Human microRNA-1245 down-regulates the NKG2D receptor in natural killer cells and impairs NKG2D-mediated functions

J. Luis Espinoza,[1] Akiyoshi Takami,[1] Katsuji Yoshioka,[2] Katsuya Nakata,[1] Tokiharu Sato,[2] Yoshihito Kasahara,[3] and Shinji Nakao[1]

[1]Department of Hematology and Oncology, Kanazawa University Hospital, Kanazawa; [2]Division of Molecular Cell Signaling, Cancer Research Institute, Kanazawa University, Kanazawa; [3]Department of Laboratory Sciences, School of Health Sciences, College of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan

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Online Supplementary Design and Methods

Cell lines

Natural killer (NK) cell lines including NK-92 and KHYG-1 (a generous gift from Dr. Y. Isobe from Juntendo University, Japan) and NKL, obtained from Dr. M. J. Robertson (Bone Marrow Transplantation Program, Indiana University, Indianapolis, IN, USA) were cultured in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 IU/mL penicillin and 100 IU/mL interleukin (IL)-2 (Millipore, Temecula, CA, USA). The NK cell line YT was kindly provided by Dr. H. Kanegane from Toyama University and the cells were cultured in RPMI 1640 + 20% FBS, 100 μg/mL streptomycin and 100 U/mL of penicillin. The K562 cells transfected with the membrane-bound form of IL-15 and human 4-1BBL (K562-mb15-41BBL cells) were generously provided by Dr. Dario Campana (St. Jude Children's Research Hospital, Memphis, TN, USA) and were cultured in RPMI 1640 supplemented with 10% FBS. The 293FT cells were purchased from Invitrogen and were maintained in D-MEM (high glucose), 10% FBS, 0.1 mM MEM non-essential amino acids, 6 mM L-glutamine, 1 mM MEM sodium pyruvate 1% and 500 μg/mL of geneticin (all from Invitrogen). HEK cells (ATCC) were cultured in RPMI supplemented with 10% FBS.

Exosome precipitation from human plasma and microRNA detection in exosomes

EDTA-blood was collected from healthy donors and from ten patients with hematologic malignancies before they started chemotherapy. All patients gave written informed consent to participate in molecular studies of this nature in accordance with the Declaration of Helsinki. The patients' details are shown in *Online Supplementary Table S1*. The samples were processed for plasma isolation immediately after collection. The isolated plasma was further centrifuged at 3,000 x g for 15 min to remove cell debris and subjected to exosome precipitation using the ExoQuick exosome precipitation kit (System Biosciences) following the manufacturer's recommendations. Total RNA was extracted from the isolated exosomes as described by Hunter *et al.*[1] Equal amounts of RNA from each sample were reverse transcribed using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems). Since there is no current consensus on the use of an internal control for real-time polymerase chain reaction (PCR) analysis of plasma exosomes, the expression of miR-1245 in these fractions relative to RNA U6 and RNA 18S, which are expressed in blood exosomes,[1] was determined using quantitative reverse transcriptase (RT)-PCR as described above.

Measurement of microRNA-1245 primary transcripts

RNA was subjected to reverse transcription using a high capacity reverse transcription kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the TaqMan gene expression master mix (Applied Biosystems) with specific probes for miR-1245 primary transcripts (pri-miR-1245) (Assay ID: Hs03305715_pri) and for human GAPDH (Applied Biosystems) in a StepOne plus RT-PCR system (Applied Biosystems). The relative levels of pri-miR-1245 normalized to GAPDH mRNA were calculated by the comparative C_T method using the relative expression function included in the StepOne v2.2 software package (Applied Biosystems). Some cells were cultured in the presence or absence of variable concentrations of one of the following recombinant cytokines transforming growth factor-β1 (TGF-β1), IL-15, interferon-γ (PeproTech Inc,) and IL-2 and harvested at several time points for RNA extraction, and were then subjected to mature miR-1245 and pri-miR-1245 quantification.

