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MicroRNA analysis of Natural Killer cell-derived exosomes: the microRNA let-7b-5p is enriched in exosomes and participates in their anti-tumor effects against pancreatic cancer cells

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ABSTRACT

Natural Killer (NK) cells are important components of the immune system in the defense against tumor growth and metastasis. They release exosomes containing proteins and nucleic acids, including microRNAs (miRNAs). NK-derived exosomes play a role in the anti-tumor NK cell function since they are able to recognize and kill cancer cells. However, the involvement of exosomal miRNAs in the function of NK exosomes is poorly understood. In this study, we explored the miRNA content of NK exosomes by microarray as compared to their cellular counterparts. The expression of selected miRNAs and lytic potential of NK exosomes against childhood B acute lymphoblastic leukemia cells after co-cultures with pancreatic cancer cells were also evaluated. We identified a small subset of miRNAs, including miR-16-5p, miR-342-3p, miR-24-3p, miR-92a-3p and let-7b-5p that is highly expressed in NK exosomes. Moreover, we provide evidence that NK exosomes efficiently increase let-7b-5p expression in pancreatic cancer cells and induce inhibition of cell proliferation by targeting the cell cycle regulator CDK6. Let-7b-5p transfer by NK exosomes could represent a novel mechanism by which NK cells counteract tumor growth. However, both cytolytic activity and miRNA content of NK exosomes were reduced upon co-culture with pancreatic cancer cells. Alteration in the miRNA cargo of NK exosomes, together with their reduced cytotoxic activity, could represent another strategy exerted by cancer to evade the immune response. Our study provides new information on the molecular mechanisms used by NK exosomes to exert anti-tumor-activity and offers new clues to integrate cancer treatments with NK exosomes.

Introduction

The tumor microenvironment (TME) is composed by cancer cells, soluble factors, elements of the extracellular matrix and different resident or infiltrating non-malignant cells that play an active role in tumor initiation and progression[1,2]. In this context, the continuous and bidirectional interactions between tumor and immune cells may promote tumor progression by impairing cellular immune responses against cancer. Although Natural Killer (NK) cells represent the first line of defense against pathogens and tumor transformation and growth, cancer cells can escape NK cell-mediated control by multiple mechanisms[3,4]. For example, cancer cells may express ligands for inhibitory receptors and down-modulate the activating receptors in infiltrating NK cells. As a consequence, tumor-associated NK cells display reduced cytotoxicity and produce low levels of pro-inflammatory cytokines[5].

During the past years, extracellular vesicles (EVs) have gained increasing attention due to their important role in extracellular signaling and their stability in blood circulation[6]. Exosomes are small EV (50–160 nm) of endosomal origin that contain proteins, DNA and RNA, including microRNAs (miRNAs)[7].

MiRNAs are single-strand RNAs of 18–22 nucleotides able to inhibit translation or to induce degradation of the target RNA[8]. During their maturation process, immature miRNAs translocate from the nucleus to the cytoplasm where they are converted to mature miRNAs by the ribonuclease III Dicer. Then, the RNA-induced silencing complex pairs miRNA to their target sequences[9]. MiRNAs may be present in exosomes. The main mechanism responsible for miRNA loading in these EVs is a sequence-based process allowing specific proteins to sort miRNA into exosomes[10]. Indeed, over-represented miRNAs in exosomes contain the so-called EXO-motifs that are recognized by RNA binding proteins such as the heterogeneous ribonucleoprotein A2B1 (hnRNPA2B1) and synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP)[11]. In addition, it has been reported a homeostatic mechanism responsible for miRNA export in exosomes that is dependent on the amount of target mRNA available in cells[12].

MiRNAs represent key players in NK cell development and function. Indeed, Dicer deletion is associated with

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impairment of NK cell development *in vivo*[13,14]. In addition, evidences showed that miRNA regulates NK cell function by targeting proteins associated with cytotoxicity or cytokine production[13,15]. Furthermore, TME can induce changes in miRNA profiles of immune cells associated with increased tumor tolerance[16]. For example, the immunosuppressive miR-183 is induced in NK cells by transforming growth factor β (TGF-β). The miR-183 targets the signaling polypeptide DNAX activating protein 12 kDa (DAP12), inducing an impairment of NK cell function[17].

Recent studies have demonstrated that NK cells release exosomes capable of recognizing and killing cancer cells by multiple mechanisms *in vitro* and *in vivo*. NK derived-exosomes (NK-exo) expressed a number of NK receptors/markers including CD56, CD69, NKG2D, NKp44, NKp46, NKp30, CD40L, PD-1, and molecules involved in tumor cell recognition and immunological synapse formation (LFA-1, DNAM1). They also carry cytotoxic proteins such as Perforin, Granzyme A-B, Fas-L and cytokines such as IFN-γ[18–20].

Interestingly, a study reported that NK exosomes carry the tumor suppressor miR-186-5p, which impairs the growth of neuroblastoma cells *in vitro* and *in vivo*[21]. Another study showed that miR-3607-3p is enriched in NK exosomes and can inhibit pancreatic cancer growth[22]. Recently, we demonstrated that NK exosomes may have cytotoxic effects on pancreatic cancer cells[23].

In this work, we explored the miRNA content in NK-exo in comparison with that of their cellular counterpart. We show that NK-exo exhibits distinct miRNA expression profiles. We found that let-7b-5p is enriched in NK-exo and can be downregulated by pancreatic cancer cells. We also provide evidence that let-7b-5p is involved in the anti-proliferative effects of NK exosomes on cancer cells. Thus, this study sheds light on novel molecular mechanisms used by NK-exo to exert anti-tumor-activity, and it may offer new clues to integrate cancer treatments with NK-exo.

Materials and methods

Cell culture and exosome isolation

NK cells were isolated from the peripheral blood of healthy volunteers admitted at blood transfusion center in Bambino Gesù Children Hospital. The study was approved by the Hospital Ethical Committee (ID: 1724/2018) and was conducted in accordance with the tenets of the Declaration of Helsinki. All donors have signed the informed consent. Briefly, peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation (Lympholyte®, Cedarlane) and NK cells isolated by RosetteSep Human NK cell enrichment cocktail (Stem Cell Technologies).

NK cells were cultured in NK MACS® medium (Miltenyi Biotec) supplemented with 10% Fetal Bovine Serum (FBS; Thermo-Fisher Scientific), 1% penicillin and streptomycin (Euroclone) in the presence of 600 U/ml IL-2 (Novartis).

Human PANC-1 pancreatic cancer and NALM-18 B-cell childhood acute lymphoblastic leukemia cell lines were cultured in RPMI medium supplemented with 10% FBS, 2 mM L-glutamine (Euroclone), 1% penicillin and streptomycin (Euroclone). MIA PaCa-2 pancreatic cell line was cultured in DMEM high-glucose medium supplemented with 10% FBS, 2 mM L-glutamine (Euroclone), 1% penicillin and streptomycin (Euroclone).

For exosome isolation, IL-2 activated NK cells were washed and seeded in round-bottom 96-wells at cell density 1.2×10^6 cells/mL in RPMI supplemented with 10% of FBS (Thermo-Fisher Scientific, Massachusetts, USA) previously depleted of bovine exosomes by ultracentrifugation (exosome-free FBS) and 600 IU/mL IL-2. Depending on the experiments, a total number of 15×10^6 or 30×10^6 NK cells in 12 mL or 24 mL of medium supplemented with exosome-free FBS were used, respectively. After 48 h, supernatants were collected and exosome isolated and quantified. Briefly, the supernatants were centrifuged at +4°C 300 g for 5 min, followed by a second centrifugation at 2000 g for 15 min. Then, supernatants are filtered by 0.22 μm pore size filters and ultra-centrifuged at +4°C (100,000 g for 70 min) (Optima MAX-XPN, Beckman, Brea CA, USA). Exosomes were washed with phosphate buffer saline solution (PBS) and undergone to a second round of ultracentrifugation (100,000 g for 70 min). Isolated exosomes were resuspended in 50 μL of PBS and indirectly quantified by Bradford Assay (Biorad, Hercules, CA, USA)[18].

Nanoparticle tracking analysis (NTA) was performed with NanoSight LM10 device by System Biosciences service. 20 μg of exosomal vesicles were diluted in PBS supplemented with protease inhibitors cocktail (Thermo Fisher Scientific) and particle Brownian movements were assessed by NTA Software. Mean exosome number/μg of protein was calculated using NTA data: $7.9 \times 10^{11} \pm 2.79 \times 10^{11}$ particles/μg protein ($n = 3$).

