Natural Killer-Derived Exosomal miR-186 Inhibits Neuroblastoma Growth and Immune Escape Mechanisms

Paolo Neviani, Petra M. Wise, Mariam Murtadha, Cathy W. Liu, Chun-Hua Wu, Ambrose Y. Jong, Robert C. Seeger, and Muller Fabbri

Abstract

In neuroblastoma, the interplay between immune cells of the tumor microenvironment and cancer cells contributes to immune escape mechanisms and drug resistance. In this study, we show that natural killer (NK) cell–derived exosomes carrying the tumor suppressor microRNA (miR)-186 exhibit cytotoxicity against MYCN-amplified neuroblastoma cell lines. The cytotoxic potential of these exosomes was partly dependent upon expression of miR-186. miR-186 was downregulated in high-risk neuroblastoma patients, and its low expression represented a poor prognostic factor that directly correlated with NK activation markers (i.e., NKG2D and DNAM-1). Expression of MYCN, AURKA, TGFBR1, and TGFBR2 was directly inhibited by miR-186. Targeted delivery of miR-186 to MYCN-amplified neuroblastoma or NK cells resulted in inhibition of neuroblastoma tumorigenic potential and prevented the TGFβ1-dependent inhibition of NK cells. Altogether, these data support the investigation of a miR-186–containing nanoparticle formulation to prevent tumor growth and TGFβ1-dependent immune escape in high-risk neuroblastoma patients as well as the inclusion of ex vivo–derived NK exosomes as a potential therapeutic option alongside NK cell–based immunotherapy.

Significance: These findings highlight the therapeutic potential of NK cell–derived exosomes containing the tumor suppressor miR-186 that inhibits growth, spreading, and TGFβ-dependent immune escape mechanisms in neuroblastoma.

Introduction

Neuroblastoma, the most common extracranial childhood cancer, is a malignancy of the embryonal sympathetic nervous system derived from highly proliferative migratory cells of the neural crest. It is characterized by a highly heterogeneous clinical behavior based on the patient’s risk at the time of diagnosis, and response to treatment depends on several factors such as age, site of primary tumor, histology, lymph node involvement, and biological features (1). Age at diagnosis is a strong prognostic factor; in fact, whereas children diagnosed under 1 year of age show survival rates of up to 95%, this drops to 68% for children ages 1 to 14 years, and overall 5-year survival for all patients regardless of risk group, is 71% (2). Based on the International Neuroblastoma Staging System (INSS), patients can be stratified in 5 different risk groups (1, 2, 3, 4, and 4S) ranging from spontaneously regressing to highly malignant and metastatic tumors (3). Regardless of staging, the amplification of the oncogene MYCN, found in 25% of cases, is the single strongest biological marker predicting poor prognosis and drug resistance (4). MYCN expression has been found to positively correlate with metastatic behavior, epithelial–mesenchymal transition (EMT), impaired immune surveillance, cell-cycle progression, and maintenance of a stem cell–like state (4). Because of its secondary structure and the lack of surfaces for small-molecule binding, efforts in therapeutically targeting MYCN have been futile, and to date myc family proteins are considered undruggable; hence, indirect targeting is currently under investigation (1, 4). Among genes highly expressed in MYCN-amplified neuroblastoma, Aurora Kinase A (AURKA) is physically associated with MYCN and promotes its stability by inhibiting its proteasome-dependent degradation (5). Therefore, AURKA has emerged as a potential target to promote MYCN downregulation; indeed, its targeting with shRNA or small molecules (e.g., MLN8237; CD532) resulted in MYCN degradation and has shown promising results in preclinical studies (5–7).

In an effort to overcome drug resistance in high-risk neuroblastoma, understanding the role of the tumor microenvironment (TME) is of extreme importance. Cancer cells are able to profoundly influence the biology of local immune cells, such as tumor-associated macrophages (TAM) and natural killer cells, by establishing immune escape mechanisms that inhibit the innate antitumoral immunity response and induce a proinflammatory and protumoral microenvironment (8).

CD163+ TAMs are the most abundant stromal component in the neuroblastoma microenvironment and are known to produce...
immunosuppressive cytokines and several factors (e.g., TGFβ, IL4, IL10, and VEGF) that support angiogenesis and metastatic potential (9). Although high infiltration of TAMs at the tumor site has poor prognostic potential, by contributing to drug resistance (9–11), abundance of activated natural killer (NK) cells in the TME cells is a favorable prognostic factor when their cytotoxic activity is sustained by a supportive cytokine profile (12). Unfortunately, in high-risk neuroblastoma, high levels of TGFβ1 have been detected in the microenvironment, causing suppression of the cytotoxicity of NK cells by downregulating the expression of cytotoxicity receptors (e.g., DNAM-1 and NKG2D) and altering their chemokine receptor repertoire (e.g., CXCR4, CXCR3, and CX3CR1; refs. 13–18). Furthermore, high local production of TGFβ1 and activation of the canonical (i.e., SMADs) and noncanonical TGFβ pathways (e.g., ROCK1 and MAPK1) promote neuroblastoma invasiveness by inducing EMT (19–21). Therefore, given the increasing evidence of the role of the TME in tumorigenesis, it is important to identify not only cancer cell–specific targets but also other microenvironment-associated factors that are responsible for the establishment of a drug-resistant phenotype. In elucidating the role of NK cells in the interplay between cancer cells and the TME, it has been recently reported that exosomes derived from NK cells exert cytotoxic activity against cancer cells (22–24). Here we show that the cytotoxicity of NK exosomes depends not only on the presence of canonical killer molecules (i.e., perforin 1, granymes A and B) but also on their nucleic acid cargo. Interestingly, we and others have shown that miRNAs can be transferred between immune and cancer cells within the TME, and that these miRNAs may target factors important for tumorigenesis and the establishment of immune escape mechanisms (8, 10). Here we show that the tumor suppressor miR-186 is present in NK-derived exosomes and that its expression is downregulated in high-risk neuroblastoma. Importantly, miR-186 is in silico predicted to target MYCN, ALUKA, and the TGFβ pathway. Ectopic delivery of miR-186 to neuroblastoma cells and to NK cells impaired the survival and migration of MYCN-amplified neuroblastoma cells both in vitro and in vivo and prevented the TGFβ1-dependent inhibition of NK cytotoxicity. Importantly, NK-derived exosomes were able to efficiently kill MYCN-amplified neuroblastoma cells regardless of the activation status of the parental NK cells. Moreover, modulation of miR-186 abundance in these exosomes altered their cytotoxic potential, suggesting that miR-186 may be at least in part responsible for their cytotoxic activity and that NK exosomes may be insensitive to TGFβ1-dependent inhibition and may represent another viable strategy to overcome immune escape mechanisms in neuroblastoma.