Reporter gene assays

A segment of the 3'UTR of the *NKG2D* gene was amplified by PCR from the cDNA of a healthy individual. The PCR was performed with forward 5'-ATTGGATCCAGATGATCAACCATCTCAATA-3' and reverse 5'-ATTGGATCCGCATGAGACTCAAGATTCTAT-3' primers. The products, which encompass nucleotides 816 to 1575 in GenBank accession n. NM_007360.2 and contain *Bam*HI sites at both the 5' and 3' ends, were digested with *Bam*HI. The resultant fragments were sub-cloned into *Bam*HI-digested pGL3-Promoter (Promega), in which the simian virus 40 promoter was replaced with the herpes simplex virus thymidine kinase promoter derived from pRL-TK (Promega), to generate pGL3-NKG2D-3'UTR. The correct orientation and nucleotide sequences of the inserted 3'UTR in the plasmid was confirmed by DNA sequencing. A reporter

plasmid lacking the miR-1245 target site was generated by overlapping PCR, as described previously,[2] using the pGL3-NKG2D-3'UTR as template. The resultant mutant plasmid, pGL3-NKG2D-3'UTR-Δ16, designated hereafter as 3'UTR-mut, has a 16-bp deletion (5'-AGGCCAGCAGATCAC/GT-3'), which corresponds to the miR-1245 target site.

Equimolar amounts of each reporter plasmid construct (pGL3-NKG2D-3'UTR, 3'UTR-mut or pGL3-TK-Luc) were transfected into NKL cells using Exfect transfection reagent (Takara Bio, Japan), or into HEK cells using lipofectamine (Invitrogen), according to the manufacturers' recommendations. The cells were co-transfected with a renilla reporter plasmid (pRL-TK) with transfection of the different constructs always running in parallel to control for transfection efficiency. The transfected cells were harvested at 48 h and the activity of both luciferase and renilla were measured using the Dual Luciferase Reporter Assay System following the manufacturer's recommendations (Promega)

Natural killer cell transduction with exogenous microRNA-1245

Fresh NK cells were transduced by lentiviral delivery of a human miR-1245 precursor microRNA overexpression construct (PMIRH1245PA-1-SBI) or a negative control construct vector (pCDH-CMV-MCS-EF1-copGFP), designated hereafter as miR-1245-vector and NC-vector, respectively, following the manufacturer's recommendations (Systems Biosciences, Mountain View, CA, ISA) with minor modifications. The plasmids were transfected into the 293FT packaging cells with the help of a packaging vector (ViraPower Packaging Mix; Invitrogen) and lipofectamine 2000 (Invitrogen) to generate packaged pseudoviral particles. Supernatants containing lentivirus particles were harvested, and stored at -80°C until use. Virus was titrated following the manufacturer's recommendations. Lentivirus particles containing miR-1245-vector or NC-vector were used to transduce primary NK cells. Since these lentiviral delivery vectors encode copGFP as a fluorescent marker, the number of positively transduced cells, which was more than 90%, was monitored with the help of inverted fluorescent microscopy. In some experiments, lentivirus particles were delivered at increasing amounts to determine dose-dependent induction of mature miR-1245 in primary NK cells, KHYG-1 and HEK cells by using quantitative RT-PCR. For experiments assessing the comparative effects on NK cells, lentivirus particles were delivered at a multiplicity of infection (MOI) of 50, corresponding to the transducing units of 50×10^6/mL. The cells were harvested 48 h after transduction and separated into two aliquots. One aliquot was used for functional assays and the other for assessing NKG2D expression by flow cytometry and quantitative RT-PCR.

