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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Correspondence:
Akiyoshi Takami, M.D., PhD, Department of Hematology and Oncology, Kanazawa University Hospital 13-1 Takaramachi, Kanazawa, 920-8641, Japan.
Phone: international +81.762652000.
Fax: international +81.762344277.
E-mail: takami@staff.kanazawa-u.ac.jp

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

NKG2D is an activating receptor expressed by natural killer and T cells, which have crucial functions in tumor and microbial immunosurveillance. Several cytokines have been identified as modulators of NKG2D receptor expression. However, little is known about *NKG2D* gene regulation. In this study, we found that microRNA 1245 attenuated the expression of NKG2D in natural killer cells.

Design and Methods

We investigated the potential interactions between the 3'-untranslated region of the *NKG2D* gene and microRNA as well as their functional roles in the regulation of NKG2D expression and cytotoxicity in natural killer cells.

Results

Transforming growth factor-β1, a major negative regulator of NKG2D expression, post-transcriptionally up-regulated mature microRNA-1245 expression, thus down-regulating NKG2D expression and impairing NKG2D-mediated immune responses in natural killer cells. Conversely, microRNA-1245 down-regulation significantly increased the expression of NKG2D expression in natural killer cells, resulting in more efficient NKG2D-mediated cytotoxicity.

Conclusions

These results reveal a novel NKG2D regulatory pathway mediated by microRNA-1245, which may represent one of the mechanisms used by transforming growth factor-β1 to attenuate NKG2D expression in natural killer cells.

Key words: NKG2D, microRNA-1245, TGF-β1.

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Introduction

NKG2D is an activating receptor expressed on natural killer (NK) cells which play a pivotal role in tumor immunosurveillance.[1-4] NKG2D is a member of the type II C-type lectin-like family of transmembrane proteins that function as both activating and co-stimulatory receptors and is constitutively expressed on most NK cells, as well on γδ T cells and some subsets of $CD4^+$ and $CD8^+$ T cells.[5] The NKG2D receptor recognizes multiple and structurally different ligands, including the MHC class I-chain related proteins (MICA and MICB) and the UL-16 binding proteins (ULBP 1-6).[6] These ligands are either absent or poorly expressed in normal tissues but are up-regulated in response to cellular stresses such as microbial infections and transformation.[1,5] Extensive research during the last few years has demonstrated the cytokine network that regulates the cell surface expression of the NKG2D receptor; however, little is known about the mechanisms that control expression of the *NKG2D* gene.

This study focused on the potential interactions between the 3'-untranslated region (3'UTR) of the *NKG2D* gene and microRNA. microRNA are endogenous, single-stranded RNA that modulate gene expression by binding to complementary sites in the 3'UTR of the target gene's mRNA. These 17-22 base oligonucleotides mediate gene regulation by either directly inducing mRNA degradation or by decreasing translational efficiency.[7,8] The data presented here identify microRNA (miR)-1245 as a novel negative regulator of NKG2D, and may clarify one of the mechanisms used by transforming growth factor-β1 (TGF-β1) to attenuate NKG2D expression.

Designs and Methods

Natural killer cell preparation and cell culture

Peripheral blood mononuclear cells were isolated from blood samples from healthy Japanese volunteers using a Lymphoprep (Pharmacia Biotech, Uppsala, Sweden) and the NK cell fraction was purified using the untouched NK isolation kit (Invitrogen). For some experiments NK cells were obtained by culturing the peripheral blood mononuclear cells from healthy donors in the presence of irradiated K562-mb15-41BBL cells in RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 100 IU/mL interleukin-2 for 10 days, as described previously.[9] These cultured peripheral blood mononuclear cells contained >95% $CD3^-CD56^+CD16^+$ NK cells and are referred to as "cultured NK cells". Details on the cell lines used in this study are given in the *Online Supplementary Design and Methods.*

Flow cytometry

CD3, CD56, CD16, CD160, MICA/B (BD Bioscience), NKG2D, NKp30, NKp44 and NKp46 (Beckman Coulter, Shizuoka, Japan) were detected by staining the cells with appropriate fluorescein isothiocyanate- or phycoerythrin-labeled monoclonal antibodies. ULBP ligand was detected by indirect staining using anti-ULBP1, ULBP2 and ULBP3 monoclonal antibodies (R&D Biosystems), followed by staining with fluorescein isothiocyanate-labeled anti-mouse IgG (BD Bioscience). Data acquisition and flow cytometry analyses were carried out on a BD FACS Calibur instrument using the CellQuest software package.

