to be involved in the activation of complement, such as CFD.

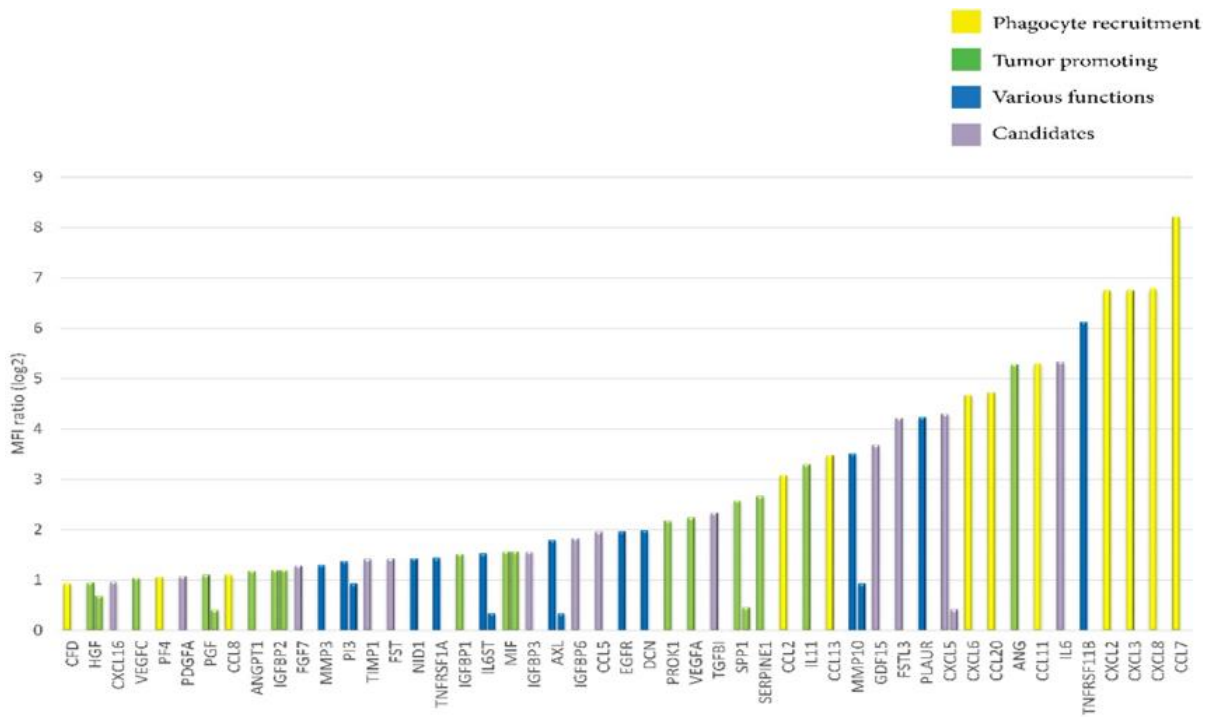


Figure 20 Functional annotation of 49 differentially secreted factors based on literature research. The bars and corresponding values are the same as in Figure 17, only annotated. Factors that were shown to be involved in immune cell recruitment, to have tumor-promoting functions, or to function as proteases, receptors or components of the extracellular matrix were excluded from further analysis. To obtain a complete picture, 13 candidate factors were looked up in gene expression data. MFI: mean fluorescence intensity.

Table 4 Twelve factors involved in phagocyte recruitment.
Protein names and proposed functions are according to www.uniprot.org.

FACTOR (gene symbol)	**PROTEIN NAME**	**PROPOSED FUNCTION**
CCL7	C-C motif chemokine 7	Chemotactic factor that attracts monocytes and eosinophils, but not neutrophils.
CXCL8	Interleukin-8	Attracts neutrophils, basophils, and T-cells, but not monocytes.
CXCL3	C-X-C motif chemokine 3	Chemotactic activity for neutrophils.
CXCL2	C-X-C motif chemokine 2	Hematoregulatory chemokine. Expressed at sites of inflammation.
CCL11	Eotaxin	Promotes the accumulation of eosinophils.
CCL20	C-C motif chemokine 20	Attracts lymphocytes and, slightly, neutrophils, but not monocytes.
CXCL6	C-X-C motif chemokine 6	Chemotactic for neutrophil granulocytes.
CCL13	C-C motif chemokine 13	Attracts monocytes, lymphocytes, basophils and eosinophils, but not neutrophils.
CCL2	C-C motif chemokine 2	Attracts monocytes and basophils, but not neutrophils or eosinophils.
CCL8	C-C motif chemokine 8	Attracts monocytes, lymphocytes, basophils and eosinophils.
PF4	Platelet factor 4	Chemotactic for neutrophils and monocytes. Released during platelet aggregation.
CFD	Complement factor D	Complement activation.

Thirteen, out of 49 factors, were shown to have tumor-promoting functions (Figure 20; Table 5), such as IL11, SERPINE1, VEGFA, PROK1, MIF, IGFBP1, IGFBP2, PGF, ANGPT1, VEGFC and HGF, and/or to be involved in angiogenesis, e.g. ANG, VEGFA, SPP1, ANGPT1, VEGFC, MIF.

Table 5 Factors with a tumor-promoting and/or pro-angiogenic effect.
Protein names and proposed functions are according to www.uniprot.org., if not otherwise stated.

FACTOR (gene symbol)	PROTEIN NAME	PROPOSED FUNCTION
ANG	Angiogenin	Angiogenic factor that supports primary and metastatic tumor growth [47].
IL11	Interleukin-11	Stimulates the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells.
SERPINE1	Plasminogen activator inhibitor 1	Serine protease inhibitor. Shown to promote metastasis in neuroblastoma [48].
SPP1	Osteopontin	Produced by various tumor cells and is suggested to be involved in angiogenesis [49].
VEGFA	Vascular endothelial growth factor A	Induces endothelial cell proliferation, promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels.
PROK1	Prokineticin-1	Directly influences neuroblastoma progression by promoting the proliferation and migration of neuroblastoma cells.
MIF	Macrophage migration inhibitory factor	It has been linked to fundamental processes such as control of cell proliferation, cell survival, angiogenesis, and tumor progression [50].
IGFBP1	Insulin-like growth factor-binding protein 1	Promotes cell migration.
IGFBP2	Insulin-like growth factor-binding protein 2	Binds to the ECM and enhances proliferation and metastatic behavior of neuroblastoma cells [51].
ANGPT1	Angiopoietin-1	Plays an important role in the regulation of angiogenesis, endothelial cell survival, proliferation, migration, adhesion and cell spreading.
PGF	Placenta growth factor	Promotes tumor cell growth.
VEGFC	Vascular endothelial growth factor C	Growth factor active in angiogenesis, and endothelial cell growth, stimulating their proliferation and migration.
HGF	Hepatocyte growth factor receptor	Increase in HGF/c-Met expression was correlated with enhanced invasiveness and activation of proteases degrading the extracellular matrix [52].

Considering their phagocyte attracting and tumor-promoting functions, these proteins were excluded from further validation assays in NB cell lines. In addition, 11 factors were excluded because of other functions (Figure 20; Table 6), such as MMP10, MMP3 and PI3 which are proteases; TNFRSF1A, EGFR, PLAUR, AXL, IL6ST and TNFRSF11B that are receptors; or components of the extracellular matrix, e.g. NID1 and DCN.

Table 6 Factors excluded as candidates based on their functions as proteases, receptors or components of the extracellular matrix.
Protein names and proposed functions are according to www.uniprot.org.

FACTOR (gene symbol)	PROTEIN NAME	PROPOSED FUNCTION
MMP10	Stromelysin-2	Can degrade fibronectin, gelatins of type I, III, IV, and V; weakly collagens III, IV, and V.
MMP3	Stromelysin-1	Can degrade fibronectin, laminin, gelatins of type I, III, IV, and V; collagens III, IV, X, and IX, and cartilage proteoglycans.
PI3	Elafin	Neutrophil and pancreatic elastase-specific inhibitor of skin.
TNFRSF1A	Tumor necrosis factor receptor superfamily member 1A	Receptor for TNFSF2/TNF-alpha and homotrimeric TNFSF1/lymphotoxin-alpha.
EGFR	Epidermal growth factor receptor	Receptor tyrosine kinase binding ligands of the EGF family.
PLAUR	Urokinase plasminogen activator surface receptor	Acts as a receptor for urokinase plasminogen activator.
AXL	Tyrosine-protein kinase receptor UFO	Receptor tyrosine kinase that transduces signals from the extracellular matrix into the cytoplasm by binding growth factor GAS6.
NID1	Nidogen-1	Sulfated glycoprotein widely distributed in basement membranes and tightly associated with laminin.
IL6ST	Interleukin-6 receptor subunit beta	Signal-transducing molecule. The receptor systems for IL6, LIF, OSM, CNTF, IL11, CTF1 and BSF3 utilize it for initiating signal transmission.
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11B	Acts as decoy receptor for TNFSF11/RANKL and thereby neutralizes its function in osteoclastogenesis.
DCN	Decorin	May affect the rate of fibrils formation.