Construction of a short hairpin RNA mciroRNA-1245 lentivirus delivery system

A target sequence of the mature human miR-1245 was inserted in the pENTR/U6 BLOCK-iT entry vector (Invitrogen) to generate a short hairpin RNA mciroRNA-1245 (shRNA-miR-1245). The plasmid construct was designed using the BLOCK-iT RNAi Designer which was obtained from Invitrogen's website. Briefly, the annealed oligonucleotides: top strand Oligo; 5'-CACCGAAGTGATCTAAAGGCCTACACGAATGTAG-GCCTTTAGATCA CTT-3' and bottom strand Oligo 5'-AAAAAAGTGATCTAAAGGCCTACATTCGTGTAG GCCTTTAGATCACTTC -3' were inserted into pENTR/U6 vector to generate pENTR/miR-1245-shRNA. Stable miR-1245-deficient NK cells were established using the BLOCK-iT Lentiviral RNAi Expression System (Invitrogen) according to the manufacturer's recommendations. A recombination reaction using LR clonase was done to transfer the pENTR/miR-1245-shRNA or the negative control pENTR/U6-GW/lacZshRNA cassette into the pLenti6/BLOCK-iT-DEST (Invitrogen) to construct pLenti6/Block-iT pENTR/miR-1245-shRNA (designated hereafter as anti-miR-1245) or pLenti6/Block-iT pENTR/U6-GW/lacZshRNA (designated hereafter as shRNA-NC). These plasmids were transfected into the 293FT packaging cells with the help of the packaging vector ViraPower Packaging Mix and lipofectamine 2000 (Invitrogen). Culture supernatants containing lentivirus, collected 48 h after transfection, were titrated following the manufacturer's recommendations and stored at –80°C until use. KHYG-1 cells were transduced with lentivirus at a MOI of 100, corresponding to the transducing units of $100\text{x}10^6$/mL, at 37°C overnight. Four days after transduction, the cells were selected in blasticidin for 3 weeks and then sub-cloned by limiting dilution. Seven blasticidin-resistant clones (anti-miR-1245, five clones and shRNA NC, two clones) were selected, expanded and screened for miR-1245 and NKG2D expression.

Transfection with a synthetic inhibitor of microRNA-1245

A synthetic miR-1245 inhibitor, anti-has-miR-1245 (catalog # MIN0005897), and a miScript Inhibitor Negative Control (Catalog # 1027271) anti-microRNA-NC) designated hereafter as antago-miR-1245 and antago-NC, respectively, were obtained from Qiagen. These synthetic antagonists were used to transfect cultured NK cells. The transfection reactions were carried out in triplicate using the Hiperfect reagent (Qiagen) following the manufacturer's recommendations. The transfection efficiency was monitored by co-transfecting the cells with a GFP-expressing plasmid and cells were examined by flow cytometry and under a fluorescence microscope. Twenty-four hours after transfection, the cells were screened for NKG2D expression and were used for functional assays. The effective inhibition of the microRNA was monitored by measuring mature miR-1245 expression in the transfected cells cultured in the presence or absence of TGF-β1.

Measurement of cytokines and granzyme B secretion

Primary NK cells (2×10^6/mL) transduced with lentiviral miR-1245 or NC-vector were suspended in RPMI 1640 + 10% FBS and 50 U/mL IL-2 and cultured for 48 h in 96-well plates coated with a mixture composed of 5 μg/mL of each of the following recombinant human NKG2D ligands: Fc chimeras, MICA, ULBP1 and ULBP2 (R&D Systems) or with human IgG. Some cells were cultured in the presence of IL-2 alone. In some experiments, the NK cells lines, NKL and KHYG-1, over-expressing miR-1245 or their corresponding wild-type counterparts, or KHYG-1 cells with miR-1245 KO (all at 1×10^6/mL) were cultured for 48 h in NKG2D ligand-coated 96-well plates, as described above. The levels of tumor necrosis factor-α, interfer-

on-γ and granzyme B in the culture supernatants were determined by enzyme-linked immunosorbent assays (ELISA) specific for tumor necrosis factor-α, interferon-γ and granzyme B (Mabtech Nacka Strand Sweden) following the manufacturer's recommendations.

Cytotoxicity assay

The cytotoxicity assay was conducted using a non-radioactive LDH cytotoxicity assay (Roche). The OUN-1 target cells were pre-incubated with 200 μg/mL of valproic acid or vehicle for 24 h. In the blocking experiments, the effector cells were incubated with 5 μg of mouse anti-NKG2D monoclonal antibodies or an equal amount of isotype control antibodies (both from R&D systems) for 30 min at 37°C before their incubation with the target cells. The target cells were then placed in the wells of a 96-well plate, and the effector cells (either NK cell lines or primary NK cells) were added to each assay well at the indicated concentrations, and were incubated at 37°C for 4 h. The supernatants were then harvested and tested according to the manufacturer's instructions.