Quantitation of NKG2D mRNA levels

Total RNA was extracted from NK cells using Isogen (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions. Complementary DNA (cDNA) synthesis was carried out using a QuantiTect Reverse Transcription kit (Qiagen Inc. Hilden Germany). Amplification of cDNA was monitored using a QuantiFast SYBR green PCR kit (Qiagen) on a StepOne plus instrument (Applied Biosystems). Predesigned specific primers for NKG2D (assay name Hs_KLRK1_1_SG, Qiagen) and a *GAPDH* primer kit (Search LC, Heidelberg, Germany) were used for mRNA quantification in each sample. The amount of *NKG2D* mRNA relative to *GAPDH* mRNA was calculated by the comparative CT method using the relative expression function included in the StepOne v2.2 software package (Applied Biosystems).

Measurement of microRNA

To detect mature miR-1245, total RNA was extracted using the Isogen LS reagent (Nippon gene) and reverse transcription was performed using a TaqMan microRNA RT kit following the manufacturer's recommendations (Applied Biosystems). The resultant cDNA was amplified using the TaqMan microRNA assay (hs-miR-1245, assay ID002823) with the TaqMan Universal PCR master mix II no UNG (Applied Biosystems). The polymerase chain reaction (PCR) and cycling parameters were set following the manufacturer's recommendations with minor modifications as follows: a 10 μL PCR contained 4.5 μL of diluted cDNA product, 1X TaqMan Universal master mix and 1X of the TaqMan microRNA assay or 1X of the U6b-specific TaqMan probe (hs-miR-U6B assay ID001093). The reactions were incubated in 96-well plates at 95°C for 10 min, followed by 44 cycles of 95°C for 15s and 58°C for 1 min. All reactions were run in duplicate in a StepOne plus RT-PCR system (Applied Biosystems). The data were analyzed with the StepOne v2.2 software package (Applied Biosystems). The relative quantities of mature miR-1245 were calculated using the comparative CT method after normalization to the expression of U6b, as reported by others.[10,11]

Exosome precipitation from human plasma and microRNA detection in exosomes

Serum exosomes were isolated from healthy donors and from ten patients with hematologic malignancies before starting chemotherapy. All patients gave their written informed consent to participate in molecular studies of this nature according to the Declaration of Helsinki. The patients and methods are described in detail in the *Online Supplementary Design and Methods*.

Reporter gene assays

The reporter gene assays were performed by constructing luciferase vectors containing wild-type or mutant 3' UTR fragments of the *NKG2D* gene. Further details are given in the *Online Supplementary Design and Methods.*

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, USA). Further details are given in the *Online Supplementary Design and Methods.*

Establishment of cell lines over-expressing microRNA-1245

NK cell sub-lines over-expressing miR-1245 derived from NK-92 and KHYG-1 cells were established by lentiviral delivery of human miR-1245 precursor microRNA overexpression construct. The

lentivirus particles were delivered at a multiplicity of infection (MOI) of 50, corresponding to the transducing units of 50×10^6/mL. The effective over-expression of miR-1245 was confirmed by quantitative reverse transcriptase (RT)-PCR.

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

A lentivirus system for delivering a target sequence of the mature human miR-1245 was constructed using the pENTR/U6 BLOCK-iT entry vector and BLOCK-iT Lentiviral RNAi Expression System (Invitrogen) and was used to establish miR-1245-deficient NK cells. Further details are given in the *Online Supplementary Design and Methods*.

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.

Measurement of cytokines and granzyme B secretion

The levels of tumor necrosis factor-α, interferon-γ and granzyme B in the culture supernatants secreted by primary NK cells or from NK cell lines in response to NKG2D receptor stimulation were measured using specific enzyme-linked immunosorbent assay (ELISA) kits (Mabtech Nacka Strand Sweden), as described in more detail in the *Online Supplementary Design and Methods*.