Flow cytometry

Flow cytometry analysis of exosomes with conjugated antibodies anti-CD81 APC-Vio770 (REA513) and anti-CD63-APC-Vio770 (H5C6; Miltenyi Biotec) was performed as already described[18]. Exosome samples were analyzed with the LX-Cytoflex instrument (Beckman Coulter) and FlowJo Software (BD Biosciences). Cytofluorimetric analysis of NK and pancreatic cancer cells was performed as described[23]. The following fluorophore-conjugated antibodies were used: anti-CD56 (REA196; Miltenyi Biotec) and anti-CD3 (UCHT1; Ancell Corporation); anti-HLA-A,B,C – PB (W6/32; Biolegend); anti-PD-L1 PE-CF594 (BD Biosciences); anti-MICA/B-PC7 (6D/4; Biolegend); anti-ULPb 2/5/6-PE (# 165903; R&D Systems).

Western blot

NK cells and derived exosomes were lysed in RIPA buffer and incubated for 30 min or 15 min in ice, respectively. Then, samples were centrifuged at 20,000 g for 20 min, lysates quantified by Bradford assay (Biorad) and 10 μg of protein lysates for each sample were analyzed by western blot (WB) as described[18]. Primary antibodies used were anti-CD63 (MX-49.1129.5), anti-CD81 (5A6; Santa Cruz Biotechnology), anti-

Calnexin (#2433; Cell Signaling) and anti-tumor susceptibility gene 101 (TSG-101; 4A10; Abcam).

Microarray analysis

High-quality total RNA (140 ng per sample) obtained from the samples of human NK cells ($n = 7$) and exosomes ($n = 7$) were used as start material for the microarray analysis. Biotin-labeled RNA was generated using the FlashTag Biotin HSR Labelling Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The biotin-labeled RNA was subsequently hybridized to a Lympholyte® miRNA 4.1 array plate (Thermo Fisher Scientific). The hybridization, wash and staining procedures were done in a GeneChip™ Instrument according to the manufacturer's protocol (Thermo Fisher Scientific). Labelling, hybridization, washing, staining and scanning procedures were performed by Eurofins Genomics microarray service. The .cel file output was used as input in the Partek Genomics Suite Software (Partek) for generation of Robust Multi-Array Average (RMA) normalized data.

RNA extraction and real time PCR

RNA extraction from purified NK exosomes and NK cells was performed with miRNeasy micro kit (Qiagen). RNA concentration and purity were evaluated by spectrophotometric analysis (Nanodrop 2000; Thermo Fisher Scientific). For miRNA expression analysis, for each sample 20 ng of RNA was reverse transcribed with miRCURY LNA RT kit following manufacturer's instructions (Qiagen). Synthetic RNA spike-in UniSP6 (Qiagen) was added to the RT reaction to normalize analytical differences between the samples. cDNA was diluted 1:30 in nuclease-free water, and 3 µL of diluted cDNA was loaded in 10 µL of final volume of real Time PCR mix by using miRCURY Sybr Green master mix plus ROX reference dye and the LNA primer mix assay (Qiagen). The following miRCURY LNA PCR assays were used: UniSP6 (Y00203954), hsa-miR-15b-5p (YP00204243), hsa-miR-20a-5p (YP00204292), hsa-miR-146a-5p (YP00204688), hsa-miR -27a-3p (YP00206038), hsa-miR-106b-5p (YP00205884), hsa-miR-16-5p (YP00205702), hsa-let-7b-5p (YP00204750), hsa-miR-92a-3p (YP00204258), hsa-miR-24-3p (YP00204260), hsa-miR-342-3p (YP00205625), SNORD44 (YP00203902), SNORA66 (YP00203905). For mRNA expression analysis, total RNA was reverse transcribed with random primers using Super Script IV first-strand synthesis system following manufacturer's instructions (Thermo Fisher Scientific). Real-time PCR was performed in 20 µL of total volume with TaqMan™ Fast Advanced Master Mix and TaqMan Gene Expression Assay (Applied Biosystems). The following TaqMan™ Gene Expression assays were used: ACTB (Hs01060665_g1), CDK6 (Hs01026371_m1), CDK4 (Hs00364847_m1), CCNA2 (Hs0099678_m1), HMGA1 (Hs00852949_g1).

PCR reactions were carried out in triplicate on a QuantStudio 12 Flex instrument using thermal cycling conditions suggested by the manufacturer (Applied Biosystems). All real-time PCR data were analyzed with baseline threshold algorithm and 2-ΔCt method (QuantStudio Real-Time PCR software, Applied Biosystems). The mean of SNORD44 and SNORA66 (in PANC-1 cells) or SNORD44 (in MIA PaCa-2 cells) expression was used for normalization of cellular miRNAs expression.

Bioinformatic analysis

Heatmap (Figure 1C) and scatter plot (Figure 2C) were generated by Partek Genomics Suite software (Partek). GO term enrichment analysis was performed using FunRich software according to[24]. An over-represented exo-motif sequence analysis was performed using the MEME suite according to the study by Bailey and Elkan[25]. Sequences of miRNA highly expressed only in exosomes (66 miRNAs) were compared to miRNA sequences highly expressed in cells and exosomes (81 + 91 miRNAs; Figure 2A) and ZOOPS (zero-or-one-occurrence) model was run to find over-presented 4 to 10 nucleotide motifs.

MiRNA target prediction was performed using miRWalk and targets predicted simultaneously by TargetScan, miRDB, miRTarBase databases were considered for further analysis[26].

Selection of cancer-related targets of miRNA was performed using the ONCO.IO database (https://onco.io/). Differential expression of MIRLET7BHG and CDK6 in pancreatic cancer patients was calculated on the basis of RNA-seq data from The Cancer Genome Atlas (TCGA) using the TNMplot.com web tool[27]. The overall survival test on pancreatic cancer patients was built using the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) on the Tumor Pancreatic adeno-carcinoma dataset (TCGA – 178 - rsem – tcgars). The Kaplan Scan feature was used to divide the samples of a dataset into two groups based on the CDK6 gene expression, with a cutoff based on the highest p-value calculated with a logrank test.

NK – pancreatic cancer cells co-culture experiments

The IL2-activated NK cells were cultured with PANC1 or MIA PaCa-2 cells at a ratio of 5:1 (NK: tumor cells) and after 5 days NK cells were separated from cancer cells by using NK cell isolation kit (Miltenyi Biotec). Purity of isolated NK cells was confirmed by flow cytometry with anti-CD56 (REA196; Miltenyi Biotec) and anti-CD3 (UCHT1; Ancell Corporation) antibodies and then seeded in an exosome-free medium for 48 h for exosome isolation.

Cytotoxic assay

NALM-18 cell line was used as target cells and labeled with Cell Tracker Green CMFDA (Thermo Fisher Scientific) according to manufacturer's instruction and seeded at density of 40,000 cells/mL in 96-well plate. The day after, cells were incubated with 20 µg/well (~1.6×10^9 exosomes/cell) of NK-exo purified from NK cells not co-cultured (control; CTRL) and after co-culture (AC) with PANC1 cells. After 24 h, Propidium Iodide (PI) was added to identify dead cells.

Cell transfection

PANC1 and MIA PaCa-2 cells were seeded in 24-well plate at a density of 40,000 cells/ml and after 24 h they were transfected by Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific). Briefly, Opti-MEM medium (Thermo Fisher Scientific) was combined with Lipofectamine™ 3000 and 60 pmol of mirVana miRNA mimic – hsa-let-7b-5p (ID: MC11050), mirVana miRNA mimic – Negative Control #1, mirVana let-7b miRNA inhibitor or mirVana miRNA inhibitor Negative Control #1 (Thermo Fisher Scientific) and incubated for 20 min to allow formation of complexes. Then, cells were incubated with liposomes containing let-7b-5p mimic, let-7b inhibitor or their respective controls, and after 24 h, cells were used for further analysis.

Cell proliferation assay

PANC1 and MIA PaCa-2 cells transfected with let-7b-5p mimic, miRNA mimic control, let-7b inhibitor, miRNA inhibitor control, incubated with NK exosomes (10 μg per 40,000 cells; ~2 × 10^8 exosomes/cell) or with an equal volume of exosome resuspension buffer (PBS) for 24 h, were washed in PBS, detached with trypsin and then cell number was determined by flow cytometry or with automated cell counter (Countess II, Thermo Fisher Scientific). Dead (PI^+ or Trypan Blue-stained) cells were excluded from cell counts.