The effector cells were co-incubated with target cells at a 5:1 effector:target ratio. These ratios were chosen after preliminary optimization experiments. The results are presented as the median values from several individuals with each individual tested in triplicate assays for the indicated effector-target cell ratio.

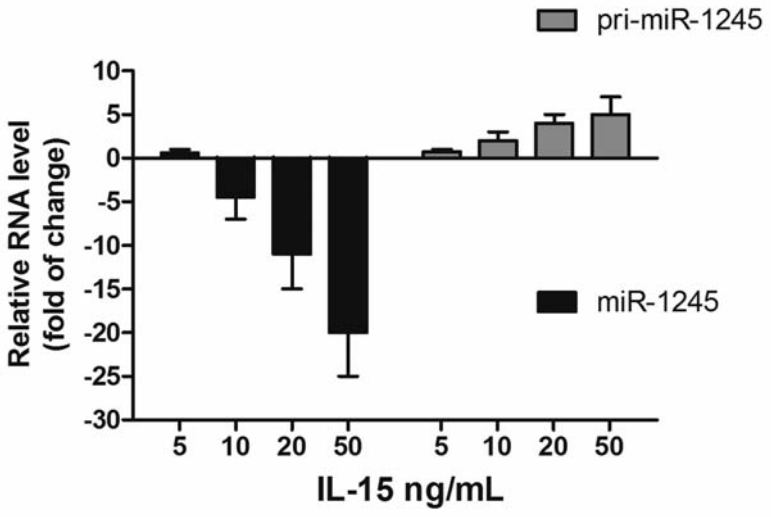
References

1. Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, et al. Detection of microRNA expression in human peripheral blood microvesicles. PLoS One. 2008;3(11):e3694.
2. Ito M, Yoshioka K, Akechi M, Yamashita S, Takamatsu N, Sugiyama K, et al. JSAP1, a novel jun N-terminal protein kinase (JNK)-binding protein that functions as a Scaffold factor in the JNK signaling pathway. Mol Cell Biol. 1999;19(11):7539-48.

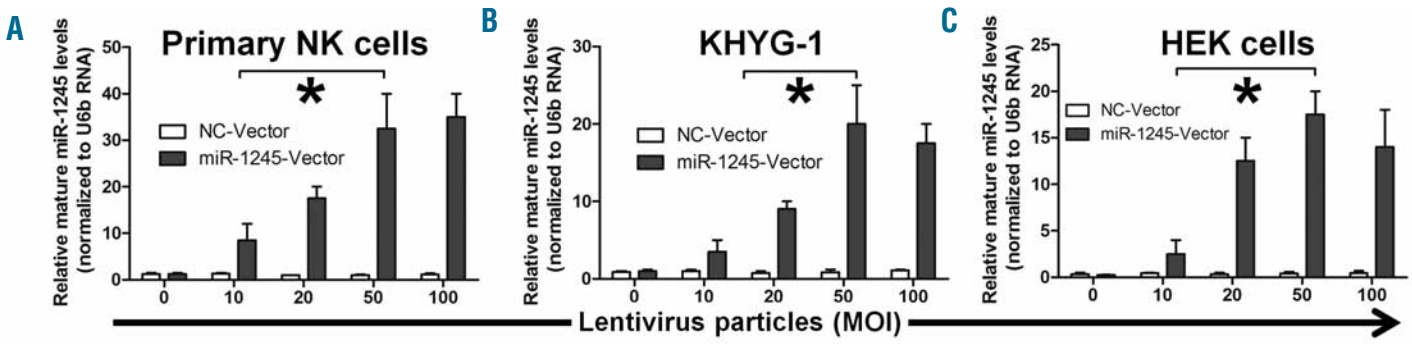
Online Supplementary Table S1. Characteristics of patients from whom plasma samples for exosome analysis were obtained.