Induction of NKG2D ligands on leukemia cells

To induce the expression of NKG2D ligands, OUN-1 leukemia cells were cultured for 24 h in the presence or absence of 200 μg/mL valproic acid, as described in a previous report.[9] The effective induction of the expression of the NKG2D ligands, MICA/B and ULBP-2, in this cell line was determined by flow cytometry.

Cytotoxicity assay

The cytotoxic activity of NK cells was measured with the LDH Non-Radioactive Cytotoxicity Assay (Roche Applied Science) using OUN-1 leukemia cells as target cells. Further details are given in the *Online Supplementary Design and Methods*.

Statistical analysis

All data are reported as means ± standard errors. When comparisons were made between two different groups, statistical significance was determined using Student's t-test. The statistical significance of multiple comparisons was determined using a one-way analysis of variance. The data were considered statistically significant when the *P* value was ≤0.05. All statistical analyses were performed using the GraphPad Prism software package (San Diego, USA).

Results

Identification of microRNA-1245 as a potential negative regulator of NKG2D in natural killer cells

Estimated to regulate more than 30% of all human genes, microRNA have emerged as important regulators of gene expression.[12] Since NKG2D is a powerful receptor modulated by numerous factors, many of which are known to induce changes in microRNA expression,[13] we hypothesized that the 3'UTR of the *NKG2D* gene may contain target sites for microRNA. To test this hypothesis, we first screened potential conserved microRNA targeting the 3'UTR of the *NKG2D* gene by using computational prediction tools. Three different online algorithms, TargetScan,[7] miTarget[14] and MicroSniper,[15] were used to demonstrate that the *NKG2D* 3'UTR contains a conserved region which represents a putative target site for miR-1245 (Figure 1A). The RNA hybrid software program, which is a computer alignment model based on the prediction of the minimal free energy of hybridization,[16] further predicted the interaction of *NKG2D* 3'UTR with miR-1245 (Figure 1B). Next, the endogenous expression of miR-1245 was assessed in NK cells to determine the functional significance of the predicted miR-1245 binding site in the 3'UTR of the *NKG2D* gene. Quantitative RT-PCR revealed that miR-1245 is expressed in fresh NK cells and was also detected in cultured NK cells (Figure 1C). Variable levels of mature miR-1245 were also detected in the four NK cell lines tested (Figure 1D). Since exosomes from serum samples have been reported to contain functional microRNA,[11,17] the expression of miR-1245 was examined in the exosomes from the plasma of patients with hematologic malignancies and from healthy donors. While miR-1245 was undetectable in the plasma exosomes of seven healthy individuals, the exosomes of three out of seven patients with non-Hodgkin's lymphoma and two out of three patients with acute myelogenous leukemia/myelodysplastic syndrome contained abundant levels of miR-1245 (Figure 1E), thus substantiating the biological relevance of this novel microRNA.

Interaction of microRNA-1245 with the 3'-untranslated region of the NKG2D gene

A luciferase reporter assay was used to directly assess whether the 3'UTR region of *NKG2D* interacts with miR-1245. A fragment of the 3'UTR region of *NKG2D* was inserted downstream of the luciferase open reading frame of the luciferase reporter vector pGL3-TK-Luc and the resultant construct was transfected into either stable miR-1245-overexpressing NKL cells or their wild-type counterparts. The presence of a *NKG2D* 3'UTR fragment in the reporter vector suppressed the luciferase activity in NKL cells but this effect was significantly greater in cells overexpressing miR-1245 (80% luciferase repression) than in the wild-type cells (44% luciferase repression) (Figure 2A). The forced expression of miR-1245 in HEK cells resulted in greater repression of luciferase activity induced by the *NKG2D* 3'UTR construct as well (Figure 2B). Remarkably, when the cells were transfected with a mutant reporter plasmid, which included a deletion of the microRNA-1245 target sequence region, there was a significant decrease in the repression of luciferase activity induced by the *NKG2D* 3'UTR fragment, which completely abolished the increased repression induced by ectopic miR-1245 expression. As expected, there were no differences in the luciferase activity in either the miR-1245-overexpressing cells, or in the wild-type cells transfected with the pGL3-TK-Luc vector (without *NKG2D* 3'UTR fragments). Taken together, these results indicate that the microRNA-1245 binding motif in the *NKG2D* 3'UTR is targeted by miR-1245.