Results

miRNA expression profiles in NK cells and exosomes

To identify the miRNA repertoire in NK exosomes, these were isolated from long-term, IL2-activated NK cell cultures by serial rounds of centrifugations, as detailed in Materials and Methods. The size of isolated vesicles was assessed by NTA (NanoSight). The typical exosomal markers including the tetraspanins CD81 and CD63 and TSG101 were analyzed by both flow cytometry and WB. The absence of contaminating intracellular protein Calnexin was also confirmed. Both size and molecular markers in the isolated vesicles corresponded to those of exosomes according to Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines (Figure 1A-B and S1)[28].

Then, we performed a comprehensive miRNA comparative analysis by microarray on seven samples of NK cells and seven samples of their derived exosomes. Out of over 2,500 mature miRNA sequences analyzed, 337 were differentially expressed (fold change <-2 o >2, False Discovery Rate p-value <0.05), of which 192 were upregulated in NK cells, and 145 were up-regulated in NK exosomes (Table S1).

Hierarchical cluster analysis using the most differentially expressed miRNAs (fold change > |10|) clearly shows that NK cells and exosome samples clustered separately (Figure 1C).

To better investigate potential differences of miRNA patterns in NK cells and their exosomes, the 100 most upregulated miRNA in NK cells or exosomes were selected for target prediction analysis, and Gene Ontology (GO) enrichment analysis was performed using FunRich software[29]. The main targeted GO categories were related to the regulation of apoptosis, intracellular and intercellular signal transduction, regulation of transcription from Polymerase II promoter and regulation of cell proliferation (Figure S2, Table S2). This analysis showed that miRNA in NK cells and NK exosomes targeted similar biological processes.

Comparative analysis of miRnas in NK cells and NK-derived exosomes

Based on microarray normalized expression signals, we focused on the most expressed miRNAs in NK cell and exosome groups. In each group, we selected mature miRNAs with the highest expression signal (over 90th percentile; 258 top-expressed miRNAs). As shown by the Venn diagram, cells and exosomes shared 156 top-expressed miRNAs, while 102 were highly expressed in cells or exosomes only, respectively (Figure 2A). In order to verify the presence of over-represented motifs in exosomal miRNAs, we performed sequence comparison analysis of miRNAs highly expressed in exosomes only versus all the miRNAs included in the Venn diagram of Figure 2A. The exo-motif discovery analysis showed an enrichment of the conserved exo-motif GGCUG in miRNA from NK exosomes (Figure 2B). This motif is recognized by SYNCRIP protein and mediates miRNA transport in exosomes[11].

We also plotted normalized expression signal in NK cells and exosomes for all 2,578 mature human miRNAs included in the microarray chip. We found that global miRNA expression in NK cells and exosomes display a positive correlation (r = 0.711; Figure 2C). The vertical line delimitates 90th percentile of cellular miRNA signals, whereas the horizontal line indicates 90th percentile of exosome miRNA signals. Since miRNAs expressed in exosomes should also be detected in parental cells (i.e., the source of exosomes), we focused our attention on two miRNA subgroups: miRNAs highly expressed in both NK cells and exosomes (quadrant I, Figure 2C) and miRNAs highly expressed in NK cells and with low expression in NK exosomes (quadrant IV, Figure 2C). Using this approach, we could select the miRNAs miR-16-5p, let-7b-5p, miR-92 a-3p, miR-24-3p, miR342-3p in the quadrant I (high in exosomes) and miR-15b-5p, miR-20 a-5p, miR-146b-5p, miR-27-3p, miR-106b-5p in the quadrant IV (low in exosomes; Figure 2C). Real-time PCR confirmed a higher expression in NK exosomes selected miRNAs in the quadrant I as compared to miRNAs of the quadrant IV (Figure 2D).

The exosomal miRNA let-7b-5p inhibits proliferation of pancreatic cancer cells

Among enriched miRNAs in NK-exo, we focused our attention on let-7b-5p, since it belongs to let-7 family of miRNAs with critical onco-suppressive function[30,31]. It plays a key role in cancer and is frequently downregulated in different tumors, including pancreatic cancer[32,33]. Thus, we asked whether
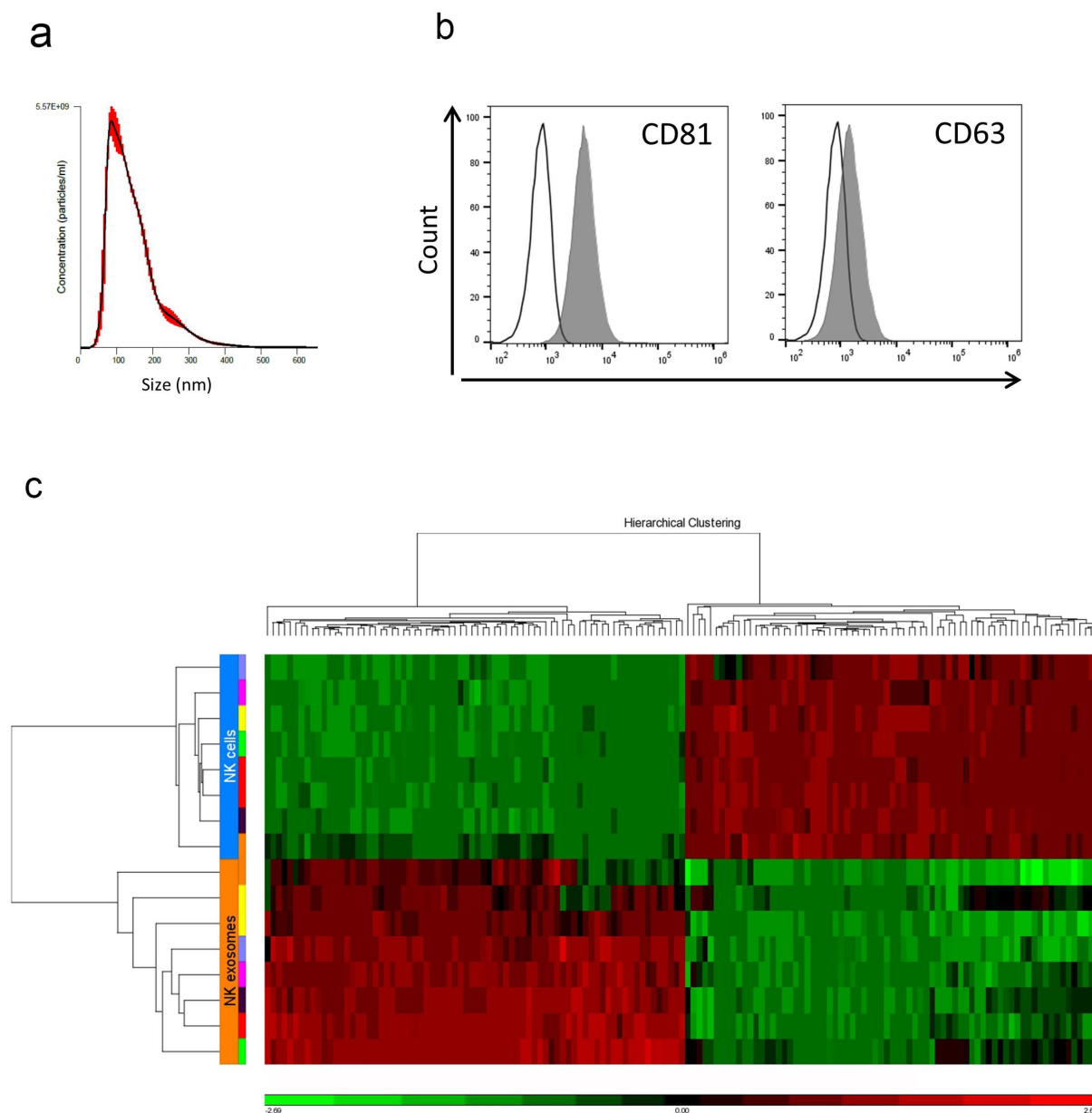
Figure 1. Comprehensive miRNA expression in NK cells and exosomes. (a) Histogram representing the size distribution of particles from NK-exo samples revealed by Nanoparticle Tracking Analysis (NTA). A representative NK-exo sample is shown. (b) Flow cytometry analysis of NK exosomes stained with anti-CD81 and anti-CD63 exosome markers. Grey histograms represent stained samples, white histograms unstained controls. A representative experiment is shown. (c) Hierarchical clustering of NK cells and exosomes samples analyzed by miRNA microarray. Rows represent samples; columns represent the top 148 mature miRnas (74 up-regulated in exosomes, 74 up-regulated in NK cells) differentially expressed between NK-exo and NK cells (FDR p-value ≤0.05).

exosomal let-7b-5p could be implicated in NK cell anti-tumor activity.