The remaining 13 factors (Figure 20; Table 7) were further analyzed to confirm their upregulation on RNA level and investigate whether they are upregulated by SCs or NB cells in co-cultures and transwell cultures, as well as to confirm the expression of the corresponding receptors on NB cell lines. The protein array data were therefore compared to gene expression data (RNA-seq) of FACS-sorted SCs and NB cells from co-cultures, transwell cultures and controls, and analyzed by Qlucore Omics Explorer.

Table 7 13 candidate factors that were further analyzed by gene expression analysis.
Protein names and proposed functions are according to www.uniprot.org., if not otherwise stated.

FACTOR (gene symbol)	PROTEIN NAME	PROPOSED FUNCTION
IL6	Interleukin-6	It induces myeloma and plasmacytoma growth and induces nerve cells differentiation. Silverman et al. [53] showed that neuroblastoma cells induce production of IL6 in stromal cells.
CXCL5	C-X-C motif chemokine 5	Shown to have an effect on nerve regeneration by inducing neurite growth [54].
FSTL3	Follistatin-related protein 3	Antagonizing protein for members of the TGF-beta family that is involved in differentiation of hematopoietic cells.
GDF15	Growth/differentiation factor 15	Shown to be secreted by Schwann cells in the lesioned peripheral nervous system [55].
TGFBI	Transforming growth factor-beta-induced protein ig-h3	Shown to significantly reduce proliferation and invasion of neuroblastomas *in vitro* and *in vivo* [56].
CCL5	C-C motif chemokine 5	Together with GPR75, may play a role in neuron survival.
IGFBP6	Insulin-like growth factor-binding protein 6	A member of the IGF system shown to be associated with the growth-arrest in neuroblastoma cells [57].
IGFBP3	Insulin-like growth factor-binding protein 3	Exhibits IGF-independent anti-proliferative and apoptotic effects mediated by its receptor TMEM219/IGFBP-3R.
FGF7	Fibroblast growth factor 7	Plays an important role in the regulation of embryonic development, cell proliferation and cell differentiation.
PDGFA	Platelet-derived growth factor subunit A	It´s expression was shown to be significantly associated with patient survival in advanced-stage tumors [58]. PDGF isoforms were also shown to induce morphological changes showing neuronal cell maturation in a wide variety of neural crest-derived human tumor cell lines [59].
CXCL16	C-X-C motif chemokine 16	It´s receptors were shown to be involved in the retention of metastatic neuroblasts in the bone marrow through interaction with CXCL16-expressing stromal cells [60].
TIMP1	Metalloproteinase inhibitor 1	Functions as a growth factor that regulates cell differentiation, migration and cell death, and activates cellular signaling cascades via CD63 and ITGB1.
FST	Follistatin	Involved in differentiation of hematopoietic cells.

Additionally, 6 neurotrophins (Table 8) known from previous research for their effects in the CNS or PNS were also considered as candidates. Since these proteins were not included on the protein arrays (PTN and EGFL8), or not shown as differentially secreted (BDNF, GDNF, CNTF and NGF), their gene expression levels were also analyzed and visualized by Qlucore.

Table 8 Factors chosen from literature research**.**
Six neurotrophins were chosen in addition to the ones identified by protein arrays. All the proposed roles are according to www.uniprot.org., unless otherwise stated.

FACTOR (gene symbol)	PROTEIN NAME	PROPOSED FUNCTION
BDNF	Brain-derived neurotrophic factor	During development, promotes the survival and differentiation of selected neuronal populations of the peripheral and central nervous systems. Participates in axonal growth, path finding and in the modulation of dendritic growth and morphology.
GDNF	Glial cell line-derived neurotrophic factor	Neurotrophic factor that enhances survival and morphological differentiation of dopaminergic neurons and increases their high-affinity dopamine uptake.
CNTF	Ciliary neurotrophic factor	CNTF is a survival factor for various neuronal cell types. Seems to prevent the degeneration of motor axons after axotomy.
NGF	Beta-nerve growth factor	Activates cellular signaling cascades through receptor tyrosine kinases to regulate neuronal proliferation, differentiation and survival.
PTN	Pleiotrophin	Secreted growth factor that induces neurite outgrowth. Binds the receptor ALK, which induces activation of the mitogen-activated protein kinase pathway, an important step in the anti-apoptotic signaling of PTN and regulation of cell proliferation.
EGFL8	Epidermal growth factor-like protein 8	Chosen based on previous research performed by Weiss, Taschner-Mandl et al. [44].

3.3 Gene expression analysis of FACS-sorted cells from co-cultivation, transwell and control cultures

3.3.1 Gene expression analysis led to selection of 8 factors

Gene expression (RNA-seq) data used in this master thesis was previously obtained by RNA-sequencing of FACS-sorted SCs and STA-NB-6 cells from the same co-culture, transwell culture and controls from which supernatants were used for protein arrays, together with additional SC samples and NB cell lines (STA-NB-2, STA-NB-7 and STA-NB-15 cells).

This RNA-seq dataset was used to analyze mRNA levels of the 13 factors selected with protein arrays and 6 additional neurotrophins selected from literature in different NB cell lines, SCs alone and one co-cultivation/transwell experiment. In addition, the expression of their corresponding receptors on different NB cell lines was investigated, since the factors can act on NB cells only if their corresponding receptors are present on these cells.

Based on the gene expression data, 2 factors, out of the 13 selected with protein arrays and literature research, were chosen as candidates, IGFBP6 and FGF7. IGFBP6 was highly expressed by

SCs, but not by NB cells (Figure 21) and the recently described receptor PHB2, an IGF signaling-independent receptor [61], was highly expressed in NB, while SCs had very low levels (Figure 21). Similarly, FGF7 was high in SCs (Figure 21) and its receptor FGFR2 [62] present at high levels on NB cells (Figure 21).

All the 6 additional neurotrophic factors, chosen from literature, were, as expected, highly expressed by SCs, with the exception of CNTF and PTN, being highly expressed by both SCs and NB cells (Figure 21). CNTFR, the receptor for CNTF, was upregulated by NB cells (Figure 21), although LIFR and IL6ST, parts of the same receptor complex [63], were upregulated by both SCs and NB cells (Figure 21). PTN binds several receptors, some of which are PTPRZ1 [64-67] and PTPRA [68], that were mainly upregulated by SCs (Figure 21), but also ALK [69, 70] that was expressed at high levels in NB cells (Figure 21).

NGFR and GFRA1, receptors of NGF and GDNF [71], respectively, were highly expressed in SCs (Figure 21), but comparison to unrelated cell types (mononuclear cells) showed that both receptors are also highly expressed by NB cells, although at lower levels as compared to SCs (data not shown). Moreover, the other receptors for NGF and GDNF, NTRK1 and RET [71], respectively, were upregulated only in NB cells (Figure 21). NGFR, like NTRK2 [71], also binds BDNF [71], which was mostly upregulated in NB cells (Figure 21). It is currently unknown to which receptor EGFL8 binds, since its structure was just recently described and it has so far unknown function [72].

Taken together the 8 selected factors showed high mRNA levels in SC samples and high expression of the corresponding receptors in NB cells making them suitable candidates for functional validation in NB cell cultures.