Inducible over-expression of microRNA-1245 in natural killer cells

To investigate the potential modulation of endogenous miR-1245 expression in NK cells, we first examined the expression of the primary gene transcript of miR-1245,

pri-miR-1245, which is the starting point in the biogenesis of mature miR-1245. Freshly isolated or activated NK cells (Figure 3A) and NK cell lines (Figure 3B) invariably expressed high levels of pri-miR-1245; these levels appeared to be higher than those of mature miR-1245, suggesting that mature miR-1245 may be markedly inducible. TGF-β1 was thought to be a candidate capable of modulating miR-1245 since it is a negative regulator of NKG2D expression[18,19] and TGF-β1 signals up-regulate other microRNA.[10,20] Primary NK cells or NK cell lines were treated with TGF-β1 and miR-1245 expression was examined. A quantitative RT-PCR analysis revealed that TGF-β1 treatment of primary NK cells induced a consistent and dose-dependent up-regulation of the level of mature miR-1245 (Figure 3C). Of note, TGF-β1 treatment did not increase the expression of pri-miR-1245. NK cell lines treated with TGF-β1 also had up-regulated mature miR-1245 levels (Figure 3D). To investigate whether the TGF-β1-mediated inducible over-expression of miR-1245 occurs at the transcriptional or post-transcriptional level, the time-based kinetic accumulation of pri-miR-1245 and mature miR-1245 in response to TGF-β1 treatment was examined in fresh NK cells derived from seven individuals. As shown in Figure 3E, the mature miR-1245 levels started increasing at 8 h and peaked at 16 h after starting TGF-β1 treatment. Conversely, no significant changes in the expression of pri-miR-1245 were detected. These results suggest that the TGF-β1-induced miR-1245 over-expression occurs at the level of post-transcriptional processing. The effect of other cytokines on the expression of miR-1245 was investigated. While the treatment of NK cells with interleukin-2 or interferon-γ had no significant effects on mature or pri-miR1245 levels, interleukin-15, which is a potent inducer of NKG2D expression, decreased the expression of mature miR1245 by 20-fold in NK cells (Figure 3F). Dose-dependent inhibition of mature miR-1245 by interleukin-15 further confirmed this finding (*Online Supplementary Figure S1*).

Effect of exogenous microRNA-1245 on NKG2D expression in natural killer cells

To investigate the effect of miR-1245 on the expression of NKG2D receptor on NK cells, fresh NK cells were transduced with a miR-1245 precursor vector (miR-1245-vector) or a negative control vector (NC-vector) using a lentiviral delivery system and the NKG2D receptor levels were