Bioinformatic analysis by miRWalk[26] showed that let-7b-5p acts on multiple genes involved in cell cycle regulation, differentiation and cancer progression. Among the validated targets identified in our analysis, we found CDK6, CCNA2, CCND1, CCND2, SOCS1, HMGA1 and AURKB, which are involved in cancer progression (Figure 3A).

Next, we investigated a possible role of let-7b-5p in NK exosome function. To this end, we first assessed let-7b-5p expression in PANC1 and MIA PaCa-2 pancreatic cancer cells upon incubation with NK-exo. After 24 h, we found an increased expression of let-7b-5p in exosome-treated pancreatic cancer cells, suggesting that this miRNA can be efficiently transferred to cancer cells by NK exosomes (Figure 3B).

NK-exo has been shown to inhibit cancer cell proliferation and viability[22]. In agreement with these data, after 24 h of incubation with NK-exo, PANC1 and MIA PaCa-2 cell numbers were significantly decreased (Figure 3C and Figure S3B). Notably, transient transfection of pancreatic cancer cells with let-7b-5p induced similar effects (Figure 3C and Figure S3A). In agreement with these data, transient transfection of pancreatic cancer cells with let-7b inhibitor produced opposite effects after 24 h, with a slight increase in cell number. Interestingly, let-7b miRNA inhibitor was sufficient to
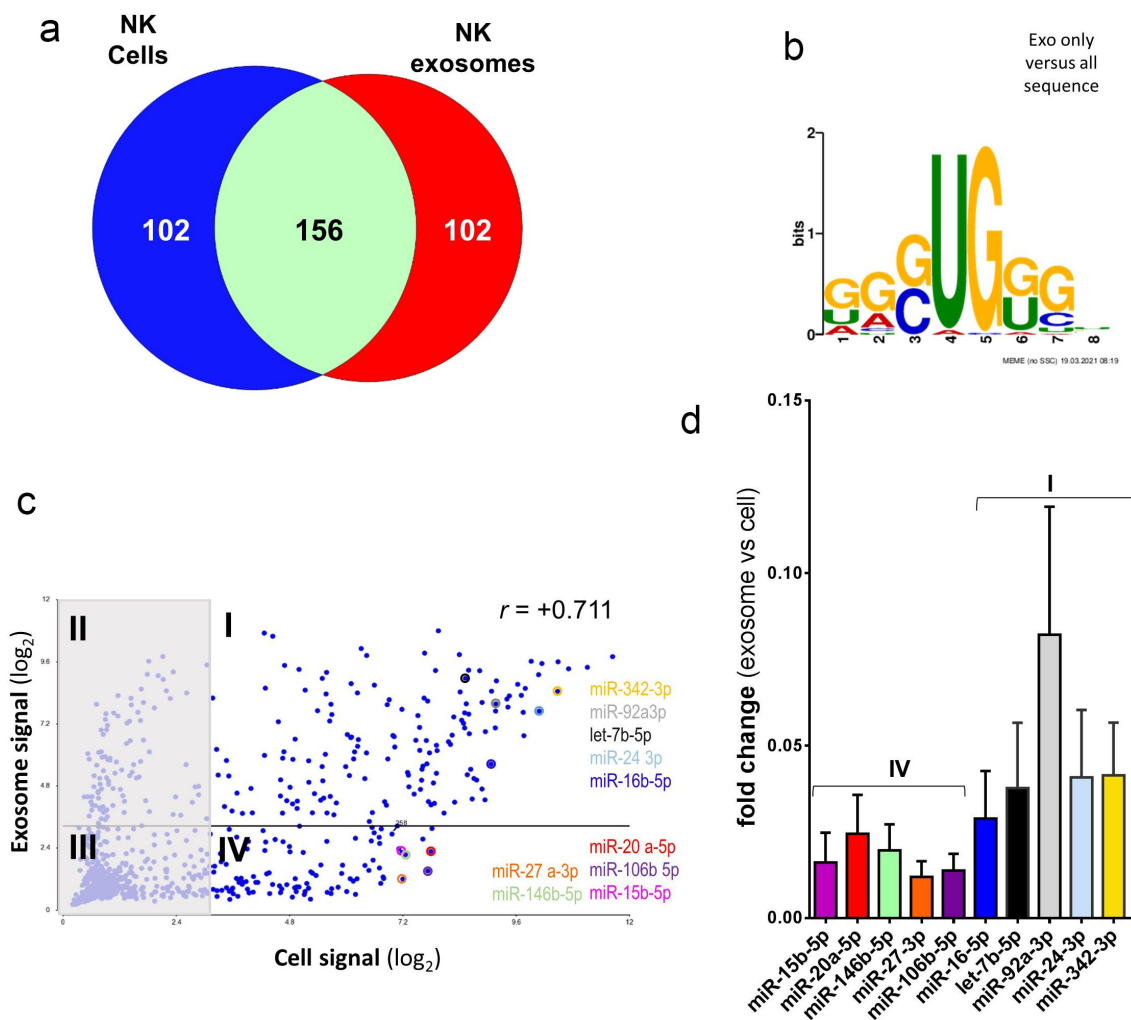
Figure 2. A restricted signature of miRnas is enriched in NK exo. (a) Venn diagram showing the number of miRnas with the highest microarray expression signal (over 90th percentile) belonging to NK cells and/or exosomes set. (b) Enrichment of the conserved exo-motif GGCUG revealed by motif-based sequence analysis of MEME suite tool[24]. Sequences of miRnas highly expressed in exosomes only were compared to all miRNA sequences highly expressed in cells and exosomes. (c) Scatter plot of the log$_2$-transformed expression signal for all the mature miRnas analyzed. Each dot represents a miRNA included in the microarray chip. X-axis reported expression in NK cells, Y-axis expression in NK-exo. The vertical axis delimitates 90th percentile of signals in NK cells, the horizontal axis delimitates 90th percentile of signals in NK-exo. Roman numerals (I-IV) identify four quadrants. We excluded from further analysis miRnas in quadrants II and III (gray zone). MiRNAs analyzed by real time PCR are indicated. (d) Real time PCR for the indicated miRnas in the same samples used for the microarray. Values are expressed as fold changes in exosome ($n = 7$) as compared to cell ($n = 7$). For each sample, the same amount of RNA was used for RT-PCR and Uni-SP6 spike-in was used as reference to normalize data. Brackets indicate miRnas belonging to quadrant I or IV. Error bars indicate SEM.

abrogate the impairment of cell proliferation observed with NK-exo treatment (Figure 3C).

Therefore, considering let-7b-5p is involved in cell cycle regulation and that NK-exo can efficiently augment let-7b-5p into target cells, the anti-proliferative effects of NK-exo could be related, at least in part, to the exosomal let-7b-5p. To confirm this hypothesis, modulation of let-7b-5p targets was analyzed in PANC1 cells following incubation with NK exosomes or upon let-7b-5p over-expression. We focused our attention on experimentally validated targets involved in cell cycle and in cancer cell proliferation (CDK6, CCNA2 and HMGA-1)[34,35]. PANC1 was incubated with NK-exo or transiently transfected with let-7b-5p mimic for 24 h and CDK6, CCNA2 and HMGA-1 expressions were assessed using Real-Time PCR. NK-exo induced significant downregulation of CDK6 in PANC1 cells, similarly to let-7b-5p miRNA mimic (Figure 3D). As expected, after 24 h of transient transfection