**Materials and Methods**

**Cell lines and primary cells**

The MYCN-amplified CHLA-136 and LAN-5 (obtained from the Children's Oncology Group) and nonamplified CHLA-255 (described in refs. 25) human neuroblastoma cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Life Technologies) containing either 10% heat-inactivated fetal bovine serum (FBS; for CHLA-255) or 20% FBS (for CHLA-136 and LAN-5) supplemented with 50 U of penicillin/mL and 0.1 mg of streptomycin/mL at 37°C in a 5% CO₂ humidified atmosphere. The CHLA-255-Fluc, CHLA-136-Fluc, LAN-5-Fluc, CHLA-136-miR-186, and CHLA-miR-136-EV cells were generated by lentiviral transduction, a firefly luciferase lentiviral vector. For NK cell propagation, peripheral blood mononuclear cells were Ficoll isolated from healthy donors and cocultured with irradiated (100 Gy) K562.mblL21 (26) at a 1:1 ratio in RPMI1640 with 10% FBS and 50 U/mL of rhIL2 (PeproTech). K562.mblL21 cells were replenished at day 7 and rhIL2 added every 3 days. At day 14, the purity of NK cells and T-cell contamination was determined by CD56/CD16/CD3 immunostaining, followed by flow cytometry analysis (Supplementary Fig. S1A). The expanded NK cells were viably frozen at day 14. Frozen aliquots of NK cells were thawed and kept in the presence of 150 U/mL rhIL2 for at least 24 hours, then washed three times in PBS and starved for at least 24 hours before the addition of 100 ng/mL of rhIL15 and/or exposure to CD56-coated miR-186–loaded nanoparticles. All cells were tested to be Mycoplasma free before performing experiments. All cells were kept in culture for 24–48 hours after splitting and before doing any experiments, so that they had time to adhere to the bottom of the flask. When they reached 80% confluence, experiments were performed with the indicated timing.

**Transfection and lentiviral transduction**

The miR-186-5p mimic (MC17153) and inhibitor (MI17153) and corresponding negative controls (Life Technologies) were packaged into artificial exosomes using DOTAP transfection reagent (Roche) according to the manufacturer’s protocol. Lentiviral pseudotyped particles were produced by Lipo negligence (2000 Life Technologies) mediated transfection of 293TN cells (System Biosciences) with the lentiviral vector, the psPAX2 packaging construct (gift from Dr Didier Trono, addgene plasmid #12260), and a plasmid carrying the G-glycoprotein of vesicular stomatitis virus (VSV-G). Viral supernatant was collected 36 hours after transfection and filtered through a 0.45-μm membrane. Cells were incubated for 6 hours with the viral supernatant and 8 μg/mL of hexadimethrine bromide (Sigma-Aldrich) and FACs sorted 48 hours after transduction.

**Immunoblotting**

Cells were lysed in RIPA buffer (150 mmol/L NaCl, 1% NP-40, 0.1% SDS, 50 mmol/l Tris [pH 8.0]) supplemented with 1 μg/mL Aprotinin, 1 mmol/L DTT, 0.2 mmol/L PMSF 1 μg/mL peptatin A. The protein concentration was determined by Bradford Protein Assay (Bio-Rad) and an equal amount of proteins was resolved by SDS gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to a standard immunoblotting protocol. For the analysis of size exclusion chromatography (SEC) eluted fractions, the proteins were isolated by trichloroacetic acid (Sigma-Aldrich) precipitation followed by acetone washes of the protein pellets. Precipitated proteins were resuspended directly in Laemmli Sample buffer and resolved by SDS gel electrophoresis. The antibodies used were as follows: rabbit polyclonal anti-Aurora A/AIK #3092, rabbit monoclonal anti-SMAD3 #9523, anti-SMAD2 #5339 (Cell Signaling); mouse monoclonal anti-Tsg101 sc-7964, anti-TGFβ RI sc-17791, anti-fibronectin sc-8422, anti-perforin 1 sc-373943, anti-HSP70 sc-24, anti-Alix sc-53540 and rabbit polyclonal anti-Calnexin sc-11397 (Santa Cruz Biotechnology, Inc.); mouse monoclonal anti-MYCN Ab16898 and rabbit polyclonal anti-TGFβ RI Ab31013 (Abcam); rabbit polyclonal anti-CD81 EXOAB-CD81A-1 (System Biosciences); mouse monoclonal anti-granzyme A #507202 and anti-granzyme B #674302 (BioLegend).
RNA isolation and qPCR

Total RNA was extracted from cells and exosomes using TRIzol reagent (Invitrogen). Reverse transcriptions were performed using the iScript cDNA Synthesis Kit (Bio-Rad) for mRNA expression, and the TaqMan miRNA Reverse Transcription kit (Life Technologies) for mature miRNA expression. Quantitative real-time PCR (qPCR) of miRNAs was performed using commercially available TaqMan MicroRNA Assay primers and probes. For mRNA qPCR, previously validated primer pairs were identified on the PrimerBank database (https://pga.mgh.harvard.edu/primerbank/index.html) and used in SYBR green chemistry-based qPCR protocols. All qPCR analyses were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with iTaq Universal SYBR Green Supermix (Bio-Rad) or TaqMan Fast PCR Advanced Master Mix (Life Technologies) formed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) or TaqMan Fast PCR Advanced Master Mix (Life Technologies) for mRNA or miRNA expression analysis, respectively. Relative mRNA or miRNA expression levels were calculated using the 2^(-ΔΔCt) method and normalized by expression levels of GAPDH mRNA or of U6 snRNA, respectively. For exosomal RNA, levels of miRNA expression were normalized by the total amount of RNA and reported as 2^-Ct. Primer sequences are reported in Supplementary Table S1.