N.	Age/Sex	Disease
NHL 1	82/F	NHL, diffuse large B cell
NHL 2	78/M	NHL, diffuse large B cell
NHL 3	71/F	NHL, T/NK cell
NHL 4	82/M	NHL, diffuse large B cell
NHL 5	67/F	NHL, follicular cell
NHL 6	64/F	NHL, follicular cell
NHL 7	68/F	NHL, lymphoplasmacytic
AML/MDS 1	42/M	AML, M2
AML/MDS 2	62/M	MDS, refractory anemia
AML/MDS 3	68/F	AML, M2

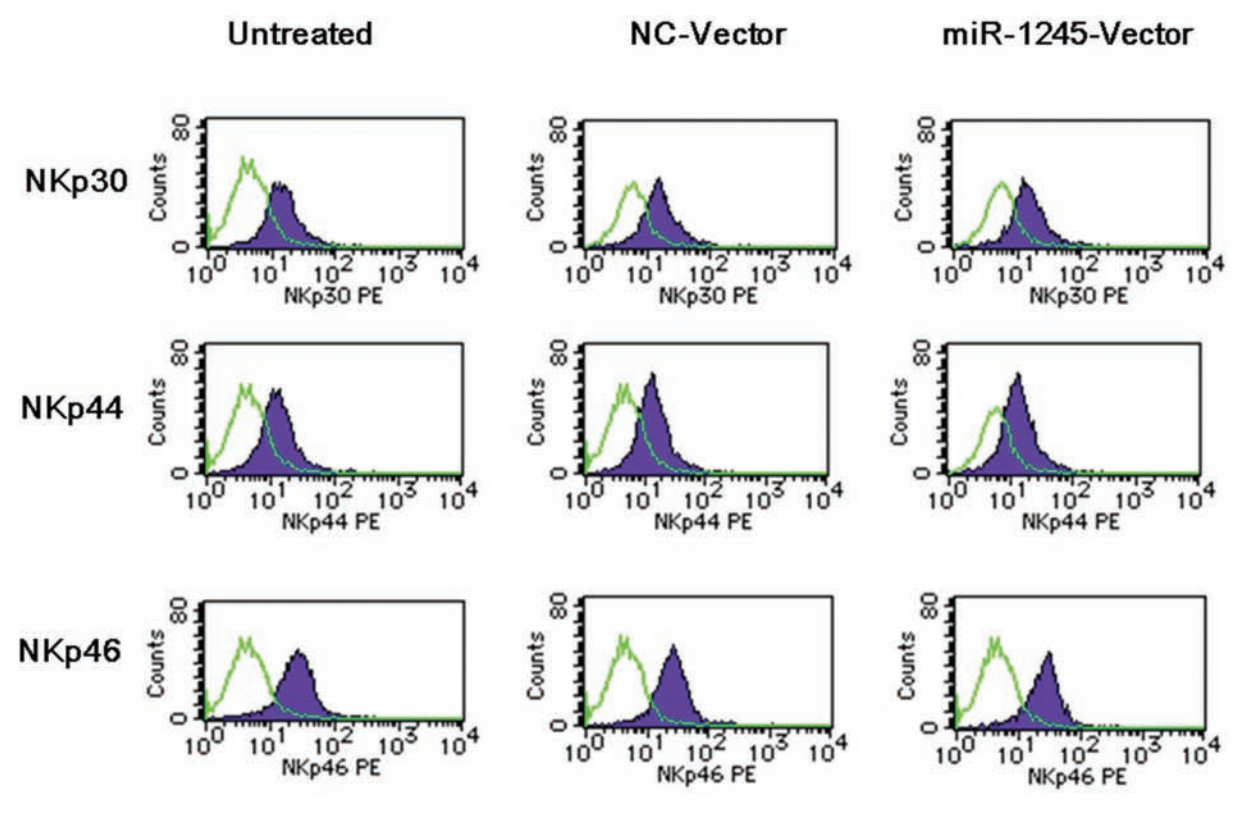
NHL: non-Hodgkin's lymphoma; AML: acute myeloid leukemia; MDS: myelodysplastic syndrome.