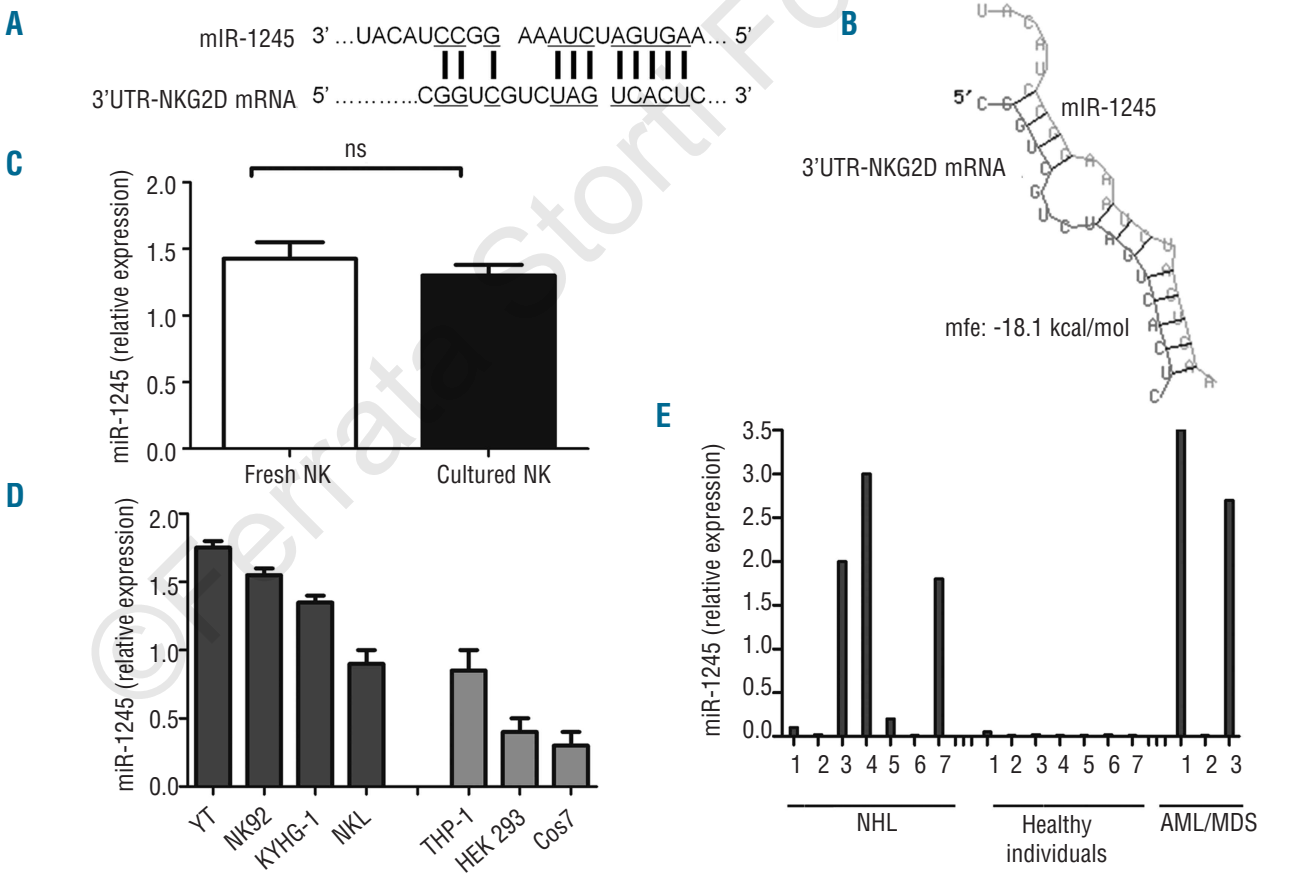

Figure 1. Identification of miR-1245 as a potential negative regulator of NKG2D in NK cells. (A) A schematic representation of the interaction between miR-1245 and its target site in the 3'UTR region of *NKG2D* mRNA. (B) Computational modeling showed the hybridization of miR-1245 and the 3'UTR region of *NKG2D* mRNA; mfe represents the calculated minimal free energy. (C) Mature miR-1245 expression in fresh or activated primary NK cells was measured by quantitative RT-PCR and normalized to U6B RNA. The data are the means ± S.E.M. (n= 13). (D) The expression of mature miR-1245 normalized to that of U6B RNA in NK cell lines YT, KYHG-1, NK92 and NKL. (E) The expression of mature miR-1245 in exosomes isolated from plasma samples from patients with non-Hodgkin's lymhoma (NHL) (n=7), acute myelogenous leukemia/myelodysplastic syndrome (AML/MDS) (n=3) and from seven healthy donors.

examined after transduction using flow cytometry. Effective over-expression of mature miR-1245 levels, which were dependent on the amount of lentivirus particles transduced, was achieved in primary NK cells, KHYG-1 cells and HEK cells as confirmed by quantitative RT-PCR (*Online Supplementary Figure S2*). The mature miR-1245 levels in NK cells transduced with a MOI of 50 were equivalent to those induced by treatment with 10 ng/mL of TGF-β1 for 16 h, and was achieved in NK cells transduced with miR-1245-vector. The over-expression of mature miR-1245 in NK cells resulted in a consistent down-regulation of the NKG2D receptor. (Figure 4A and 4B).