Luciferase reporter assays

For the luciferase reporter assays, we used the CHLA-255 neuroblastoma cell line. 2 x 10^6 cells in 200 μL were transfected with the indicated reporter plasmids and miR-186 or scrambled control at a final concentration of 100 nmol/L. Transfections were carried out by electroporation in a GenePulser Xcell electroporation system (Bio-Rad) with the following settings: exponential; 250 V, 975 μF, Ω 4 mm. Transfected cells were then incubated for 48 hours and both firefly and renilla luciferase activities were assessed using the Dual-Luciferase Reporter Assay protocol in a GloMax-Multi luminometer (Promega). The renilla luciferase activity was normalized by firefly luciferase activity and expressed as change relative to the value of the negative control, which was set as 1.

Growth curves

Neuroblastoma cells/well (25 x 10^3–50 x 10^3 cells) were seeded in a 12-well plate; after overnight incubation, the cells were transfected with 100 nmol/L of scrambled or miR-186 mimic in triplicates in IMDM with 1% BSA. Viable cells were detected by trypan blue dye exclusion assays and counted with a TC10 automated cell counter (Bio-Rad) at the indicated time points.

Migration and wound-healing assay

LAN-5 cells (1 x 10^3) transfected with 100 nmol/L of scrambled or miR-186 mimic were seeded on top of 8-μm polycarbonate transwell inserts. Migration assays were carried out in 2% FBS/IMDM. SDIF1t (100 ng/mL) was added to the bottom well. After 4 hours, the top side of the transwell was wiped to remove nonmigrated cells, and the migrated cells were stained with the Protocol Hema 3 staining kit (Fisher HealthCare). Stained membranes were mounted on slides, and visualized at a 10 x magnification; 9 representative fields per membrane were acquired, and the number of cells was averaged across three replicates.

For the wound-healing assay, LAN-5 or CHLA-136 cells were grown to confluency in 6-well plates and transfected with 100 nmol/L of scrambled or miR-186 mimic. The monolayers were scratched with a pipette tip and cultured for 6 days. Images of the scratches were acquired at time 0, and at 3 and 6 days (4 fields/well) and the average distance of cell migration determined by Imagel software. The migration rate was expressed as the size of the scratch 6 days after culture relative to the size at time zero.

Flow cytometry analysis

Cells were stained with the following antibodies: PE anti-GD2, PE/Cy7 anti-CD56 (NCAM), PE anti-CD16 (3G8), FITC anti-CD3 (HIT3a; BioLegend); and PE anti-CXCR4 (BD Biosciences). Stained cells were analyzed in a BD LSR II flow cytometer (BD Biosciences).

Imaging flow cytometry

The RNA cargo of NK exosomes was stained with the SYTO RNASelect Green Fluorescent dye and the excessive unincorporated dye was removed with Exosome Spin Columns following the manufacturer’s protocol (Thermo Fisher). CHLA-136 and LAN-5 cells were exposed to stained and NK exosomes for 18 hours and acquired on the Imagestream X Mark II imaging flow cytometer and analyzed with the IDEAS software (Amnis Corporation). Cells were visualized at a 40 x magnification and excited with a 488-nm laser; the emission from internalized exosomes was detected by 533/55 bandpass filter, and brightfield images were used to determine the cell boundaries.

Constructs and mutagenesis

psiCHECK-2 luciferase reporter vectors. Potential miR-186 target sequences were in silico predicted at www.microrna.org or www.targetscan.org. The full-length 3’UTRs of MYCN, AIURKA, and fragments (containing a potential miR-186 target sequence) of TGFBR1 (nucleotides 3551–4166), SMAD2 (nucleotides 601–821), and SMAD3 (nucleotides 843–1037; 1357–1573; and 2278–2508) 3’UTRs were PCR amplified from LAN-5 genomic DNA and cloned between the Xhol and NotI restriction sites of the psiCHECK-2 dual-luciferase reporter vector (Promega). Cloning primer sequences are reported in Supplementary Table S1. The psiCHECK-2-TGFBR2-3UTR-wild-type (WT) construct was a gift from Robert Blelloch (University of California, San Francisco, CA; Addgene plasmid # 31882, ref. 27). The miR-186 7/8-mer seed regions in the 3’UTRs of MYCN, AIURKA, TGFBR1, and TGFBR2 were deleted with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent).

pSBE4. The SBE4-Luc smad-reporter plasmid was a gift from Bert Vogelstein (Johns Hopkins University, Baltimore, MD; Addgene plasmid # 16495; ref. 28).

Lentiviral pCDH vectors. The human full-length MYCN and AIURKA were PCR amplified from LAN-5 cDNA and cloned between the XbaI and EcoRI sites of the pCDH-CMV-MCS-EF1-copGFP (CD511B-1) lentiviral vector (System Biosciences).

Anti-CD56 or anti-GD2 antibody-conjugated anionic lipopolyplex nanoparticles

The preparation of antibody-conjugated nanoparticles has been previously described (29). The DSPE-PEG2000 maleimide-conjugated antibodies used to coat the surface of the lipopolyplex nanoparticles were as follows: LEAF purified anti-CD56 (clone HCD56, 318324; BioLegend) and chimeric anti-GD2/CH14.18 (United Therapeutics Corp).
NK exosome isolation by SEC

For NK exosome preparation, NK cells were cultured for 48 hours in 10% FBS/RPMI medium that had been cleared of bovine exosomes by an ultracentrifugation step for 1 hour at 100,000 × g. The supernatant was centrifuged at 300 × g for 10 minutes, followed by centrifugation at 3,000 × g for 10 minutes. The clarified supernatant was then concentrated to approximately 500 μL on a 100 KD Amicon Ultra Centrifugal filter (Millipore). The NK exosomes were then isolated from the concentrated supernatant by SEC. Sphacryl S-300 High Resolution (GE Healthcare) was packed on a glass econo-column chromatography column (Bio-Rad; 10 cm height, 1.5 cm diameter). The column was washed with 0.32% sodium citrate in PBS and the supernatant was loaded onto the column and allowed to enter the resin by gravity flow. The eluate was collected in 23 fractions of 15 drops (~500 μL) on a Model 2110 Fraction Collector (Bio-Rad). For each fraction, the presence of exosomes was determined by nanoparticle tracking analysis, protein concentration and anti-CD81, anti-calnexin, anti-TSG101, and anti-fibronectin immunoblotting. The exosome-containing fractions were then further concentrated to 1/100th of the original supernatant.