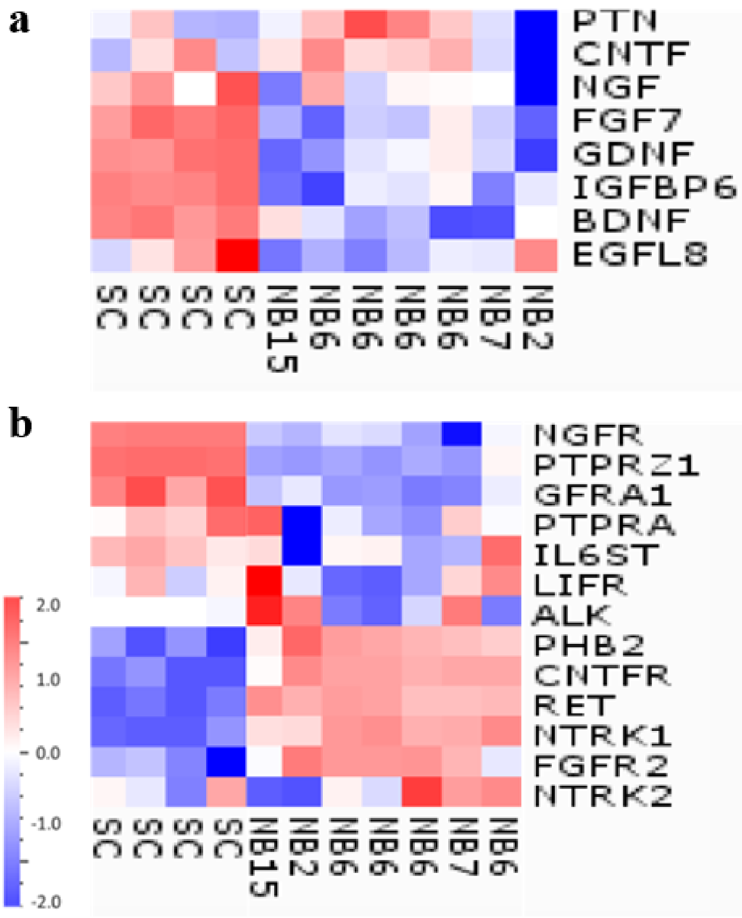


Figure 21 Heatmaps showing RNA expression levels of the 8 selected Schwann cell-secreted proteins and their corresponding receptors in different neuroblastoma and Schwann cell samples.

a) Expression levels of the selected factors, b) expression levels of the corresponding receptors. The receptors correspond to their ligands as follows: BDNF – NGFR, NTRK2, ; GDNF – GFRA1, RET; CNTF – LIFR, CNTFR, IL6ST; NGF – NGFR, NTRK1; PTN –ALK, PTPRZ1, PTPRA; IGFBP6 – PHB2; FGF7 – FGFR2. The receptor for EGFL8 is unknown. Samples were abbreviated according to cell type for simplicity. SC: Schwann cell; NB: neuroblastoma.

3.4 Functional validation of 8 candidate factors

In order to functionally validate the 8 candidate factors, their corresponding recombinant proteins were added at different concentrations, separately and in combination, to cell media of two NB cell lines derived from aggressive tumors, STA-NB-6 and STA-NB-10. The STA-NB-6 cell line was previously demonstrated to be sensitive to direct/indirect interaction with SCs in co-cultures, and STA-NB-10 to be less sensitive (manuscript in preparation). Flow cytometry-based assays were used to measure the reduction of cell proliferation and induction of cell differentiation of NB cells, as well as to define the concentration and combination needed to induce these effects.

3.4.1 Flow cytometry-based cell proliferation and differentiation rate measurement confirmed the proposed effects of selected proteins on STA-NB-6 cells

In these experiments, NF200 expression, as marker for neuronal differentiation, and EdU incorporation, as indicator of proliferative activity, were measured by flow cytometry-based assays.

First, STA-NB-10 cells were cultivated in the presence of candidate neurotrophic/neuritogenic factors for 17 days. In previous co-culture experiments with SCs, the STA-NB-10 cell line showed only moderate responsiveness regarding inhibition of proliferation, and did not show any responsiveness concerning differentiation (manuscript in preparation). Similarly, there was no significant effect of any of the factors at the tested concentrations with regard to cell proliferation or differentiation on the STA-NB-10 cell line. Some of the factors, e.g. IGFBP6, BDNF, CNTF, GDNF and PTN, even slightly increased proliferation (Figure 22), although not significantly, while EGFL8 slightly decreased cell proliferation on day 17. Combination of all 8 factors also had no significant effect, although a slight decrease of cell proliferation is visible on day 17 (Figure 22).

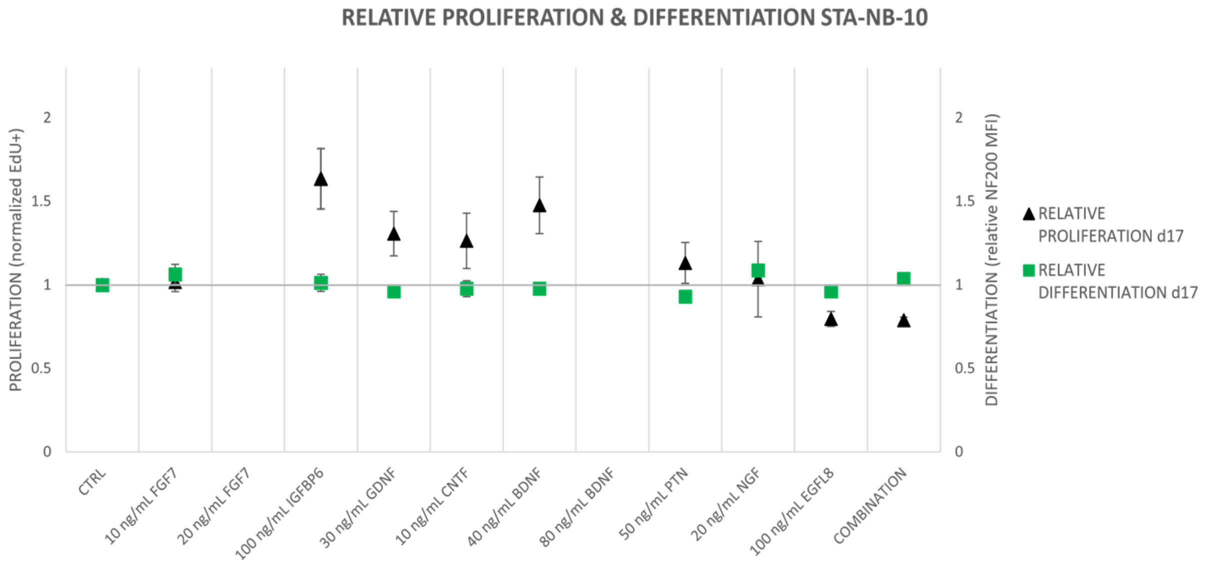


Figure 22 Relative proliferation and differentiation rates of the STA-NB-10 cell line cultivated in the presence or absence (control) of chosen recombinant proteins for 17 days.
Mean+/-SEM values of normalized EdU incorporation data and normalized NF200 MFI values from 3 independent experiments were calculated to analyze the relative effect on cell proliferation and neuronal-like differentiation, respectively. The single factors showed no significant effect on cell differentiation at these concentrations, while IGFBP6, GDNF, CNTF, BDNF and PTN even slightly increased cell proliferation. The combination of all factors slightly decreased cell proliferation on day 17, but had no effect on cell diferentiation. EGFL8 data was derived from one experiment and was thus not considered for statistical analysis, although a slight decrease of cell proliferation is visible on day 17. CTRL: control; MFI: mean fluorescence intensity.

Second, STA-NB-6 cells were cultured in the presence of 8 candidate factors, separately or in combination, for 8 and 17 days, respectively. This cell line showed strong responsiveness to SCs in co-cultures already on day 8 in previous experiments, which was even more pronounced on day 17 (manuscript in preparation). However, addition of factors demonstrated no significant changes of proliferation (Figure 23a), nor differentiation (Figure 23b) on day 8. Significantly decreased proliferation in the presence of NGF (20 ng/mL) and PTN (50 ng/mL) (Figure 23a) was shown on day 17, and increased neuronal-like differentiation in the presence of PTN on day 17 (Figure 23b). NGF also had a pronounced effect on cell differentiation, although it was not statistically significant (Figure 23b).