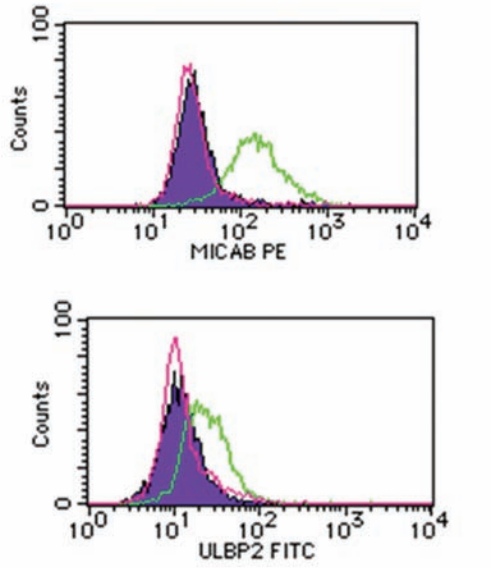
Online Supplementary Figure S1. The expression of miR-1245 and pri-miR-1245 in NK cells in response to stimulation with interleukin-15 (IL-15). Primary NK cells were cultured for 24 h with the indicated concentrations of IL-15. Total RNA was extracted and the levels of mature miR-1245 and pri-miR-1245 were measured by RT-PCR. The data are the means ± S.E.M. from three independent experiments using cells from healthy donors (n=3).



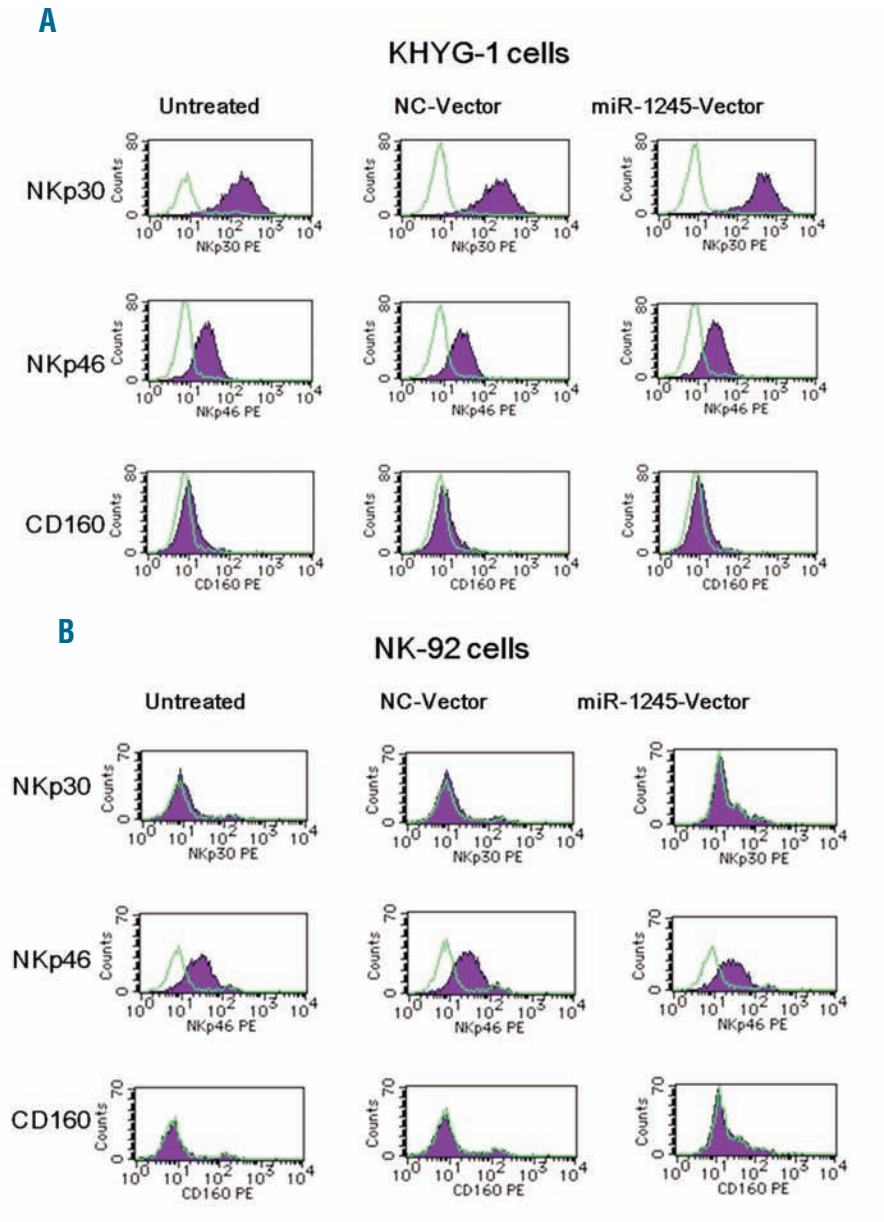
Online Supplementary Figure S2. The relative expression of mature miR-1245 in (A) fresh NK cells derived from five donors or in (B) KHYG-1 or (C) HEK cells transduced with increasing amounts of lentiviral particles containing a human miR-1245 precursor vector (miR-1245-vector) or a negative control vector (NC-vector). The data are the means ± S.E.M. from three independent experiments.