Transmission electron microscopy

Exosomes isolated by SEC were precipitated with ExoQuick (System Biosciences) following the manufacturer’s instruction, to obtain a discreet pellet. Pelleted exosomes were immediately fixed in 2.5% glutaraldehyde. The exosomes were then post-fixed with 1% Osmium Tetroxide, dehydrated with alcohols, embedded in a mold with eponate resin and cured overnight in an oven. Ultra-thin (80–100 nm) sections were cut at with a diamond knife, and onto copper grids with formvar. The grids are stained with uranyl acetate and lead citrate to give the images more contrast. Samples were imaged with an FEI Morgagni transmission electron microscope with Olympus imaging software.

NK cytotoxicity assay

Frozen aliquots of 15-day-old K562.mbIL21-expanded aNK cells were thawed and cultured in 10% FBS/RPMI1640 with 150 U/mL of rhIL-2 for at least 48 hours. For the cytotoxicity assays, NK cells were washed twice in PBS and starved from growth factors in 10% FBS/RPMI1640 for 24 hours. NK cells were then treated with 100 ng/mL of rhIL-15, and/or 10 ng/mL of rh TGFβ1, and/or anti-CD56 labeled miR-186 carrying nanoparticles. The cytotoxicity was assessed by seeding CHLA-136-Fluc cells (2 × 10^5 cell/well) into individual wells of a 96-well plate together with a 2-fold serial dilution of an NK cell line. The eluate was weighed, and homogenized; the expression of miR-186 in livers was detected as a non-GD2–expressing tissue control. For survival studies, mice were treated for up to 60 days and humanely euthanized according to the IACUC guidelines.

Statistical analysis

Statistical data are presented as mean ± standard deviation. Statistical significance was calculated by two-tailed Student t test. The survival analysis was calculated by the Kaplan–Meier method and the log-rank test. The GraphPad Prism version 6.0h software was used for statistical analyses.

Results

NK cells secrete cytotoxic exosomes that carry miR-186

NK cells were isolated and expanded from the peripheral blood from healthy donors and their purity confirmed by immunophenotyping after 14 days in coculture with the feeding cell line K562.mbIL21 (Supplementary Fig. S1A). To isolate extracellular vesicles, the supernatant of NK cells was concentrated and processed by SEC. The presence of extracellular vesicles in the SEC fractions was confirmed by nanosight tracking analysis (Fig. 1A, left). Most particles were detected in fractions 13 and 14, had a median diameter of 92.45 nm, and had a structure revealed by transmission electron microscopy consistent with the expected size and morphology of exosomes (Supplementary Fig. S1B; ref. 30). Moreover, immunoblots were performed to further characterize the particles following the guidelines of the International Society of Extracellular Vesicles (30). Accordingly, NK-derived exosomes were positive for the tetraspanin transmembrane marker CD81, the cytosolic proteins TSG101 and HSP70, the endosomal associated protein ALIX, and the extracellular protein fibronectin and negative for the intracellular protein calnexin, used as a cell contamination marker (Fig. 1A, right).

When assessed in cytotoxicity assays, IL15-activated NK cells and their exosomes were able to kill neuroblastoma cells in a dose-dependent manner (Fig. 1B); interestingly, although the cytotoxicity of NK cells starved from growth factors or treated with TGFβ1 was inhibited, their exosomes showed killing equal to exosomes derived from activated NK cells (Fig. 1B). Notably, exosomes derived from inactivated NK cells had low levels of the killer molecules perforin 1, granzyme A, and granzyme B, while still retaining their cytotoxic activity (Fig. 1C), suggesting that the cytotoxic activity of NK exosomes may be, in part, dependent on other factors (e.g., exosomal miRNAs and/or mRNAs) other than
Exosomal miR-186 Circumvents Immune Escape Mechanisms

Figure 1.

A, Left, particle number (by nanosight tracking analysis) and total protein concentration in SEC fractions 9 through 23. Right, immunoblotting showing the expression of CD81, fibronectin, TSG101, calnexin, Alix, and HSP70 in SEC fractions 9 through 25; NK cell lysate (lane 1) was used as a control. B, Cytotoxic potential of NK cells or NK exosomes assessed by luciferase reporter cytotoxicity assays. CHLA-136-Fluc cells (target cells) were exposed to increasing numbers of NK cells (effector cells) for 6 hours or increasing NK exosome particle numbers for 24 hours. NK cells were starved from growth factors or IL15 activated for at least 48 hours or inhibited by TGFβ1 treatment for 4 days; exosomes were isolated from the corresponding supernatants by SEC. Cytotoxicity is expressed as a percent change of relative lumintometry units (RLU) compared with the untreated control. C, Immunoblotting showing the expression of perforin 1, granzyme A, and granzyme B in NK exosomes derived from NK cells starved from growth factors or IL15 treated for 48 hours; densitometry numbers underneath blots are expressed as arbitrary units normalized to levels of TSG101, used as loading control for the exosomes. D, Comparison of cytotoxic potential of exosomes derived from NK cells or K562.mbIL21 cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Student t test, two-tailed.