with let-7b inhibitor, CDK6 was increased. Interestingly, let-7b inhibition overwhelmed the negative regulation of NK-exo on CDK6 expression (Figure 3D). The expression of CDK6 was in agreement with proliferation data (Figure 3C and 3D). When MIA PaCa-2 cells were used as model, CDK6 transcript resulted poorly expressed by real-time PCR ($C_t > 32$) without any clear modulation after let-7b mimic or NK exosomes (data not shown). Nevertheless, MIA PaCa-2 highly expressed CDK4, a CDK6 functional homologue targeted by let-7b[34]. In MIA PaCa-2, incubation with NK exosomes induced a significant down-regulation of CDK4, whereas only a slight decrease of this transcript was observed in PANC-1 (Figure S3C). CCNA2 in PANC1 were significantly down-regulated in cells transfected with let-7b-5p, whereas a trend of decrease of HMGA1 but not a significant down-regulation was observed in PANC1 cells transfected with let-7b-5p or exposed to NK exosomes. Similar results were obtained for MIA PaCa-2
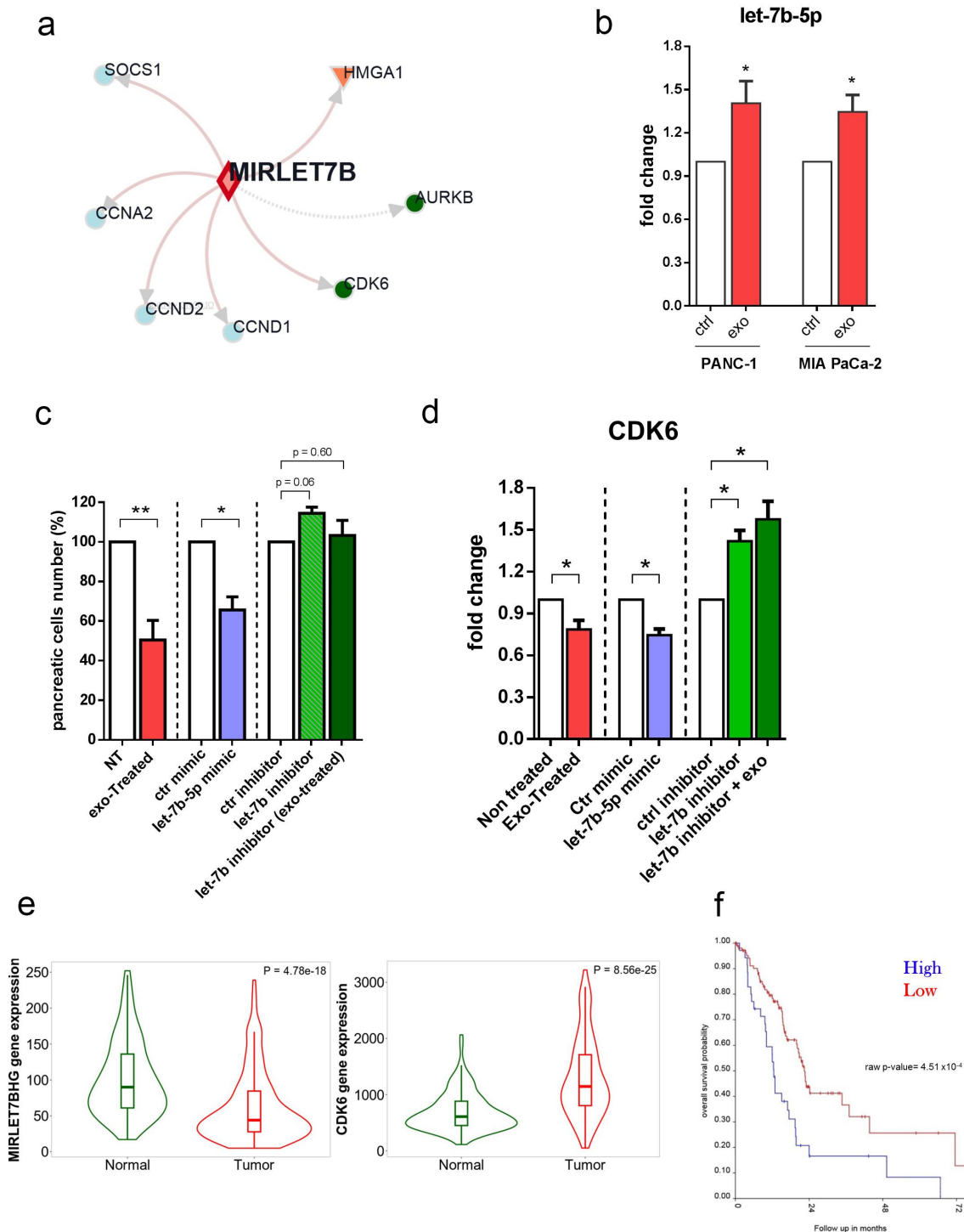
Figure 3. The NK exosomal miRNA let-7b-5p inhibits proliferation of pancreatic cancer cells. (a) Graph of let-7b-5p targets involved in cancer progression/cell proliferation elaborated by using the ONCO.IO database tool. (b) Expression of let-7b-5p in PANC1 cells and in MIA PaCa-2 cells after 24 hours of incubation with NK-exo (exo; 10 µg per 40,000 cells; ~2 × 10^8 exosomes/cell) and without NK-exo (ctrl) measured by real time PCR. Values are expressed as $2^{-\Delta Ct}$. SNORD44 was used for normalization in MIA PaCa-2 ($n = 3$). The mean of SNORD44 and SNORA66 expression was used for normalization in PANC-1 ($n = 3$) bars indicate SEM. (c) Number of live PANC1 ($n = 5$) cells after NK-exo treatment (10 µg per 40,000 cells; ~2 × 10^8 exosomes/cell) or transfection with let-7b-5p miRNA mimic ($n = 4$) or with let-7b miRNA inhibitor in absence or in combination with NK-exo treatment ($n = 3$). Percentages of proliferating live cells were calculated to their respective control, arbitrarily set as 100%.; * p-value ≤0.05; ** p-value ≤0.01 (Student's t-test). (d) CDK6 expression measured by real time PCR in PANC1 untreated and NK-exo-treated ($n = 5$), or transfected with control mimic, let-7b-5p miRNA mimic ($n = 4$), control miRNA inhibitor or let-7b miRNA inhibitor in absence or in combination with NK-exo treatment ($n = 3$). Values are expressed as fold change relative to their control. ACTB was used as endogenous control to normalize expression data. Bars indicate SEM. * p-value ≤0.05; ** p-value ≤0.01 (Student's t-test). (e) RNA-seq expression data of MIRLET7BHG (let-7b-5p host gene, left panel) and CDK6 gene (right panel) in normal ($n = 252$) and in pancreatic cancer samples ($n = 177$) obtained from TCGA dataset using TNMplot.com web tool[26]. (f) Overall Survival at 72 months of pancreatic cancer patients divided for high expression (blue; $n = 35$) or low expression (red; $n = 111$) of CDK6 gene. Kaplan–Meier curves were built by the R2: Genomics Analysis and Visualization Platform on TCGA − 178 dataset. p-value = $4.5×10^{-4}$ (logrank test).

(Figure S3 D-E). These data show that NK exosomes can participate to repressing CDK4/CDK6 in target cells via let-7b-5p.

We also evaluated the expression of CDK4/CDK6 in the TGCA dataset using the TNMplot.com tool[27]. In accordance with different reports[36–38], we found higher levels of CDK4/CDK6 in pancreatic cancer as compared to non-tumoral samples (Figure 3E and Figure S3F). We also analyzed the expression of the let-7b-5p host gene MIRLET7BHG in pancreas tumors on publicly available TCGA datasets using the TNMplot.com platform[27]. As shown in Figure 3E, MIRLET7BHG expression in pancreas tumors was lower as compared to non-tumor samples, suggesting that downregulation of let-7b-5p could represent a pathogenic event in pancreatic cancer.

Furthermore, the overall survival analysis of pancreatic cancer patients using R2 Genomics Analysis and Visualization Platform highlighted that high levels of CDK6 had worse overall survival as compared to patients with low CDK6 (Figure 3F).

Taken together, these experiments suggest that NK exosomes could affect pancreatic cancer cell proliferation by transferring let-7b-5p that in turn acts on its target gene CDK6. Moreover, in pancreatic cancer patients, let-7b-5p is frequently downregulated, while its target CDK6 is upregulated and its expression is associated with worse overall survival.

Pancreatic cancer cells modulate miRNA expression in NK-exo

While NK-exo can exert cytotoxic effects on cancer cells, on the other hand, cancer cells could exert inhibitory effects on NK cells. Thus, we investigated whether the pancreatic cancer cell line PANC1 could induce changes in the miRNA content and in the functional properties of NK exosomes. To this aim, NK cells were co-cultured with PANC1 cancer cells for 5 days. Then, PANC1-"conditioned" NK cells were isolated, and their purity was confirmed (Figure S4A). Conditioned NK cells were seeded in an exosome-free medium and, after 48 h, NK-exo was isolated (Figure 4A).