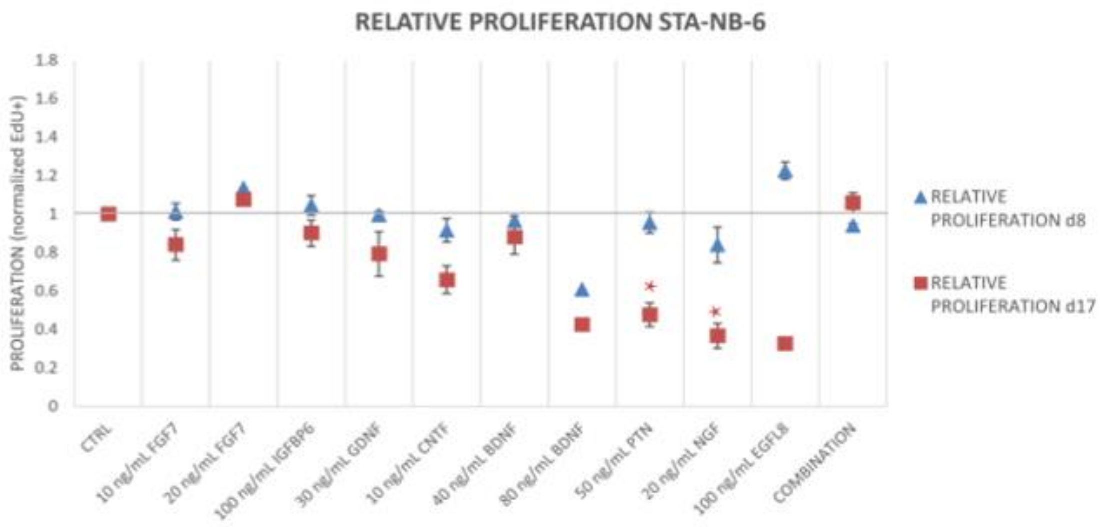
For PTN, two cell populations were clearly visible by flow-cytometry analysis of differentiation (Figure 24a, differentiation, PTN), one expressing low levels of NF200 and the second high levels, probably representing more differentiated (mature) NB cells. Neuronal-like differentiation induced by PTN and NGF, as compared to CTRL, was also confirmed by axonal outgrowth, as visualized by phase contrast microscopy (Figure 24b, NGF; PTN; CTRL).

All other factors, except FGF7 and BDNF, showed a trend towards increased differentiation (Figure 23b) and decreased proliferation (Figure 23a) on day 17 at the initial tested concentrations, 10 and 40 ng/mL, respectively. For FGF7 and BDNF, that did not show any effect on cell proliferation or differentiation at initial concentrations, concentration was doubled in additional experiments, and BDNF showed a more pronounced effect at 80 ng/mL on both cell proliferation (Figure 23a) and differentiation (Figure 23b), when compared to the lower concentration used. FGF7, however, failed to reduce cell proliferation or induce differentiation even at higher concentration, 20 ng/mL (Figure 23a, b).

Even though only one experiment was performed with EGFL8 (100 ng/mL), it showed an unexpected effect on day 8, with an increase of cell proliferation (Figure 23a) and decrease of cell differentiation (Figure 23b), while on day 17, a pronounced effect in decreasing cell proliferation (Figure 23a) and increasing cell differentiation (Figure 23b).

Surprisingly, combination of all factors at tested concentrations had no significant effect on cell proliferation at both time-points (Figure 23a), while cell differentiation was affected on day 17, although not significantly (Figure 23b).

a



b

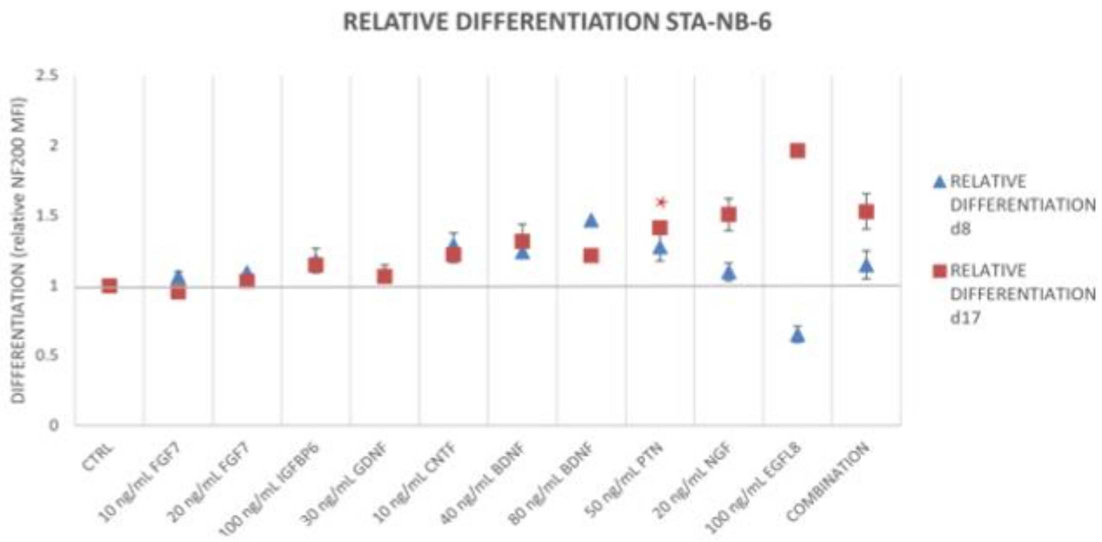


Figure 23 Relative cell proliferation and differentiation rate of the STA-NB-6 cell line, 8 or 17 days, respectively, after the addition of recombinant proteins.

a) Mean+/-SEM values of normalized EdU incorporation data from 3 independent experiments were calculated to analyze the relative effect on cell proliferation. Most of the factors, except NGF and EGFL8, showed no effect on day 8. NGF and PTN significantly decreased cell proliferation on day 17 at tested concentrations. All factors, except FGF7 and BDNF, showed a tendency towards decreased proliferation at initial tested concentrations, while for FGF7 and BDNF the initial concentration was doubled. FGF7 failed to decrease cell proliferation even at the double concentration, while BDNF showed a pronounced effect both on day 8 and day 17. Combination of all factors had no effect on day 8, nor day 17. EGFL8, although only tested in one experiment and thus not considered for statistical analysis, showed an unexpected effect on day 8 with an increase of cell proliferation, and a pronounced decrease on day 17.
b) Mean+/-SEM values of normalized NF200 MFI values from 3 independent experiments were calculated to analyze the relative effect on cell differentiation. None of the factors showed a significant effect on day 8, while PTN showed a significant effect in increasing cell differentiation on day 17 at the tested concentration. NGF also had a pronounced effect on cell differentiation, although it was not significant. All factors, except FGF7, showed a tendency towards increasing cell differentiation at tested concentrations on day 17. EGFL8 showed an unexpected effect, with a decrease of cell differentiation on day 8 and a pronounced increase on day 17. Asterisks indicate statistically significant differences compared to CTRL, $^*p \leq 0.05$. CTRL: control; MFI: mean fluorescence intensity.