on classic NK cytotoxic proteins. To prove that the exosome-dependent cytotoxicity is NK exosome specific and not due to the presence of K562.mbIL21-derived exosomes, K562 were isolated and tested in cytotoxicity assays: as expected, the exosomes derived from this unrelated, noncytotoxic, myeloid leukemia cell line did not exert any cytotoxic effect on target cells, compared with exosomes derived from IL15-activated NK cells (Fig. 1D). To elucidate the potential role of the miRNA payload in NK exosomes, we profiled the top miRNAs represented in the NK exosomes. Among these, the tumor suppressor miR-186-5p (NR_029707) was predicted in silico to target factors important for the survival of neuroblastoma cells (e.g., MYCN and AURKA) and several components of the TGFβ pathway (e.g., TGFB1, TGFB2, SMAD2, and SMAD3; Table 1), which is involved in the...
Table 1. In silico–predicted targets of miRNAs found in NK exosomes

<table>
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<tr>
<th>miRNA</th>
<th>Ct</th>
<th>MYCN</th>
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**Table 1.** In silico–predicted targets of miRNAs found in NK exosomes

**miRNA**

- **hsa-miR-17-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-166-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-155-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-122-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-16-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-19b-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-484-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-146b-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-24-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-150-5p**: Predicted targets of miRNAs found in NK exosomes
- **hsa-miR-342-5p**: Predicted targets of miRNAs found in NK exosomes

**Ct**: Ct value for each miRNA

**MYCN**: Predicted targets of miRNAs found in NK exosomes

**TGFBR1**: Predicted targets of miRNAs found in NK exosomes

**SMAD2**: Predicted targets of miRNAs found in NK exosomes

**SMAD3**: Predicted targets of miRNAs found in NK exosomes

**AURKA**: Predicted targets of miRNAs found in NK exosomes

**NOTE**: Text appearing in bold highlights the miRNA that has been further considered in this study.

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TGFβ-dependent inhibition of NK cytotoxicity, a common immune escape mechanism in neuroblastoma.

**miR-186-5p expression is low in high-risk and MYCN-amplified neuroblastoma and correlates with the expression of NK activation markers**

Because miR-186 has been previously described as a tumor suppressor, we evaluated its expression in a publicly available RNA-seq data set containing 498 primary neuroblastoma samples (FDA SEQC NB) with the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl; ref. 31). Patients were stratified by risk (high-risk: stage 4 disease in patients older than 18 months at diagnosis and patients of any age and stage with MYCN-amplified tumors), by MYCN-status, or by INNS ranking (32). We found miR-186 expression to be significantly downregulated in high-risk versus low-risk, MYCN-amplified versus nonamplified, and stage 4 versus the lower-risk stages (Fig. 2A). Remarkably, patients with low expression of miR-186 showed significantly shorter event-free survival and overall survival probability, consistent with a role of miR-186 as a bona fide tumor suppressor (Fig. 2B; refs. 33–40).

In another publicly available data set (GEO#: GSE16237), RNA from 51 neuroblastoma samples was analyzed by Affymetrix array, and miR-186 was found to positively correlate with the NK activation markers NK22D and DNAM-1 (Fig. 2C), suggesting that high expression of miR-186 may be associated with the presence of activated NK cells in neuroblastoma. Moreover, analysis of the expression of NK22 and DNAM-1 revealed that their expression is significantly downregulated in the higher-risk categories of neuroblastoma and that their low expression is a poor prognostic factor in neuroblastoma (Supplementary Fig. S1C), underscoring the importance of NK cell activation in the neuroblastoma microenvironment. Interestingly, significantly lower expression of miR-186 was detected in TGFβ-treated NK cells compared with IL15-activated NK cells (Fig. 2D), suggesting that the expression of miR-186 may be inhibited by TGFβ signaling and may correlate with TGFβ-regulated NK cytotoxicity. Moreover, we confirmed that NK exosomes carry miR-186 (Fig. 2E) and deliver their RNA cargo (including miR-186) to the MYCN-amplified neuroblastoma cell lines CHLA-136 and LAN-5 (Fig. 2F and G). Interestingly, when CHLA-136 and LAN-5 cells were exposed to control K562.mbl21 exosomes, we did not see an increase of miR-186, suggesting that this miR-186 is specifically delivered by NK exosomes and not by exosomes derived from the feeding K562.mbl21 cells (Fig. 2G).

**miR-186-5p targets neuroblastoma oncogenes**

To evaluate the role of miR-186 in MYCN-amplified neuroblastoma, we transiently transfected a mimic miR-186 or a scrambled sequence into the MYCN-amplified CHLA-136 or LAN-5 cell lines by treatment with artificial exosomes (Dotap-miR-186 or Dotap-sc). Elevated miR-186 expression was detected up to 72 hours after transfection compared with the scrambled control (Supplementary Fig. S2A). We then found that ectopic expression of miR-186 was inducing downregulation of the miR-186–predicted targets MYCN, AURKA, TGFBR1, and TGFBR2 at the protein but not mRNA level (Fig. 3A; Supplementary Fig. S2B). Thus, to assess whether miR-186 could directly prevent their mRNA translation, we performed luciferase reporter assays in the easily transfectable CHLA-255 neuroblastoma cell line. Notably, the expression of the luciferase reporter was inhibited upon miR-186 transfection when the WT 3'UTRs of MYCN, AURKA, TGFBR1, and TGFBR2 were cloned downstream of the renilla luciferase cDNA (Fig. 3B). Importantly, when the predicted seed sequence was deleted from the 3'UTRs, the inhibitory effect of miR-186 was lost, indicating that miR-186 directly recognizes the target sequence and prevents MYCN, AURKA, TGFBR1, and TGFBR2 translation (Fig. 3B; Supplementary Fig. S2C). Next, to evaluate whether direct miR-186–dependent downregulation of TGFBR1 and TGFBR2 was sufficient to inhibit TGFβ1-dependent signaling, we used a Smad-reporter assay and found that TGFβ1 was unable to induce Smad-dependent transcription in cells that had been transfected with miR-186 (Fig. 3C; ref. 28).
Figure 2.
A, Expression of miR-186 in the GSE62564 RNA-seq data set representing 498 neuroblastoma patients stratified by low-risk versus high-risk; MYCN nonamplified (NA) versus amplified (A); INSS staging. B, miR-186–based event-free survival (left) and overall survival (right) probability analysis (n = 498). C, Correlation between miR-186 and NKG2D (KLRK1, \( r^2 = 0.495; ** \)) and DNAM-1 (CD226, \( r^2 = 0.602; *** \)) in 61 patients represented in the GSE62537 Affymetrix gene-expression array data set. D, Detection of miR-186 by qRT-PCR in NK cells treated for 96 hours with rhIL15 (100 ng/mL) and rhTGFβ1 (10 ng/mL) as indicated. E, Particle number (by nanosight tracking analysis), total protein concentration, and miR-186 expression (by qRT-PCR) in SEC fractions 9 through 23 (same samples of Fig. 1A). F, Delivery of the exosomal RNA cargo into CHLA-136 and LAN-5 cells exposed to SYTO-RNASelect-labeled NK exosomes for 18 hours. G, Detection of miR-186 by qRT-PCR in CHLA-136 or LAN-5 cells exposed to NK or K562.mbIL21 exosomes for 18 hours. *, \( P < 0.05 \); **, \( P < 0.001 \). Student t test, two-tailed.
To further demonstrate the direct targeting of MYCN and AURKA by miR-186 in neuroblastoma cells, we cloned their coding sequence lacking the 3’UTR into a lentiviral vector and transduced CHLA-136 and LAN-5 cells; in both cell lines, the lack of 3’UTR was sufficient to rescue the expression of MYCN or AURKA when the cells were transfected with Dotap-miR-186 (Fig. 3D).