The over-expression of miR-1245 in primary NK cells did not reduce the expression of other activator receptors, including NKp30, NKp44 and NKp46, in primary NK cells indicating the specificity of miR-1245 in the NKG2D down-regulation (*Online Supplementary Figure S3*). Next, quantitative real-time PCR was used to measure NKG2D mRNA levels in NK cells transduced with miR-1245 in order to identify the suppressive mechanism used by this particular microRNA, since microRNA can suppress gene expression by inhibiting translation or by inducing mRNA degradation. There were significantly lower levels of NKG2D transcripts in NK cells transduced with the miR-1245-vector in comparison with the untreated cells or the NC-vector transduced cells ($P<0.05$) (Figure 4C)

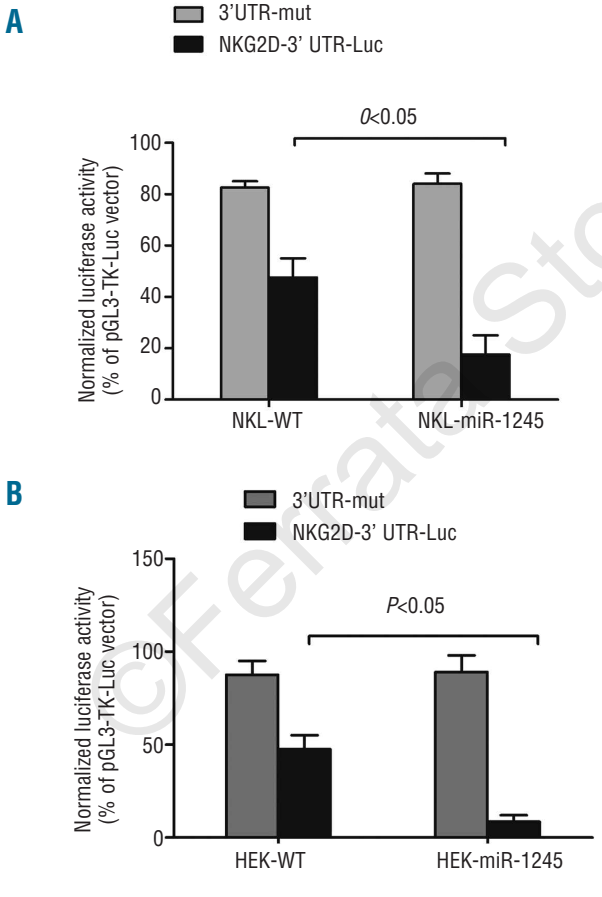

Figure 2. The interaction of miR-1245 with the *NKG2D* gene 3'UTR. (A) NKL cells or (B) HEK cells overexpressing miR-1245, or their wild type (WT) counterparts, were transfected with a luciferase expression vector (pGL3-TK-Luc), or with constructs including the *NKG2D* 3'UTR (NKG2D-3' UTR-Luc) or with a luciferase expression vector that included the *NKG2D* 3'UTR with a 16-bp deletion of the miR-1245 targeting site (3' UTR-mut). The firefly luciferase activities measured 48 h after transfection were normalized to the Renilla luciferase expression and the mean activities ± S.E.M. from three independent experiments are shown.

To substantiate whether the down-regulation of NKG2D by miR-1245 in NK cells has functional significance, the NKG2D-mediated effector functions were assessed in NK cells over-expressing miR-1245. Previous studies demonstrated that *in vitro* engagement of the NKG2D receptor by recombinant NKG2D ligands is sufficient to deliver activator signals leading to cytokine release from the NK cells.[21] To examine whether the NKG2D down-regulation induced by miR-1245 has functional implications, primary NK cells from seven healthy donors transduced with the miR-1245-vector, and their counterparts transduced with the NC-vector, were cultured in plates coated with a mixture of recombinant NKG2D ligands or on plates coated with mouse IgG, and the levels of tumor necrosis factor-α in the cell culture supernatants were measured by an ELISA. The NK cells over-expressing miR-1245 secreted significantly lower levels of tumor necrosis factor-α in response to NKG2D engagement with recombinant NKG2D ligands compared to the NK cells transduced with the NC-vector (Figure 4D). Consistent with these results of the cytokine secretion assay, the miR-1245 over-expressing NK cells displayed significantly lower cytotoxicity against the myeloid leukemia OUN-1 cells treated with valproic acid, an inducer of NKG2D ligands in this cell line (*Online Supplementary Figure S4*), compared with the NK cells transduced with NC-vector (Figure 4E). There was no difference in the killing effect between NK cells and miR-1245-transduced NK cells when the target cells were pretreated with vehicle alone, or when the effector NK cells were pretreated with anti-NKG2D monoclonal antibodies, thus suggesting that this effect was ligand-dependent. Taken together, these results suggest that the NKG2D expression regulated by microRNA-1245 in NK cells impairs the cytotoxic activity of NK cells.