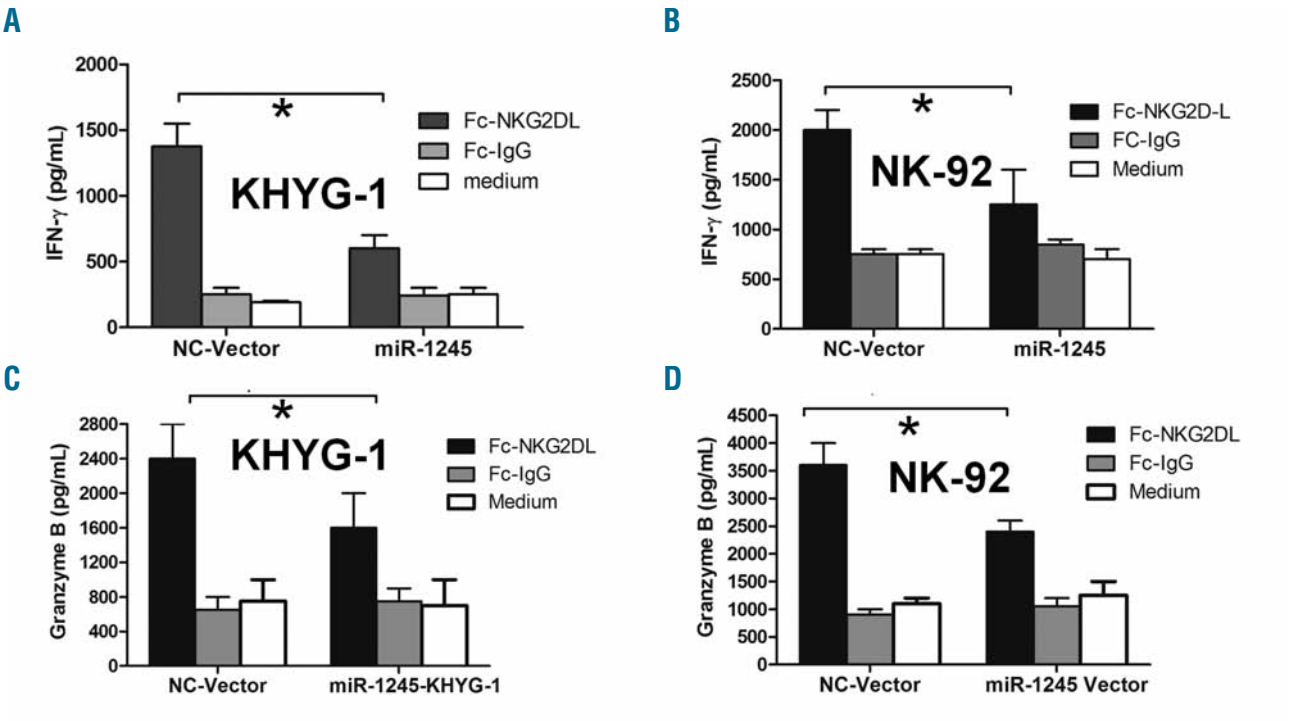
Online Supplementary Figure S3. The expression of activator receptors on NK cells transduced with miR-1245 precursor or a NC-vector or on their wild-type counterpart assessed by flow cytometry. Representative results using cells from the same donor are shown.