Ectopic expression of miR-186 inhibits the growth and migration of MYCN-amplified neuroblastoma cell lines

Figure 3. A, Immunoblotting for MYCN, AURKA, TGFBR1, and TGFBR2 in CHLA-136 and LAN-5 cells treated with Dotap-miR-186 or Dotap-Scr for 48 or 72 hours, respectively; densitometry numbers underneath blots are expressed as arbitrary units normalized to levels of β-ACTIN, used as a loading control. B, Luciferase reporter assays of CHLA-255 cells electroporated with the mature miR-186 (or a scrambled SCR control) and the psiCHECK-2 vector carrying the WT or seed-sequence deleted (Del) 3’UTR of MYCN, AURKA, TGFBR1, or TGFBR2. C, Luciferase reporter assay of CHLA-255 cells electroporated with the mature miR-186 (or a scrambled SCR control) and the pSBE4-luc vector and treated with TGFβ1 where indicated. D, Immunoblotting for MYCN and AURKA in CHLA-136 cells transduced with the lentiviral vectors containing the full-length coding sequence of MYCN or AURKA without their 3’UTR and treated with Dotap-miR-186 or Dotap-Scr for 24 hours; densitometry numbers underneath blots are expressed as arbitrary units normalized to levels of β-ACTIN, used as a loading control. **, P<0.01; ***, P<0.001. Student’s t test, two-tailed. n.s., nonsignificant.

A nanoparticle modulating miR-186 in NK cells counteracts the TGFβ-dependent immune escape mechanism in neuroblastosoma

Because we showed that miR-186 expression is low in high-risk neuroblastoma patients and that its expression may correlate with the activation of NK cells, we sought to investigate its role in the biology of NK cells. To efficiently transfect NK cells, we utilized anionic lipopolyplex nanoparticles (NP), a liposomal formulation that has been previously successfully utilized to transduce synthetic miRNAs or antagomiRNAs in human primary cells with high efficiency both in vitro and in vivo (29, 41). Importantly, these
nanoparticles have been shown to direct their cargo to specific target cells when labeled with an appropriate pegylated antibody (41). Nanoparticles coated with an anti-CD56 or an unrelated IgG were loaded with the miR-186 mimic or a scrambled control and exposed to NK cells in vitro; as expected, CD56 significantly and specifically enhanced the delivery of miR-186 in the target NK cells, compared with IgG-NP (Fig. 5A). We also utilized CD56-NPs carrying an anti-miR-186 antagomiRNA and found that expression of miR-186 was significantly reduced in the target cells (Fig. 5A). Importantly, a significant upregulation and down-regulation of miR-186 was also detected in the corresponding NK exosomes (Fig. 5A).

Figure 4.
A, Growth curves of CHLA-136 and LAN-5 cells treated with Dotap-miR-186 or Dotap-Scr and followed for up to 72 hours. B, Wound-healing assays of CHLA-136 and LAN-5 cells treated with Dotap-miR-186 or Dotap-Scr. The gap size after 6 days is expressed as a percentage of the gap size at time zero. C, Boyden chamber migration assay of LAN-5 cells treated with Dotap-miR-186 or Dotap-Scr and exposed to SDF1α for 4 hours; the absolute number of migrated cells is reported. D, Expression of MMP-12 and vimentin by SYBR green qRT-PCR in CHLA-136 or LAN-5 cells treated with Dotap-miR-186 or Dotap-Scr.

\* P < 0.05; \*\* P < 0.01; \*\*\* P < 0.001. Student t test, two-tailed.
We then investigated whether upregulation of miR-186 was able to target the TGFβ pathway in NK cells, and we found that upon CD56-NP-miR-186 treatment, TGFBR1, TGFBR2, and SMAD3 were downregulated in the NK cells, suggesting that miR-186 is able to efficiently inhibit TGFβ signaling in activated NK cells (Fig. 5B).