As shown in Figure 4B, cytotoxicity of NK-exo isolated from PANC-1 conditioned NK cells (after co-culture; AC) was significantly lower than that of exosomes isolated from non-co-cultured NK cells (CTRL), with a reduction of 60–80%. This data suggests that pancreatic cancer cells can affect NK-exo function by reducing their cytotoxic potential.

We evaluated whether the low cytotoxicity of AC NK-exo could be related to altered miRNA expression. Thus, we compared the miRNA expression in CTRL and AC NK-exo by Real-Time PCR. After co-culture with PANC-1 cells, we found that there was an overall significant reduction of all the NK exosomal miRNAs tested (Figure 4C). A trend of miRNA down-regulation, in particular for miR-16-5p and miR-24-3p, occurred also performing similar co-culture experiments with MIA PaCa-2, although we only found a mild decrease of miRNA cargo upon NK conditioning with these cells (Figure S4B).

We also analyzed by cytofluorimetric analysis the expression of HLA-I and PD-L1 on the surface of PANC-1 and MIA PaCa-2 cells. These molecules, which can negatively regulate NK cell activity, were expressed on both cell lines, although they were detected at higher levels in MIA PaCa-2. The cell lines also expressed comparable levels of the NKG2D ligands MICA/B, whereas ULBp 2/5/6 ligands were more expressed in MIA PaCa-2 cells (Figure S4C). These data suggest that pancreatic cancer cells may impair the functional activity of NK-exo by inducing downregulation of their shuttled miRNAs.

Discussion

In the present study, we show that NK-derived exosomes may substantially differ in their miRNA cargo from miRNAs present in NK cells. Importantly, they contain miRNAs that exert anti-tumor activity by inhibiting cancer cell proliferation. Moreover, we provide evidence that NK cell exposure to cancer cells significantly hampers the miRNA composition and cytolytic function of NK-exo.

In view of the potential anti-tumor activity of NK-exo, we assessed their miRNA content and compared it to that of the parental cells. The global analysis highlighted a positive correlation between the expression of exosomal and cellular miRNAs. Although the majority of exosomal miRNAs reflects the miRNA content of the cells, we also found distinct miRNA signatures in NK cells and exosomes differences, which may reflect sequence-based regulating processes as well as partially passive process[39,40]. Of note, miRNAs that were significantly up-regulated in NK-exo were enriched in the GGCUG motif, a sequence favoring miRNA shuttling into exosomes[11].

A miRNA expressed in exosomes should also be detected in parental cells (i.e., the source of exosomes). Thus, we focused on miRNAs that are consistently expressed in NK cells. We focused on miRNAs with the highest expression signals detected by microarray, since they have more chance to be biologically relevant. Considering the ascendant ranking of expression signal in NK cells, we selected only miRNAs over the 90th percentile (Figure 2C, quadrant I and IV). Within this pool, we could confirm the expression of selected miRNAs by real-time PCR. As selection criteria, we chose miRNAs with low ID numbers because of their solid experimental validation, whereas the existence of several recent miRBase entries with high ID numbers is supported by NGS only[41]. According to microarray, we found a higher expression in NK-exo of the miRNA signature miR-342-3p, miR-92a-3p, miR-24-3p, miR-16-5p and let-7b-5p.

Next, we asked whether the functional properties of NK-exo and their miRNA cargo could be affected by exposing NK cells to pancreatic cancer cells, as it may occur in vivo in the TME. We show that NK co-cultures with PANC-1 pancreatic cancer cells can substantially reduce the cytotoxicity and the miRNA content of NK-exo. It is conceivable that this event may indeed represent a further immunosuppressive mechanism used by cancer cells to hamper the NK-mediated antitumor activity. In a previous study, we demonstrated that NK exosomes are equipped with several molecules involved in NK function and DNAM-1 appeared to play a substantial role in NK exosome-
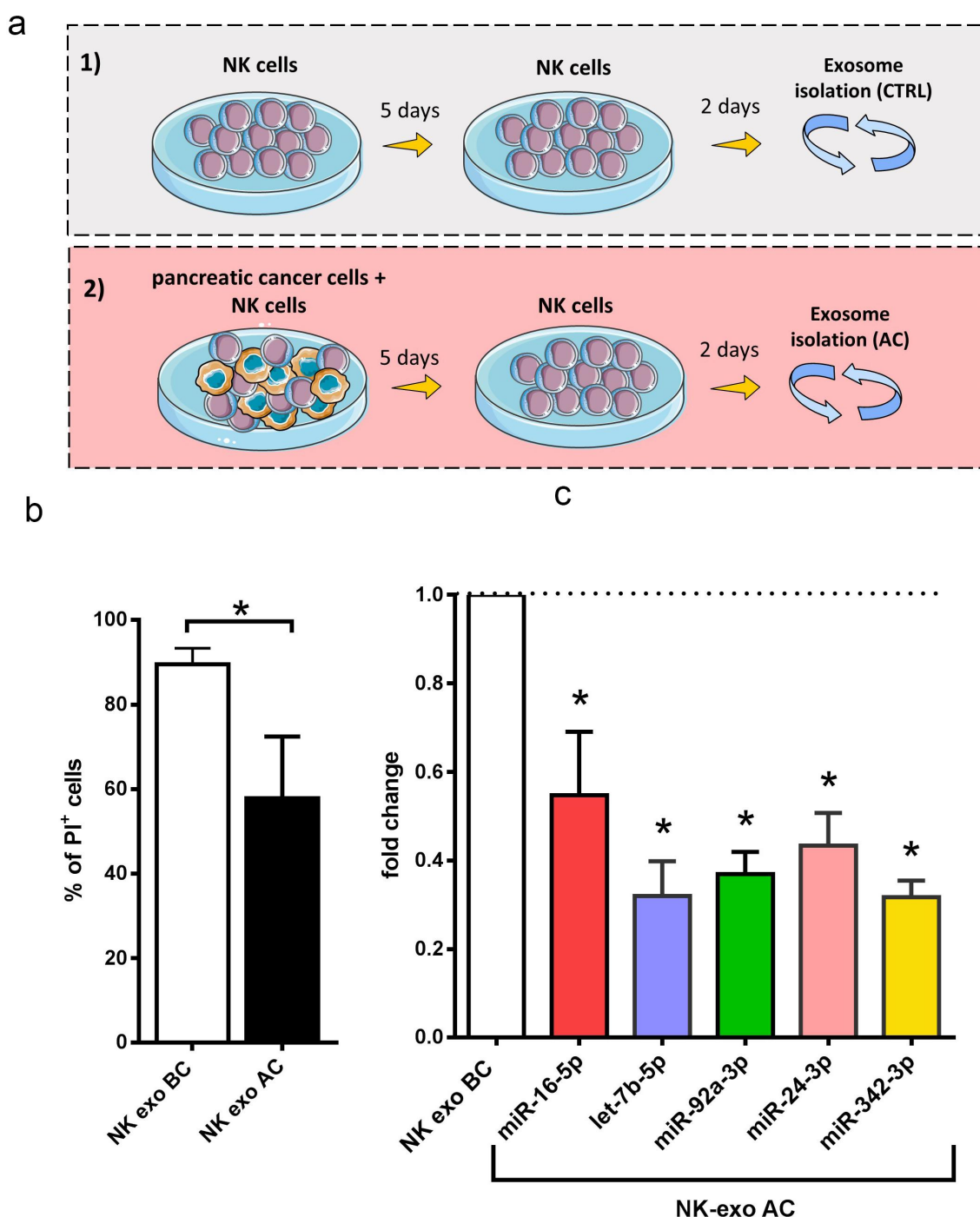
Figure 4. Lytic activity and miRNA content of NK-exo are affected by co-culture with pancreatic cancer cells. (a) Picture of the experimental design: 1) as control, NK cells were purified and grown in medium supplemented with exosome-depleted FBS. NK-exo were isolated by ultracentrifugation after 2 d. 2) After 5 d of NK-PANC1 cells co-culture, NK cells were purified, seeded after 2 d NK-exo were obtained as in 1). (b) Percentages of dead NALM-18 target cells (PI⁺) measured by flow cytometry after incubation (24 hours) with exosomes derived from NK cells not co-cultured (CTRL) or after PANC1 co-culture (AC). 20 μg of exosomes/10,000 cells (~1.6 × 10⁹ exosomes/cell). Bars indicate SEM. * p-value ≤0.05 (Student's t-test). (c) Real time PCR of the indicated miRnas in NK-exo derived from NK cells after PANC1 co-culture (NK exo AC). Values are expressed as fold change with respect to exosomes from NK cells not co-cultured (NK exo CTRL). Bars indicate SEM. * p-value ≤0.05 (Student's t-test; $n = 4$).