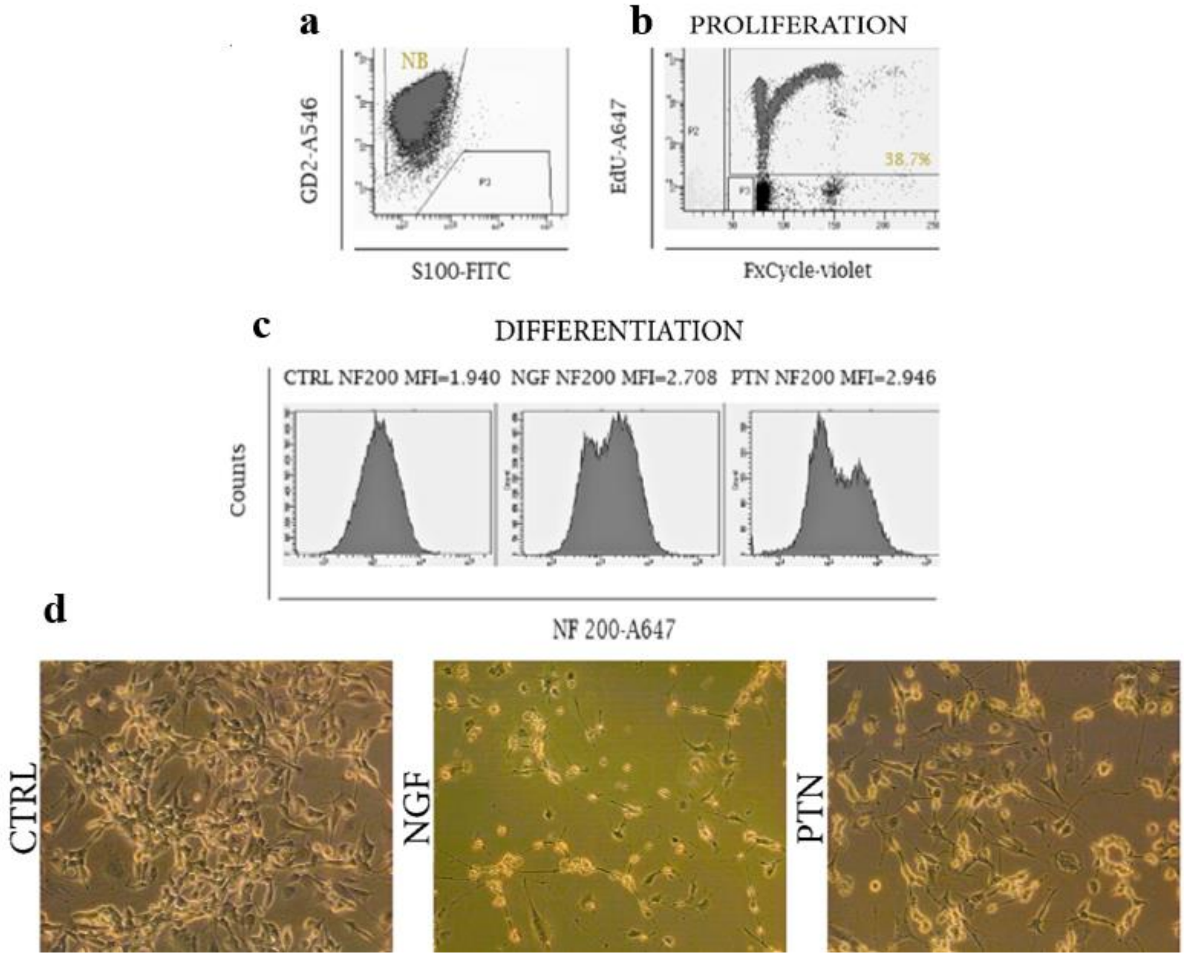


Figure 24 Representative flow cytometry plots and microscopic images.
a) Representative plot of the flow-cytometry gating strategy. Neuroblastoma cells are detected as GD2-A546 positive cells. b) The proliferation rate of neuroblastoma cells is measured as the percentage of EdU incorporation, while c) the differentiation rate as the expression value of the NF200 marker. NF200-APC histograms show MFI values for STA-NB-6 cells cultured in the presence NGF or PTN for 17 days, respectively, as compared to untreated control. On the histogram for PTN (right), two cell populations are clearly visible - one expressing low levels of NF200, presented by the lower peak, and the second high levels, presented with the higher peak, probably representing more differentiated neuroblastoma cells. This is also visible on the histogram for NGF (middle), although less, whereas not visible at all for control cells (left). d) Representative phase contrast microscopy images of cultures. The prolongation of neural processes upon cultivation in the presence of PTN and NGF is clearly visible when compared to control. The microscopic images in d) and NF200-APC histograms in c) are not from the same experiments. NB: neuroblastoma cells; CTRL: control; MFI: mean fluorescence intensity value.

3.5 Pilot study: Isolation of Schwann cells from mouse peripheral nerve tissue and co-cultivation with the STA-NB-6 cell line

A pilot experiment was conducted to assess whether it is possible to culture SCs isolated from mouse peripheral nerves, and whether their *in vitro* co-cultivation with human NB cells will have an effect on NB cell proliferation and differentiation. The human SC isolation procedure was successfully adapted to mouse peripheral nerve tissue and the mouse SC could be cultivated and further co-cultivated with the STA-NB-6 cell line (Figure 25a) in the same manner as with human SCs, although the number of mouse SCs obtained was rather low. Furthermore, it was possible to measure the rate of STA-NB-6 cell proliferation in co-cultures with mouse SCs (Figure 25b), proving that the isolation and cultivation of SCs from mouse peripheral nerve tissue and co-cultivation with human NB cells is feasible. However, no effect on NB proliferation was observed, probably due to the very low numbers of mouse SCs used in this experiment.

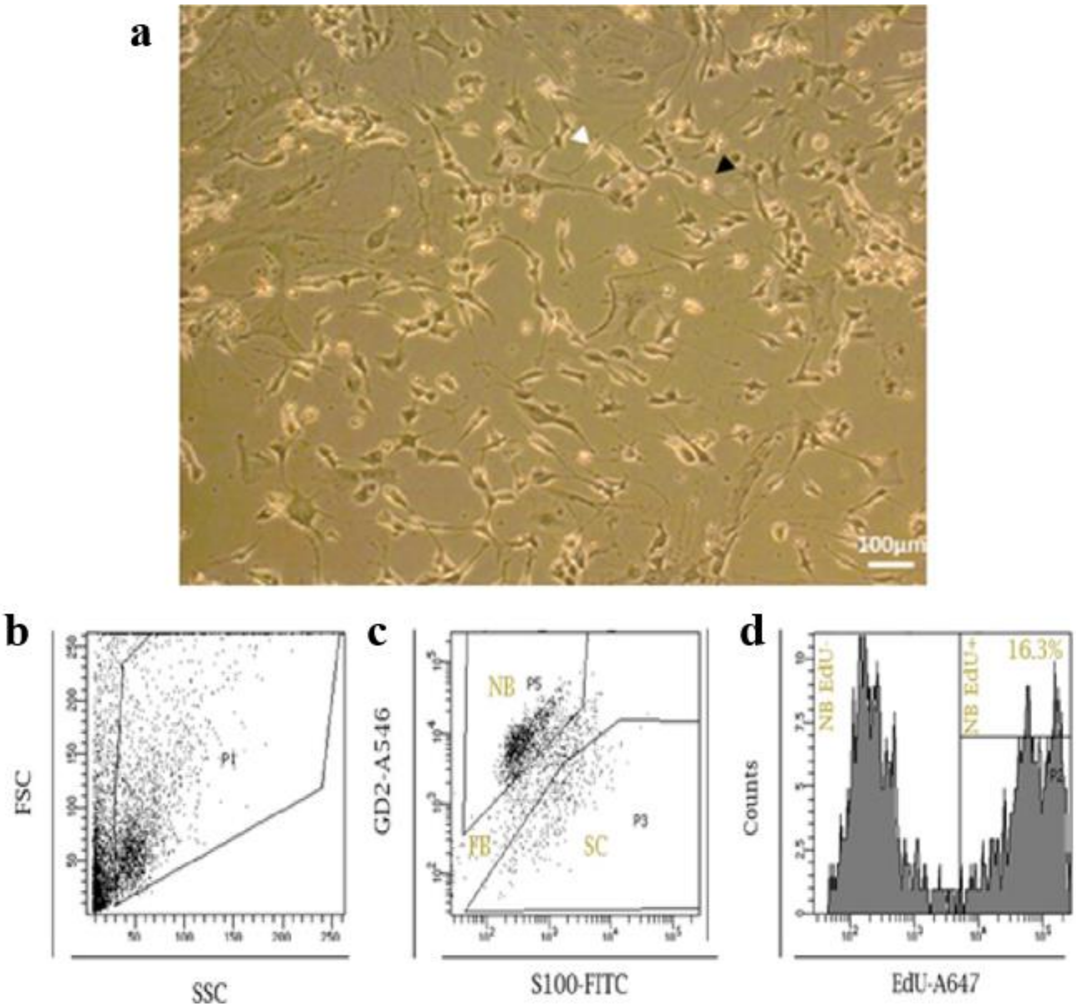


Figure 25 Co-cultivation of mouse Schwann cells with the STA-NB-6 cell line and flow cytometry measurement of the cell proliferation rate of STA-NB-6 cells.

a) Microscopic image of the mouse Schwann cell/STA-NB-6 co-cultivation. The white arrow indicates Schwann cells, and the black one neuroblastoma cells. Due to the low number of Schwann cells obtained by isolation from mouse peripheral nerve tissue, the number of neuroblastoma cells added was also lower. The gating strategy of the flow cytometry cell proliferation rate measurement can be seen on flow cytometry plots, with all cells shown on b) the FSC/SSC plot; c) separate gating into GD2+S100- neuroblastoma cells, GD2-S100+ Schwann cells and GD2-S100- fibroblasts on the GD2/S100 plot; d) an univariate (single-parameter) histogram showing incorporation of EdU for measurement of cell proliferation with a peak for GD2+S100- NB cells negative for EdU incorporation, on the left side, and EdU positive cells, on the right. NB: neuroblastoma cell; FB: fibroblasts; SC: Schwann cells.