Indeed, whereas TGFβ treatment was inhibiting the cytotoxicity of IL15-activated NK cells treated with CD56-NP-Scr, TGFβ was unable to inhibit NK cells treated with CD56-NP-miR-186 (Fig. 5C), supporting the hypothesis that delivery of miR-186 to NK cells may circumvent the TGFβ-dependent immune escape mechanism in high-risk neuroblastoma and restore NK cytotoxicity in patients. To investigate the role of miR-186 in the cytotoxicity of NK exosomes, we isolated NK exosomes from the supernatant of NK cells treated with CD56-NP-antagomiRNA anti-miR-186 (50 nmol/L) or its scrambled control (Fig. 5D; ref. 22).
Targeted delivery of miR-186 in an *in vivo* orthotopic model of neuroblastoma impairs the growth of cancer cells and improves survival

Having shown that forced expression of miR-186 in MYCN-amplified neuroblastoma cell lines effectively impaired their proliferation and migration potential, we sought to determine whether delivery of this miRNA *in vivo* could represent a possible new anticancer strategy. We utilized an orthotopic mouse model of neuroblastoma, in which we surgically injected the GD2-positive and MYCN-amplified CHLA-136-Fluc cells in the left kidney of NSG mice and started treatment 4 days later. As treatment, we utilized anti-GD2-coated NPs to specifically deliver the mature miR-186 to cancer cells. These NPs have previously been shown to have high bioavailability and a good safety profile in immune-competent mice (29). We first tested these NPs *in vitro* and found that miR-186 was specifically delivered to CHLA-136-Fluc cells by GD2-NP-miR186 compared with the IgG-NP-miR186, GD2-NP-Scr, and IgG-NP-Scr controls (Supplementary Fig. S3B).

In a first *in vivo* experiment, 19 tumor-bearing mice were randomized and treated with IgG-NP-Scr (*n* = 5), IgG-NP-miR186 (*n* = 4), GD2-NP-Scr (*n* = 5), and GD2-NP-miR186 (*n* = 5) intravenously 3 days a week. Tumor growth was monitored weekly by *in vivo* imaging for 30 days after transplantation, and we found that (after 26 days of treatment) the luminescent signal from the mice treated with GD2-NP-miR186 was significantly lower compared with all control groups (Fig. 6A). Importantly, mice treated with GD2-NP-miR186 had significantly longer survival (median: 60 days) compared with mice treated with IgG-NP-Scr (median: 45 days), IgG-NP-miR186 (median: 47 days), and GD2-NP-Scr (median: 42 days). Two identical independent *in vivo* experiments were then performed with the same treatment groups as above (all groups, *n* = 10 total each group), images were taken 24 days after transplantation, all mice were euthanized 30 days after surgery, tumors derived from mice treated with GD2-NP-miR186 showed markedly lower weight and size (Fig. 6B, left, and Supplementary Fig. S4). Furthermore, tumor-bearing kidneys and the contralateral healthy kidneys were homogenized to extract RNA and analyze the expression of miR-186 by qPCR; as expected, miR-186 was efficiently and specifically delivered by GD2-NP-miR186 to the tumor site and not to the healthy kidney when compared with control groups (Fig. 6C, middle and right).
Discussion

In order to successfully eradicate malignancies and overcome drug resistance, a two-pronged approach is required: (i) directly targeting of the cancer cells to lead to efficient eradication of the primary tumor and (ii) dissecting the biology of the tumor-supportive microenvironment to negate protumoral and prometastatic mechanisms (8, 42). In high-risk neuroblastoma, the MYCN amplification found in cancer cells represents a major, yet undruggable, culprit and the targeting of its physically related partner, Aurora Kinase A, has shown promising preclinical results (4–6). Several TME-dependent mechanisms of drug resistance have been described in neuroblastoma; for example, TAM-derived exosomes deliver miR-155 to neuroblastoma and induce drug resistance by downregulating the telomerase inhibitor TERTF1 (10); high infiltration of TAMs and elevated local production of the inflammatory cytokine IL6 by monocytes has been reported to contribute to a prometastatic phenotype and to induce drug resistance (43). Importantly, elevated IL6 and TGFβ expression in the TME activated the STAT3 and SMAD2/3 pathways in NK cells and suppressed activation of NK cell cytotoxicity, and IFNγ, granzyme A and B secretion, and perforin expression and has been reported to induce inhibition of the innate antitumor activity of NK cells (13, 14). NK cells infiltrate the neuroblastoma microenvironment. Metelitsa and colleagues showed that 53% of primary neuroblastomas were infiltrated with CD1d-restricted Vε24-Jα18-invariant NK cells (iNKT) and were most abundant in MYCN-nonamplified tumors expressing CCL2 (44). Although the distribution of NK cells in relation to other immune cells within the neuroblastoma microenvironment is currently unknown, NK cells are specialized for the killing of cancer cells, and clinical trials are ongoing to test NK cell–based therapy for the treatment of several malignancies, including neuroblastoma. The existence of an immune-suppressive microenvironment in neuroblastoma is relevant not only because it induces inhibition of the innate antitumor response of NK cells but also because it could hinder the efficacy of antibody-based therapies that rely on antibody-dependent cellular cytotoxicity (ADCC) mediated by NK cells (14, 43). Moreover, we and others have recently reported that exosomes derived from NK cells expanded in vitro can be isolated in large quantities, contain NK markers and killer proteins, and show cytotoxic activity against a variety of cancer cell lines (i.e., the murine melanoma cell line B16F10 and MM501mel, the leukemia and lymphoma cell lines Jurkat, K562, and DAUDI and the breast cancer cell line SKBR3; refs. 22–24). Here we show that exosomes derived from growth-factor–starved or TGFβ-inactivated NK cells retain their cytolytic activity, suggesting that factors other than NK-specific killer molecules, such as their nucleic acid cargo, may be also required for their targeting activity (Fig. 1B and C). In this study, we focused on the potential role of exosomal miRs. Notably, extracellular miRNAs can be transferred between immune and cancer cells within the TME and they may have a role in targeting factors important for the survival and propagation of cancer cells and for the establishment of immune escape mechanisms (8, 10). As global regulators of gene expression, miRNAs can simultaneously target multiple genes and have often been shown to be dysregulated in cancer and specifically in neuroblastoma (45). Among the miRNAs highly represented in the NK exosome cargo, we found the tumor suppressor miR-186 (Table 1; Fig. 2E).