mediated cytotoxic effects on cancer cells[18]. However, we did not observe significant changes in the expression of DNAM-1 and other effector molecules in NK exosomes after co-culture with pancreatic cancer cells (data not shown). The concomitant reduction of both cytolytic activity and miRNA cargo in NK-exo was suggestive of the role of exosomal miRNAs in the NK-exo cytotoxicity. Indeed, previous reports have suggested that NK cells can exert antitumor effects through exosome-mediated delivery of miRNAs. Sun et al. reported that in pancreatic cancer miR-3607-3p derived from NK-exo can inhibit cancer progression[22]. We detected only a weak expression signal for miR-3607-3p both in NK cells and exosomes, and we did not find differential expression in NK-exo. This discrepancy could depend on different methodological approaches and/or on the NK culture conditions, which may significantly affect miRNA expression profiles. Sun et al. investigated

a single miRNA in NK EVs obtained from resting NK cells after 24 h of *in vitro* culture in serum-free media; we explored the entire miRNome in exosomes purified from IL2-activated NK cells cultured in exosome-free serum supplemented media.

Exosomes can exert their effects on target cells in multiple ways, including transfer of their content into target cells. Active miRNA transfer from exosomes to target cells has been well described and represents a mechanism allowing genetic transfer between cells[42]. Remarkably, the exosomal miRNA subset identified in our study includes let-7b-5p, a member of let-7 family with a critical role in oncosuppression. Upon incubation with NK exosomes, pancreatic cancer cells increased their expression of let-7b-5p, suggesting a miRNA transfer from NK-exo into cancer cells. One of the most important biological effects mediated by let-7 family is the inhibition of cell proliferation[30]. Accordingly, ectopic expression of let-7b-5p in PANC-1 cells induced inhibition of cell growth, as well as down-regulation of the let-7-b target CDK6, whereas let-7b-5p inhibition induced opposite effects. Interestingly, the incubation of pancreatic cancer cells with NK-exo induced inhibitory effects is similar to those observed upon ectopic expression of let-7b-5p, whereas let-7b inhibition abrogated this effect, suggesting that exosomal let-7b-5p may play a role in this process.

Down-regulation of let-7b targets, especially CCNA2 and HMGA1, resulted in more efficiency upon mimic transfection (characterized by a higher let-7b up-regulation) as compared to exosome treatment. Despite these data, NK-exo exerted a stronger inhibition on PANC-1 cell proliferation than the ectopic expression of let-7b-5p by transfection. These results suggest that exosomes use multiple ways to reduce proliferation of target cancer cells, and let-7b-5p is only one of the players involved in the process. Future studies are necessary to identify other molecules in exosome cargo with anti-tumor effects (e.g., proteins, miRNAs or other nucleic acids).

CDK6 resulted the most down-regulated let-7b-5p target in PANC-1 cells upon NK-exo treatment. However, in MIA PaCa-2 cells, CDK6 was barely detectable and NK-exo or let-7b ectopic expression failed to down-regulate it. Cell cycle in this pancreatic cell line appeared more CDK4-dependent since CDK4 was highly expressed. Of note, CDK4 was negatively regulated by let-7b-5p and associated to reduced cell growth. CDK4 and CDK6 are G1 kinases regulating the transition from G1 to S phase and have been identified as a major driver in cancer progression[43]. CDK4 and CDK6 up-regulation has clinical impact in human cancers. Indeed, by exploring public datasets, we found that CDK4/6 are up-regulated in pancreatic cancers, whereas let-7b-5p expression exhibited an opposite expression pattern. These data highlight the potential relevance of CDK4/6-let-7 axis in pancreatic cancer proliferation and progression. Recently, our group showed that NK exosomes have cytotoxic effects on PANC-1 cells[23]. Thus, the induction of cell death and the down-regulation of cell cycle genes by let-7b-5p transfer could represent two mechanisms by which NK exosomes counteract tumor growth. On the other hand, our data also indicate that alterations in the miRNA cargo of NK-exo may represent a novel strategy exerted by cancer to evade immune response.

The identification of specific molecules in NK exosomes with anti-tumor activity is important also in the broad perspective of tumor therapy, potentially including adult and pediatric cancers. For example, NK exosomes loaded with let-7a-5p, a let-7 family member sharing high sequence similarity with let-7b-5p, was found to accumulate in tumor tissue and inhibit cancer cell proliferation in a mouse model of neuroblastoma[44].

In conclusion, this study sheds light on the contribution of miRNAs contained in NK-exo to the anti-tumor effects and provides clues for the development of novel therapeutic tools.

Data availability statement

Microarray data are available in the public, open access repository Gene Expression Omnibus (GEO), with GEO accession number *GSE186056*, at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186056.

Notes on contributions

ADP performed experiments, analyzed and interpreted data and wrote the article. AP designed and performed experiments, interpreted data and wrote the article. PF, FB and SS performed experiments. NT performed experiments and revised the manuscript. LQ revised the manuscript. PV and LM interpreted data and revised the manuscript.

References

1. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423–1437. doi:10.1038/nm.3394.
2. Wang M, Zhao J, Zhang L, Wei F, Lian Y, Wu Y, Gong Z, Zhang S, Zhou J, Cao K, et al. Role of tumor microenvironment in tumorigenesis. J Cancer. 2017;8(5):761–773. doi:10.7150/jca.17648.
3. Groth A, Kloss S, von Strandmann EP, Koehl U, Koch J. Mechanisms of tumor and viral immune escape from natural killer cell-mediated surveillance. J Innate Immun. 2011;3(4):344–354. doi:10.1159/000327014.
4. Cantoni C, Wurzer H, Thomas C, Vitale M. Escape of tumor cells from the NK cell cytotoxic activity. J Leukoc Biol. 2020;108(4):1339–1360. doi:10.1002/JLB.2MR0820-652R.

5. Paul S, Kulkarni N, Shilpi LG, Lal G. Intratumoral natural killer cells show reduced effector and cytolytic properties and control the differentiation of effector Th1 cells. Oncoimmunology. 2016;5(12): e1235106. doi:10.1080/2162402X.2016.1235106.

6. Kalluri R, LeBleu VS. The biology, function, and biomedical applications of exosomes. Sci. 2020;367(6478). doi:10.1126/science. aau6977.

7. Mashouri L, Yousefi H, Aref AR, Ahadi AM, Molaei F, Alahari SK. Exosomes: composition, biogenesis, and mechanisms in cancer metastasis and drug resistance. Mol Cancer. 2019;18(1):75. doi:10.1186/s12943-019-0991-5.

8. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol. 2019;20(1):21–37. doi:10.1038/s41580-018-0045-7.

9. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–233. doi:10.1016/j.cell.2009.01.002.

10. Groot M, Lee H. Sorting mechanisms for MicroRNAs into extracellular vesicles and their associated diseases. Cells. 2020;9(4):1044. doi:10.3390/cells9041044.

11. Santangelo L, Giurato G, Cicchini C, Montaldo C, Mancone C, Tarallo R, Battistelli C, Alonzi T, Weisz A, Tripodi M, et al. The RNA-Binding protein SYNCRIP is a component of the hepatocyte exosomal machinery controlling MicroRNA sorting. Cell Rep. 2016;17(3):799–808. doi:10.1016/j.celrep.2016.09.031.

12. Squadrito ML, Baer C, Burdet F, Maderna C, Gilfillan GD, Lyle R, Ibberson M, De Palma M. Endogenous RNAs modulate microRNA sorting to exosomes and transfer to acceptor cells. Cell Rep. 2014;8 (5):1432–1446. doi:10.1016/j.celrep.2014.07.035.

13. Pesce S, Greppi M, Ferretti E, Obino V, Carlomagno S, Rutigliani M, Thoren FB, Sivori S, Castagnola P, Candiani S, et al. miRnas in NK cell-based immune responses and cancer immunotherapy. Front Cell Dev Biol. 2020;8:119. doi:10.3389/fcell.2020.00119.

14. Bezman NA, Cedars E, Steiner DF, Blelloch R, Hesslein DG, Lanier LL. Distinct requirements of microRnas in NK cell activation, survival, and function. J Immunol. 2010;185(7):3835–3846. doi:10.4049/jimmunol.1000980.