4 DISCUSSION

SCs are suggested to be essential for the benign clinical behavior of ganglioneuroma, acting via neuritogenic, anti-proliferative and pro-apoptotic signals. The exact mechanism of this unique tumor-stroma crosstalk is, however, still unknown. In this study, SC-secreted factors and corresponding receptor expression was investigated using protein arrays and RNA-sequencing data. This study identified PTN as a potential factor involved in the SC-NB interaction. The *in vitro* experiments conducted here showed that addition of 50 ng/mL of recombinant PTN to cell media for 17 days causes a reduction of cell proliferation and induction of neuronal-like differentiation of a NB cell line derived from an aggressive tumor, STA-NB-6. The growth-impairing effect of PTN was demonstrated *in vitro* by cultivation of STA-NB-6 cells in the presence of the recombinant protein and measured by flow cytometry-based readout assays. In addition to PTN, NGF was also shown to have a significant proliferation-reducing effect on STA-NB-6 cells, which confirms previous investigations of NGF in NTRK1/TrkA over-expressing neuroblastoma cells [58, 73].

4.1 PTN-a mediator of the SC-NB interaction

In this study, PTN was recognized as a potential factor involved in the SC-NB interaction. Recombinant PTN was shown to decrease proliferation and induce neuronal-like differentiation of STA-NB-6 cells *in vitro*. This sheds new light on its role in disease and tumorigenesis, since it is considered to be a tumor growth- and angiogenesis-inducing factor [74, 75], and makes PTN suitable for further testing in other NB cell lines, as well as other tumor types.

PTN is a secreted neurite outgrowth-promoting factor usually associated with the extracellular matrix that acts in several tissues through different receptors. In the CNS, it is widely expressed during development [76] and it regulates neurogenesis [77], neural migration [78] and differentiation [79, 80]. The expression in the adult brain is constricted to certain regions, such as the hippocampus [81], where PTN is involved in learning and long-term memory processes [82]. In the PNS on the other hand, its function has so far mostly been investigated in the context of nerve regeneration after peripheral nerve injury [15, 83]. The possibility that PTN is secreted by tumor-associated SCs in ganglioneuroma and may thus be linked to the benign phenotype of these tumors was, until now, not thoroughly interrogated.

It is well known that PTN is associated with the pathogenesis of neurodegenerative diseases, as well as with inflammation and cancer development. Unlike some tumors, such as breast carcinoma, in which PTN is highly expressed and implicated as a tumor-growth factor [84, 85], in neuroblastoma, low levels of PTN are correlated to poor prognosis [86]. Interestingly, PTN expression was shown to be significantly higher in favorable types of neuroblastoma than in unfavorable ones [86-88]. In line with the findings demonstrated in this study, Nakagawara et al. hypothesized in their work that it is possible that PTN 'acts on neuroblastoma cells to potentiate the neuronal and/or Schwannian differentiation' [86]. Since ganglioneuromas are composed of few fully differentiated ganglionic cells that are surrounded by a dense SC stroma, it is very likely that the highly expressed PTN in ganglioneuromas originates from SCs. Transcriptome analysis of the SC fraction of ganglioneuromas could clarify this open question. In the gene expression data presented in this work, PTN was expressed by SCs, but also by NB cells. It was previously observed that highly aggressive NB cells, especially those with *MYCN* amplification, express much lower amounts of PTN than cells without *MYCN* amplification [86, 87]. Similarly, this study showed slightly higher expression of PTN by the *MYCN* non-amplified STA-NB-6 cell line, while STA-NB-7 and STA-NB-15, both *MYCN*-amplified cell lines, presented very low PTN expression.

The preliminary *in vitro* co-cultivation data suggesting the tumor growth-inhibiting and differentiation-inducing effect of SCs on NB cells (manuscript in preparation), together with the validation of the anti-tumor effect of PTN on STA-NB-6 cells presented in this work, indicate that PTN might indeed be one of the factors involved in the SC-NB interaction that provoke neuroblastoma maturation.

4.2 PTN signaling in neuritogenesis and neuroblastoma

PTN's neurite-outgrowth abilities were demonstrated to be conducted through the ALK (anti-anaplastic lymphoma kinase) receptor [89]. Although ALK was shown to be more active in favorable neuroblastoma tumor types than in unfavorable ones [87], its activation is considered to frequently initially occur in neuroblastoma oncogenesis [87]. In 8-10% of neuroblastoma patients, ALK activation is due to activating point mutations in its tyrosine kinase domain [90, 91]. It is speculated, however, that ALK activation in many tumor types, as well as neuroblastoma, depends on the activity of a ligand [87, 90, 92].

ALK has two known ligands belonging to the same family of growth factors, PTN and MK (midkine) [93]. MK was shown to compete with PTN for ALK binding [70]. Opposite to PTN, high MK levels in NB are correlated with poor prognosis [68, 86], and MK was shown to be expressed relatively weakly in ganglioneuroma [86]. Moreover, MK occurs frequently and at high levels in all stages of neuroblastoma [86, 93], especially in undifferentiated and aggressive neuroblastomas, and *MYCN* amplified tumor cells [86]. ALK activation and overexpression were shown to be concomitant with MK upregulation in neuroblastoma [87]. The growth-impairing effects of PTN on STA-NB-6 cells demonstrated in this work, suggests that the two ligands, although sharing structural similarities

[93], might have opposite effects on ALK activation in neuroblastoma, which may depend on the equilibrium of both factors in the tumor microenvironment.

In the *MYCN* amplified NB cell lines used in this study, i.e. STA-NB-7 and STA-NB-15, ALK expression was elevated as compared to the *MYCN* non-amplified STA-NB-6 cell line, in which it was low, even though PTN had a significant effect on this cell line. Phosphorylation of ALK in cells should be evaluated in future studies to investigate ALK activation, since it was shown that the ligand-induced ALK activation might be mediated through alternative mechanisms [94], suggesting that PTN signaling might be conducted through various receptors. Another receptor that binds PTN is PTPRZ1 (protein tyrosine phosphatase receptor type Z1) [64, 65], which was shown to be predominantly expressed in the CNS [95]. PTN acts as an inhibitory ligand for PTPRZ1 by inducing oligomerization of the receptor, thereby preventing its tyrosine phosphatase activity [96]. During reparative re-myelination in the CNS, PTN acts through PTPRZ1 to promote oligodendrocyte differentiation [67]. In this study, PTPRZ1 was shown to be highly upregulated by SCs, in which it was shown to act as the putative F3 receptor [97]. This receptor has, so far, not been investigated in NB, which might be interesting to pursue in future studies, since it was showed that PTN stimulates tyrosine phosphorylation of ALK through the PTN/PTPRZ1 signaling pathway [98]. An alternative receptor that can interact with PTN is PTPRA (protein tyrosine phosphatase receptor type A, or LRP) [68, 99]. This receptor was shown to be upregulated and involved in the DMSO-induced neuronal differentiation of N1E-115 neuroblastoma cells [100]. High PTPRA expression in SCs was demonstrated in this study, but also in STA-NB-7 and STA-NB-15, *MYCN* amplified cell lines, suggesting that PTN might act through PTPRA in *MYCN* amplified cell lines.

Other reported receptors that could be analyzed in further experiments are neuropilin-1 [75], N-syndecan [101], integrin α4β1, α6β1 [102]. Altogether, PTN signaling is most likely conducted through multiple receptors, and PTN and corresponding receptor expression analysis should be expanded to other cell lines to investigate the mechanism involved.

4.3 Confirmation of the growth-impeding effects of NGF

The results of this study demonstrated that the patient-derived NB cell line, STA-NB-6, expresses TrkA and responds to NGF stimulation by decreased proliferation, neuronal differentiation and neurite outgrowth. This is also in concordance to previous knowledge, since TrkA activation, besides inducing differentiation, can inhibit angiogenesis, mediate apoptosis and cause growth arrest [58]. Interestingly, NTRK1 (TrkA), high-affinity receptor for NGF, has similar expression patterns as PTN in NB. Ganglioneuromas and favorable NBs were shown to express high levels of TrkA [36, 103]. TrkA expression is also very low or absent in *MYCN*-amplified cell lines [37, 73]. In this study the *MYCN* non-amplified cell line, STA-NB-6, had higher TrkA expression levels than the *MYCN*-

amplified STA-NB-15, explaining at least in part the ability of the STA-NB-6 cell line to respond to SCs.

4.4 Examination of the SC secretome

The comprehensive analysis of extracellular factors produced by human SCs performed in this study revealed 49 differentially secreted proteins in co-cultures/TW cultures compared to NB CTRL, and 10 proteins compared to SC CTRL, suggesting that the majority of factors are secreted by SCs. Moreover, the heatmap and PCA plot presenting protein array data demonstrated that all cultures containing Schwann cells, including co-cultures, transwell cultures and Schwann cell controls, cluster together and distantly from NB-containing samples, thereby, again suggesting a dominant contribution of Schwann cells to the analyzed secretome. These results, thus, together with the various roles that SCs exert in physiological and pathological conditions [4, 12, 13, 16] known from literature, justify the lower number of proteins presented in co-cultures/TW cultures when compared to SC CTRL.