In a 2007 study of the prognostic relevance of miRNA expression in neuroblastoma, Chen and colleagues showed that miR-186 is downregulated in MYCN-amplified tumors. This miRNA has recently been shown to behave mainly as a tumor suppressor in prostate cancer (targeting Y1 and CDK6, ref. 46), in renal cell carcinoma (targeting SENP1; ref. 47), in cervical cancer (competing with the oncogenic lncRNA ANRIL; ref. 48), in non–small cell lung cancer (targeting MAP3K2 and ROCK1; refs. 35, 39), in hepatocellular carcinoma (targeting YAP1; ref. 36), in multiple myeloma (targeting Jagged1; ref. 37), in esophageal squamous cell carcinoma (targeting SKP2; ref. 38) and in glioblastoma multiforme (targeting FGF2 and ReLA; ref. 33). Limitations of this study are related to the multiplicity of targets for a given miRNA, and the plethora of miRNAs contained as a cargo of extracellular vesicles. Although our data support a role for miR-186 as a bona fide tumor suppressor in neuroblastoma, miRNA-based treatment approaches are hindered by the promiscuity of miRNA target genes. Moreover, further studies are necessary to study the reciprocal interactions of exosomal miRNAs within each other and how such interactions affect the phenotype of the recipient cells. Of note, a few studies have reported miR-186 as an onco-miRNA by inducing repression of FOXO1 in endometrial cancer, of CYLD in melanoma, and of FAM134B in colorectal cancer (49–51). In this study, we show that in neuroblastoma, miR-186 is downregulated in high-risk patients, and its low expression represents a poor prognostic factor (Fig. 2A and B). Moreover, its levels directly correlate with the expression of two NK activation markers NKG2D and DNAM-1, whose low expression represents a poor prognostic factor (Fig. 2A and B). miR-186 directly recognizes a seed sequence in the 3'–5'UTR of MYCN, AURKA, and the TGFβ pathway (Table 1; Supplementary Fig. S2C; ref. 22). Here, we show that miR-186 directly recognizes a seed sequence in the 3'–5'UTR of MYCN, AURKA, TGFBR1, and TGFBR2 miRNAs and induce down-regulation of the encoded proteins and of the TGFβ pathway in the MYCN-amplified CHLA-136 and LAN-5 cells (Fig. 3; Supplementary Fig. S2). Consistent with the role of miR-186 as a tumor suppressor, ectopic expression of this miRNA resulted in impaired in vitro proliferation of MYCN-amplified cell lines and inhibition of their migration potential (Fig. 4A and B). Forced expression of miR-186 also resulted in downregulation of MMP12 and Vimentin. Interestingly, serum MMP12 is a negative prognostic marker in cancer and has been previously shown to be induced by TGFβ1 and to correlate with enhanced tumor invasiveness (52, 53). Moreover, vimentin, a mesenchymal marker of metastatic potential, is also induced by TGFβ, suggesting that inhibition of the TGFβ pathway by miR-186 in neuroblastoma cells may hinder the EMT process (Fig. 4B–D; ref. 53). Furthermore, we show that in vivo–targeted delivery of miR-186 to neuroblastoma cells is feasible by packaging in liposome-based nanoparticles coated with an anti-GD2 antibody. The in vivo administration of these anionic nanoparticles has been previously investigated in preclinical models of acute myeloid leukemias and found to have promising safety profile (29, 41). Delivery of miR-186 to the GD2-positive CHLA-136 cell lines (Fig. 6A; Supplementary Fig. S3B) implanted in an orthotopic model of neuroblastoma resulted in specific delivery of miR-186 to the tumor-bearing kidney with significant reduction of tumor burden and enhanced survival (Fig. 6).

Because we showed that miR-186 was detected in the cytotoxic exosomes derived from activated NK cells and that this miRNA induces inhibition of TGFβ signaling by downregulating TGFBR1 and TGFBR2 (Figs. 2 and 3), we sought to identify the role of miR-186 in NK cells. By utilizing nanoparticles coated with an...
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anti-CD56 antibody, we delivered the mimic miR-186 and its inhibitor to NK cells, and consequently their exosomes, and confirmed downregulation of the TGFβ receptors when miR-186 is overexpressed (Fig. 5A and B). Importantly, ectopic expression of miR-186 in NK cells prevented the inhibition of their cytotoxicity upon treatment with TGFβ (Fig. 5C), suggesting that delivery of this miRNA to tumor sites may not only inhibit the growth of the neuroblastoma cancer cells, but also counteract the TGFβ-dependent immune escape mechanism that hampers the efficiency of ADCC-based therapies. Importantly, although the exosomes derived from activated NK cells showed significant cytotoxic activity against the CHLA-136 MYCN-amplified cell line, the exosomes derived from NK cells inactivated by growth-factor withdrawal or TGFβ treatment retained their cytotoxic activity (Fig. 1B). Interestingly, NK exosomes derived from growth-factor-starved or TGFβ-treated NK cells showed a reduction in killer molecules (e.g., perforin 1, granzymes A and B), but retained equal levels of miR-186, suggesting that this miRNA (and potentially other RNA molecules carried by exosomes) may have a fundamental role in mediating the cytotoxicity of NK-derived exosomes. Accordingly, when miR-186 expression was inhibited in the exosomes after treatment of the parental NK cells with an anti-miR-186, their cytotoxic activity was significantly reduced (Fig. 5D).

In conclusion, we have provided evidence that targeted delivery of miR-186 in high-risk neuroblastoma is feasible and may lead to inhibition of tumor growth and spreading, and evasion of a common immune escape mechanism by which the tumor itself is able to circumvent the innate ability of NK cells to target cancer cells. Interestingly, although the use of anti-CD2 antibody is common in the treatment of neuroblastoma, the CD56 antigen is expressed by both neuroblastoma cells and NK cells, suggesting that this marker may be used to concurrently deliver miR-186 to NK and to the cancer cells (54). Moreover, the discovery that NK exosomes are cytotoxic and retain their killing ability even in an immunosuppressive microenvironment supports the notion of including ex vivo–derived NK exosomes as a potential tool alongside NK cell–based immunotherapy.

Disclosure of Potential Conflicts of Interest
M. Fabbri, R.C. Seeger, and P. Neviani have ownership interest in a patent application to be filed. No potential conflicts of interest were disclosed by the authors.

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