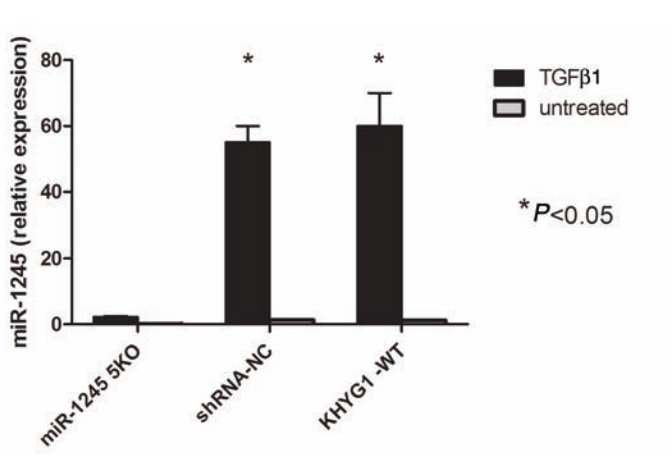
Online Supplementary Figure S4. The expression of the NKG2D ligands ULBP-2 and MICA/B on the surface of OUN-1 cells cultured for 24 h in the presence or absence of valproic acid.



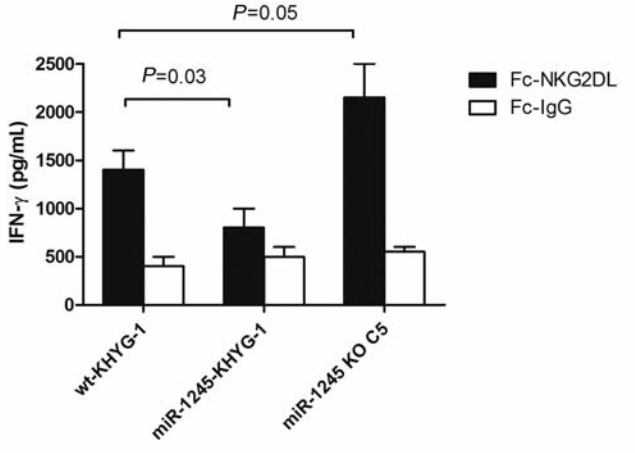
Online Supplementary Figure S5. The expression of activator receptors on (A) KYHG-1 cells or (B) NK-92 cells transduced with miR-1245-vector or a NC-vector or on their wild-type counterpart. Representative results for each cell line are shown.



Online Supplementary Figure S6. (A) KHYG1 cells and (B) NK-92 cells over-expressing miR-1245 or their counterparts transduced with NC-vector were cultured for 48 h in plates coated with recombinant NKG2D ligands (NKG2D-L). The levels of interferon-γ (IFN-γ) in the culture supernatants were determined using an ELISA. (C) KHYG1 cells and (D) NK-92 cells over-expressing miR-1245 or their counterparts transduced with NC-vector were cultured as in (A) and the levels of granzyme B in the culture supernatants were determined using an ELISA. The data are the means ± S.E.M. from three independent experiments. *indicates $P<0.05$.



Online Supplementary Figure S7. miR-1245 5KO KHYG-1 cells or their counterparts transduced with shRNA-NC, or wild-type KHYG-1 cells, were cultured for 24 h in the presence or absence of TGF-β1 (10 ng/mL), and the levels of mature miR-1245 normalized to that of U6b RNA were measured by quantitative RT-PCR. The data are the means ± S.E.M. from three independent experiments.



Online Supplementary Figure S8. miR-1245 5KO KHYG-1 cells or their counterparts transduced with shRNA-NC, or wild-type (wt) KHYG-1 cells, were cultured for 48 h in 96-well plates coated with recombinant NKG2 ligands (NKG2D-L). The levels of interferon-γ (IFN-γ) in the culture supernatants were determined using an ELISA. The data are the means ± S.E.M. from three independent experiments.