Most of the 49 identified proteins had functions already attributed to SCs, but some of them have not been implicated in SC function yet. Among those, 2 potentially neuritogenic factors, IGFBP6 and FGF7, were tested in *in vitro* assays. FGF7 failed to reduce cell proliferation or induce differentiation at both concentrations tested, 10 and 20 ng/mL, respectively. IGFBP6, however, showed a slight decrease of proliferation and increase of differentiation at a concentration of 100 ng/mL. IGFBP6 was in previous research mostly examined in the context of its IGF-II binding abilities and was demonstrated to inhibit NB cell growth *in vitro* and *in vivo* [57, 104]. However, an IGF-independent role of IGFBP6 was recently discovered [61], involving signaling through PHB-2. PHB2 was upregulated in all NB cell lines analyzed in this work, suggesting that in addition to PTN and NGF, IGFBP6 is a potential SC-secreted protein that can affect NB growth and differentiation.

Evaluation of 6 additional proteins known from literature, revealed EGFL8 as a potential proliferation-reducing and neuritogenesis-inducing factor. Only one experiment with EGFL8 was performed as part of this master thesis. Nevertheless, EGFL8 showed a pronounced effect on STA-NB-6 cells at a concentration of 100 ng/mL. EGFL8 was recently described to be produced by repair type SCs upon injury and in culture [44]. Its low expression was shown as an unfavorable prognostic marker for colorectal and gastric cancer, respectively [72, 105]. However, the knowledge on its exact function is still very limited, and it has mostly been correlated with EGFL7, which shares similar structure and molecular weight in mice [106]. EGFL7 was shown to exhibit its neuritogenic activity on neural stem cells by enabling neural differentiation in the brain through inhibition of NOTCH [107]. EGFL8 might act in a similar way in the PNS, in promoting cell cycle arrest and axonal outgrowth. Since inhibition of NOTCH in NB was shown to induce cell cycle arrest and tumor shrinkage in xenograft mice [108], its potential role in neuronal differentiation via NOTCH should be further investigated.

EGFL8 and PTN could not be directly detected in the SC secretome because their antibodies were not included on the arrays used for analysis. Their levels in SC supernatants should be quantified by e.g. ELISA assays. Two additional co-cultivation experiments using the STA-NB-6 cell line were performed as part of this thesis and their supernatants were stored for future quantification of secreted factors, while the cells were FACS-sorted for RNA isolation and the RNA will be further sequenced.

NGF, BDNF, GDNF, CNTF were not identified by the protein arrays as differentially secreted between the co-cultivation/TW experiments and controls, probably because of their very low concentrations in cell supernatants. It is known, however, that Schwann cells are a rich source of NGF [109], BDNF and CNTF [4, 110], as well as GDNF [111], which is why they were chosen for the analysis. Thus, although easy to handle and appropriate for a comprehensive analysis, a limitation of the cytokine antibody arrays is low sensitivity.

Future experiments should investigate receptors and downstream pathways activated by PTN, IGFBP6 and EGFL8 in NB cells, resp., e.g. by introducing receptor-blocking antibodies together with transcriptomic analysis of cells cultivated in the presence or absence of these factors. Additionally, titration experiments are required to determine a possible concentration dependent effect on NB differentiation, proliferation and/or apoptosis.

4.5 SC recruitment plays an important role in tumor maturation

Similar experiments were recently conducted by Pajtler et al. who induced differentiation of TrkA-expressing NB cells by addition of SC-conditioned media to cells in culture [112]. However, their goal was different. They aimed at identifying key molecules upregulated by NB cells that would have the ability of SC attraction, i.e. proliferation and migration [112]. They identified NRG1 and furthermore, showed that it induces secretion of NGF by SCs [112], consequently leading to neuroblastoma cell differentiation. In agreement with this, substantial upregulation of NGF by SCs upon co-cultivation was here presented. This corroborates the hypothesis, that the SCs in favorable neuroblastoma, i.e. ganglioneuroma, are reactivated and recruited to the tumor site, where they are able to proliferate considerably, explaining their predominance in these tumors [22, 32, 113].

The human SC-NB co-cultivation model developed by the Tumor Biology group, CCRI, Vienna (manuscript in preparation), might be used in future studies for an in-depth research of these pathways with the aim of gaining insight into yet unknown SC-attracting/proliferation inducing molecules and exploring the SC-NB cross-talk.

4.6 The therapeutic aspect

Despite the extensive research that has been conducted in the last 2 decades, the 5-year overall survival rate of high-risk NB patients, that are over 1 year of age and present with metastatic disease, is still around 40-50% [18, 114]. Agents intending to induce neuroblastoma differentiation, such as 5-bromo-2'-deoxyuridine [115], 13-cis-retinoic acid [116] or agents that increase intracellular calcium [117] were already tested in clinics. Identification of biologicals and their application in neuroblastoma treatment has, however, been hampered by lack of knowledge and challenges in delivering growth factors, due to the severe side effects arising with systematic application, as was the case with the administration of NGF in numerous disorders [118, 119].

One example of a differentiation-inducing protein is PEDF. PEDF was identified in SC supernatants and later shown to be present in mature ganglionic cells and SCs in ganglioneuroblastomas and ganglioneuromas analyzed by immunostaining [39]. Moreover, recombinant PEDF administered in low doses had the ability to induce NB cell differentiation *in vivo* and *in vitro*, as well as increase the numbers and survival of Schwann cells [39]. Hence, the authors suggested that its clinical administration could stimulate a multifaceted antitumor feedback loop with the potential to limit tumor growth and indicated that additional SC-derived differentiating agents are yet to be discovered [39]. These findings are in line with the results presented in this work, which propose PTN, IGFBP6 and EGFL8 as potential anti-neuroblastoma components of the SC secretome. The discovered growth-impairing effects of PTN, IGFBP6 and EGFL8 form basis for further *in vivo* experiments. This may contribute to the development of new therapies for aggressive neuroblastoma.

4.7 An *in vivo* mouse model for studying SC-NB interaction

A pilot experiment showed that it is feasible to isolate and culture SCs from mouse peripheral nerves according to the protocol established for human SCs. Further, they were successfully co-cultivated *in vitro* with human STA-NB-6 cells, and mouse SC and NB cell proliferation was measured in the co-cultivation experiment. The number of SCs obtained was, however, very low, since the small size of mouse peripheral nerves made the isolation challenging. Thus, for future experiments, SCs from several animals may be pooled to allow e.g. *in vitro* co-cultivation experiments. Moreover, culture conditions need adaptation. Nevertheless, the pilot experiment shows that it would be feasible to establish a NB xenograft model to validate the *in vitro* findings and test the effect of PTN, and other factors, on neuroblastoma growth *in vivo*.

Neuroblastoma xenograft models in athymic (nu/nu) mice have so far been used for *in vivo* studies of the SC-NB interaction [112, 120]. Pajtler et al. inoculated a mixture of rat SCs and NB cells, suspended in matrigel, subcutaneously (s.c.) in the flank of mice [112]. In this way, they could show that NTRK1 induction in neuroblastoma xenografts mixed with primary SCs significantly reduces tumor growth *in vivo* [112]. On the other hand, Liu et al. injected NB cells intrafascicularly to show that mSCs can infiltrate the tumor and promote human neuroblast differentiation, induce apoptosis, as well as inhibit proliferation and angiogenesis [120].

Xenograft models have also been used to test the effect of SC-secreted factors, such as PEDF and SPARC [38, 39]. Crawford et al. treated fully established tumors with recombinant PEDF injections s.c. into 3 sites/tumor/day, which they continued for 4 days [39]. They were able to show that even a short treatment with recombinant PEDF causes local tumor differentiation [39]. An interesting method was used by Chlenski et al., in which purified SPARC was delivered continuously for 3 weeks with s.c. implanted osmotic pumps [38]. SPARC was released at a rate of 62.5 ng/h [38]. In this manner, they showed complete cessation of tumor growth for the first 2 weeks in SPARC-treated mice, while, during the third week, a slight increase in tumor size was observed, with the tumor volume still being significantly smaller than in control animals [38].

Continuous delivery of recombinant protein neuritogens, would be suitable also in the case of the newly identified PTN and other factors. According to the *in vitro* findings in this study, treatment should be carried out for at least 17 days and daily doses adjusted to prior dose-response testing. Continuous treatment via pumps should be compared with intra-tumoral application to identify the optimal way of application. Furthermore, sets of experiments with varying concentrations could be conducted 2 or 3 weeks after injection of NB-provoking cells, when the tumor is established, to test the effect on tumor growth, in combination with experiments immediately after injection. In this way, it could also be tested if PTN has an influence on tumor incidence.