15. Xu SJ, Hu HT, Li HL, Chang S. The role of miRnas in immune cell development, immune cell activation, and tumor immunity: with a focus on Macrophages and Natural Killer cells. Cells. 2019;8 (10):1140. doi:10.3390/cells8101140.

16. Yang N, Zhu S, Lv X, Qiao Y, Liu Y-J, Chen J. MicroRNAs: pleiotropic regulators in the tumor microenvironment. Front Immunol. 2018;9:2491. doi:10.3389/fimmu.2018.02491.

17. Donatelli SS, Zhou JM, Gilvary DL, Eksioglu EA, Chen X, Cress WD, Haura EB, Schabath MB, Coppola D, Wei S, et al. TGF-β–inducible microRNA-183 silences tumor-associated natural killer cells. Proc Natl Acad Sci U S A. 2014;111 (11):4203–4208. doi:10.1073/pnas.1319269111.

18. Di Pace AL, Tumino N, Besi F, Alicata C, Conti LA, Munari E, Maggi E, Vacca P, Moretta L. Characterization of human NK cell-derived exosomes: role of DNAM1 receptor in exosome-mediated cytotoxicity against tumor. Cancers. 2020;12 (3):12. doi:10.3390/cancers12030661.

19. Lugini L, Cecchetti S, Huber V, Luciani F, Macchia G, Spadaro F, Paris L, Abalsamo L, Colone M, Molinari A, et al. Immune surveillance properties of human NK cell-derived exosomes. J Immunol. 2012;189(6):2833–2842. doi:10.4049/jimmunol.1101988.

20. Wu CH, Li J, Li L, Sun J, Fabbri M, Wayne AS, Seeger RC, Jong AY. Extracellular vesicles derived from natural killer cells use multiple cytotoxic proteins and killing mechanisms to target cancer cells. J Extracell Vesicles. 2019;8:1588538. doi:10.1080/20013078.2019.1588538.

21. Neviani P, Wise PM, Murtadha M, Liu CW, Wu CH, Jong AY, Seeger RC, Fabbri M. Natural Killer–derived exosomal miR-186 inhibits Neuroblastoma growth and immune escape mechanisms. Cancer Res. 2019;79(6):1151–1164. doi:10.1158/0008-5472.CAN-18-0779.

22. Sun H, Shi K, Qi K, Kong H, Zhang J, Dai S, Ye W, Deng T, He Q, Zhou M, et al. Natural Killer Cell-Derived Exosomal miR-3607-3p Inhibits Pancreatic Cancer Progression by Targeting IL-26. Front Immunol. 2019;10:2819. doi:10.3389/fimmu.2019.02819.

23. Fiore PF, Di Pace AL, Conti LA, Tumino N, Besi F, Scaglione S, Munari E, Moretta L, Vacca P. Different effects of NK cells and NK-derived soluble factors on cell lines derived from primary or metastatic pancreatic cancers. Cancer Immunol Immunother. 2022;72(6):1417–1428. Nov Online ahead of print. doi:10.1007/s00262-022-03340-z.

24. Pathan M, Keerthikumar S, Ang CS, Gangoda L, Quek CY, Williamson NA, Mouradov D, Sieber OM, Simpson RJ, Salim A, et al. FunRich: an open access standalone functional enrichment and interaction network analysis tool. Proteomics. 2015;15 (15):2597–2601. doi:10.1002/pmic.201400515.

25. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol. 1994;2:28–36.

26. Sticht C, De La Torre C, Parveen A, Gretz N, Campbell M. miRwalk: an online resource for prediction of microRNA binding sites. PLos One. 2018;13(10):e0206239. doi:10.1371/journal.pone.0206239.

27. Bartha Á, Gyorffy B. Tnmplot.com: a web tool for the comparison of gene expression in normal, tumor and metastatic tissues. Int J Mol Sci. 2021;22(5):22. doi:10.3390/ijms22052622.

28. Thery C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of thE international society for extracellular vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles. 2018;7(1):1535750. doi:10.1080/20013078.2018.1535750.

29. Fonseka P, Pathan M, Chitti SV, Kang T, Mathivanan S. FunRich enables enrichment analysis of OMICs datasets. J Mol Biol. 2021;433(11):166747. doi:10.1016/j.jmb.2020.166747.

30. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res. 2007;67(16):7713–7722. doi:10.1158/0008-5472.CAN-07-1083.

31. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, et al. Let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell. 2007;131(6):1109–1123. doi:10.1016/j.cell.2007.10.054.

32. Lee J, Lee HS, Park SB, Kim C, Kim K, Jung DE, Song SY. Identification of circulating serum miRnas as novel biomarkers in pancreatic cancer using a penalized algorithm. Int J Mol Sci. 2021;22(3):22. doi:10.3390/ijms22031007.

33. Torrisani J, Bournet B, du Rieu MC, Bouisson M, Souque A, Escourrou J, Buscail L, Cordelier P. Let-7 MicroRNA transfer in pancreatic cancer-derived cells inhibits in vitro cell proliferation but fails to alter tumor progression. Hum Gene Ther. 2009;20 (8):831–844. doi:10.1089/hum.2008.134.

34. Schultz J, Lorenz P, Gross G, Ibrahim S, Kunz M. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res. 2008;18(5):549–557. doi:10.1038/cr.2008.45.

35. Schubert M, Spahn M, Kneitz S, Scholz CJ, Joniau S, Stroebel P, Riedmiller H, Kneitz B. Distinct microRNA expression profile in prostate cancer patients with early clinical failure and the impact of let-7 as prognostic marker in high-risk prostate cancer. PLos One. 2013;8(6):e65064. doi:10.1371/journal.pone.0065064.

36. Knudsen ES, Kumarasamy V, Chung S, Ruiz A, Vail P, Tzetzo S, Wu J, Nambiar R, Sivinski J, Chauhan SS, et al. Targeting dual signalling pathways in concert with immune checkpoints for the treatment of pancreatic cancer. Gut. 2021;70(1):127–138. doi:10.1136/gutjnl-2020-321000.

37. Dong S, Huang F, Zhang H, Chen Q. Overexpression of BUB1B, CCNA2, CDC20, and CDK1 in tumor tissues predicts poor

survival in pancreatic ductal adenocarcinoma. Biosci Rep. 2019;39 (2):39. doi:10.1042/BSR20182306.

38. Azzariti A, Bocci G, Porcelli L, Fioravanti A, Sini P, Simone GM, Quatrale AE, Chiarappa P, Mangia A, Sebastian S, et al. Aurora B kinase inhibitor AZD1152: determinants of action and ability to enhance chemotherapeutics effectiveness in pancreatic and colon cancer. Br J Cancer. 2011;104(5):769–780. doi:10.1038/bjc.2011.21.

39. Pigati L, Yaddanapudi SC, Iyengar R, Kim DJ, Hearn SA, Danforth D, Hastings ML, Duelli DM. Selective release of microRNA species from normal and malignant mammary epithelial cells. PLos One. 2010;5(10): e13515. doi:10.1371/journal.pone.0013515.

40. Villarroya-Beltri C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vázquez J, Martin-Cofreces N, Martinez-Herrera DJ, Pascual-Montano A, Mittelbrunn M, Sánchez-Madrid F, et al. Sumoylated hnRNPA2B1 controls the sorting of miRnas into exosomes through binding to specific motifs. Nat Commun. 2013;4(1):2980. doi:10.1038/ncomms3980.

41. Alles J, Fehlmann T, Fischer U, Backes C, Galata V, Minet M, Hart M, Abu-Halima M, Grässer FA, Lenhof H-P, et al. An estimate of the total number of true human miRnas. Nucleic Acids Res. 2019;47(7):3353–3364. doi:10.1093/nar/gkz097.

42. Valadi H, Ekstrom K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRnas and microRnas is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–659. doi:10.1038/ncb1596.

43. Fassl A, Geng Y, Sicinski P. CDK4 and CDK6 kinases: from basic science to cancer therapy. Science. 2022;375(6577):eabc1495. doi:10.1126/science.abc1495.

44. Wang G, Hu W, Chen H, Shou X, Ye T, Xu Y. Cocktail strategy based on NK cell-derived exosomes and their biomimetic nano-particles for dual tumor therapy. Cancers (Basel). 2019;11(10):11. doi:10.3390/cancers11101560.