5 CONCLUSION

This study identified PTN as a potential factor involved in the Schwann cell-neuroblastoma interaction that causes decrease of cell proliferation and increase of cell differentiation when added to cell media of STA-NB-6 cells at a concentration of 50 ng/mL for 17 days. NGF was confirmed as a neuritogenic factor in STA-NB-6 cells at a concentration of 20 ng/mL. Additionally, IGFBP6 and EGFL8 were identified as potential SC-secreted proteins that affect STA-NB-6 cell growth. The combination of all factors (PTN, NGF, IGFBP6, EGFL8, BDNF, GDNF, CNTF and FGF7) had no significant effect on STA-NB-6 cell proliferation and differentiation. There was no significant effect of any of the factors at the tested concentrations on cell proliferation or differentiation on the STA-NB-10 cell line.

6 SUMMARY

6.1 SUMMARY

Schwann cells are essential for the maturation process of benign forms of neuroblastoma, ganglioneuroma, acting via neuritogenic, anti-proliferative and pro-apoptotic signals. The goal of this master thesis was to identify Schwann cell-secreted proteins involved in the cross-talk with neuroblastoma cells, and define the concentration and combination needed to induce a growth-impairing effect in neuroblastoma cells. Eight factors were chosen based on protein array results, RNA-sequencing data and literature research: IGFBP6, FGF7, CNTF, PTN, NGF, BDNF, GDNF and EGFL8. They were functionally validated in vitro by cultivating two neuroblastoma cell lines derived from aggressive tumors, STA-NB-6 and STA-NB-10, in presence of corresponding recombinant proteins. The effects were measured by flow cytometry. PTN, IGFBP6 and EGFL8 were identified as Schwann cell-secreted proteins that cause reduction of cell proliferation and induction of neuronal-like differentiation of STA-NB-6 cells, when individually added to culture media for 17 days. NGF was confirmed as a neuritogenic factor of STA-NB-6 cells. The combination of all factors had no significant effect on STA-NB-6 cells. The factors had no significant effect on STA-NB-10 cells. This work forms basis for further in vitro and in vivo experiments to address the effect of these factors on neuroblastoma growth in xenograft models.

6.2 SAŽETAK

Schwannove su stanice neophodne za proces sazrijevanja benignih tipova neuroblastoma, ganglioneuroma, djelujući kroz neuritogenične, anti-proliferativne i pro-apoptostske signale. Cilj ovog diplomskog rada bio je identificirati proteine koje izlučuju Schwanove stanice, a koji sudjeluju u njihovoj interakciji sa stanicama neuroblastoma, te definirati koncentraciju i kombinaciju potrebnu za inhibiciju rasta stanica neuroblastoma. Usporedbom rezultata dobivenih pomoću analize proteina i podataka dobivenih sekvenciranjem transkriptoma s podacima iz literature, izabrano je osam faktora: IGFBP6, FGF7, CNTF, PTN, NGF, BDNF, GDNF i EGFL8. Za funkcionalnu potvrdu njihova djelovanja in vitro, odgovarajući su rekombinantni proteini dodani u medij korišten za kultiviranje dviju staničnih linija neuroblastoma porijeklom iz agresivnih tumora, STA-NB-6 i STA-NB-10, te je učinak izmjeren pomoću protočne citometrije. Ovo je istraživanje identificiralo PTN, IGFBP6 i EGFL8 kao proteine koje izlučuju Schwannove stanice, koji, kada su individualno dodani staničnom mediju tijekom 17 dana, smanjuju proliferaciju i potiču diferencijaciju STA-NB-6 stanica. NGF je potvrđen kao neuritogenični faktor STA-NB-6 stanica. Kombinacija svih navedenih faktora nije imala značajni učinak na STA-NB-6 stanice. Faktori nisu imali značajan učinak na STA-NB-10 stanice. Ovaj rad daje osnovu za daljnja in vitro i buduća istraživanja in vivo, za ispitivanje učinka navedenih faktora na rast neuroblastoma metodom staničnog eksplantata.

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8 CURRICULUM VITAE

8.1 EDUCATION

2013-2017 Master of Molecular Biology, University of Zagreb, Croatia

- Semester abroad at the University of Natural resources and life sciences (BOKU), Vienna, 2/2015-7/2015
- Scientific observer at the Institute of Cancer Research (ICR), Vienna, 7/2015-8/2015
- Internship at the Institute for Children's Cancer Research (CCRI), Vienna, 9/2015-9/2016
- Master thesis: Analysis of the anti-proliferative effects of the Schwann cells on neuroblastoma cells – Advisors: Assoc. Prof. Peter F. Ambros, PhD and doc.dr.sc Petra Korać

2010-2013 Bachelor of Molecular Biology, University of Zagreb, Croatia

- Bachelor thesis: Metastasis of Cancer Cells - Advisor: doc.dr.sc. Inga Marijanović

2006-2010 Classical Gymnasium, Zagreb, Croatia

8.2 PUBLICATIONS

Machine Learning Framework incorporating Expert Knowledge in Tissue Image Annotation. Florian Kromp, Inge Ambros, Tamara Weiss, Dominik Bogen, Helena Dodig, Maria Berneder, Teresa Gerber, Sabine Taschner-Mandl, Peter F. Ambros, Allan Hanbury. Paper accepted as part of the 23rd International Conference on Pattern Recognition, December 4-8, 2016, Cancún, México.

Deciphering the Cross-Talk between Schwann Cells and Neuronal Cells Identifies Epidermal Growth Factor Like Domain 8 (EGFL8) to Affect Neuronal Differentiation in Nerve Repair and Neuroblastic Tumors. Tamara Weiss, Sabine Taschner-Mandl, Helena Dodig, Fikret Rifatbegovic, Andrea Bileck, Christian Frech, Max Kauer, Reinhard Windhager, Hugo Kitzinger,Chieh-Han Tzou, Christopher Gerner, Inge M Ambros, Peter F. Ambros, 2017, manuscript in preparation.

8.3 PROFESSIONAL EXPERIENCE

Laboratory Exercises Assistant for the Molecular genetics course, Faculty of Science, Zagreb, summer semester, 2014

Scientific Observer, **ICR**, Vienna, 7/2015-8/2015
Mice handling, AOM/DSS model for CRC-inducement in mice, real-time qPCR, IHC, quant. histomorphometry

Intern, **CCRI**, Tumor Biology Group, Vienna, 9/2015-9/2016
Isolation of Schwann cells (SC) from human and mouse PN tissue, SC culturing, human neuroblastoma cell-culturing, co-culturing, transwell cultures, protein extraction, Western blot, cytokine antibody arrays, RNAseq data analysis with Qlucore Omics Explorer, flow cytometry and FACS, ChIP, data presentation on seminars/conferences, InDesign

ADDITIONAL: **Promoter** of food supplements in pharmacies, Almagea, Zagreb, 2/2014-11/2014 / **Head of Public Relations** for Visioneers, a non-profit student organization formed as part of a business course at the WU, Vienna, 3/2015-10/2015

8.4 CONFERENCES/PRESENTATIONS

inPharma OMEGA-3, September 12, 2014, Zagreb, Croatia participant, as part of the Almagea team

HDIR-3: "FROM BENCH TO CLINIC" **-** The 3rd meeting with international participation, November 6-7, 2014, Zagreb, Croatia – participant, as student

29th Annual pediatric oncology meeting of the Kind-Philipp Foundation, June 1-3, 2016, Wilsede, Germany– oral presentation: Schwann cells secrete factors that impair neuroblastoma growth, part of the Solid Tumors/Brain Tumors –Biology, Genetics and New Therapeutic Strategies session

8.5 AWARDS/FELLOWSHIPS

Rector's prize awarded by the University of Zagreb
For the organization of 'Night of Biology', a scientific popular manifestation of the Faculty of Science, Zagreb, 2012

Erasmus+ program

- Fellowship granted for an exchange semester at the University of Natural resources and life sciences (BOKU), Vienna, 2015
- Fellowship granted for an internship at the Institute for Children's Cancer Research (CCRI), Vienna, 2/2015-9/2016

8.6 LANGUAGES

CROATIAN (native speaker) **/ ENGLISH** (C1.1) / **GERMAN** (B 2.2) / **FRENCH** (A1)