Natural killer cell lines over-expressing microRNA-1245 down-regulate NKG2D expression

NK cell sub-lines derived from NK-92 and KYHG-1 cells lines were established by lentivirus delivery of the miR-1245-vector. The effective over-expression of miR-1245 in NK cell lines resulted in a significant decrease in cell surface NKG2D expression in KHYG-1 and NK-92 cells (Figure 5A and 5B) and reduced NKG2D transcripts (Figure 5C), indicating that miR-1245 is a negative regulator of NKG2D expression. The over-expression of miR-1245 in these cell lines was not associated with a reduction in the expression of other activator receptors (*Online Supplementary Figure S5*). Notably, the KHYG-1 and NK-92 cells over-expressing miR-1245 secreted significantly lower levels of interferon-γ and granzyme B in response to NKG2D receptor engagement by NKG2D ligands (*Online Supplementary Figure S6*).

Knocking down microRNA-1245 in natural killer cells resulted in higher NKG2D expression and relative resistance to the effect of transforming growth factor-β1

miR-1245-deficient NK cell lines were established by lentivirus delivery of the anti-miR-1245 to substantiate the role of miR-1245 in the regulation of NKG2D expression in NK cells. A KYHG-1 clone was identified, in which effective miR-1245 knockdown was consistently achieved as demonstrated by quantitative RT-PCR. In contrast to their wild-type counterpart or in clones transduced with shRNA-NC, these cells did not express miR-1245 even in the pres-

ence of TGF-β1 treatment (*Online Supplementary Figure S7*). Interestingly miR-1245 5KO cells expressed higher levels of NKG2D receptor at both protein (Figure 6A) and transcript levels (Figure 6B). In addition, although TGF-β1 treatment also resulted in the down-regulation of NKG2D receptor in miR-1245 5KO cells, this effect was less notable than in wild-type cells (Figure 6C) or in those cells transduced with shRNA-NC (*data not shown*). Moreover, miR-1245 5KO cells secreted higher levels of interferon-γ in response to NKG2D receptor engagement by NKG2D ligands compared to KYHG-1 cells transduced with shRNA-NC or with their wild-type counterpart (*Online Supplementary Figure S8*). These results suggest that NKG2D down-regulation induced by TGF-β1 in NK cells involves the induction of miR-1245.

To address whether the effects of knocking down miR-1245 are also observed in primary NK cells, cultured NK cells from four healthy individuals were transiently transfected with a synthetic antagonist of miR-1245 or a negative control antago-NC. The antago-miR-1245- transfected cells up-regulated NKG2D receptor (Figure 6D) and were less sensitive to the effects of TGF-β1 treatment on the down-regulation of NKG2D expression compared to their antago-NC-transfected counterparts (Figure 6E).

Discussion

Normal cells require a protective mechanism against NKG2D receptor overactivation to prevent self-attack, since NKG2D is implicated in certain autoimmune conditions.[22] This study has identified a novel mechanism for the modulation of *NKG2D* gene expression in NK cells. Several *in vitro* approaches demonstrated that miR-1245 interacts with the 3'UTR region of *NKG2D*, and that this interaction resulted in the down-regulation of NKG2D expression in NK cells, thus leading to an impairment in NKG2D-mediated immune functions, including less effective cytotoxicity and lower cytokine secretion.

One question remains with regard to how miR-1245 contributes to regulating the expression of NKG2D *in vivo*. The constitutive expression of miR-1245 in NK cells suggests that it may represent an endogenous autoregulatory mechanism to maintain NKG2D expression at physiological levels. NK cells are a pivotal source of endogenous TGF-β1, and this immunosuppressive cytokine secreted from NK cells represses NK cell functions, including the down-regulation of the NKG2D expression, in a paracrine/autocrine manner,[18,23,24] thus indicating that TGF-β1 plays an important role as negative feedback to prevent overstimulation of NK cell functions. The current findings suggest that miR-1245 induc-

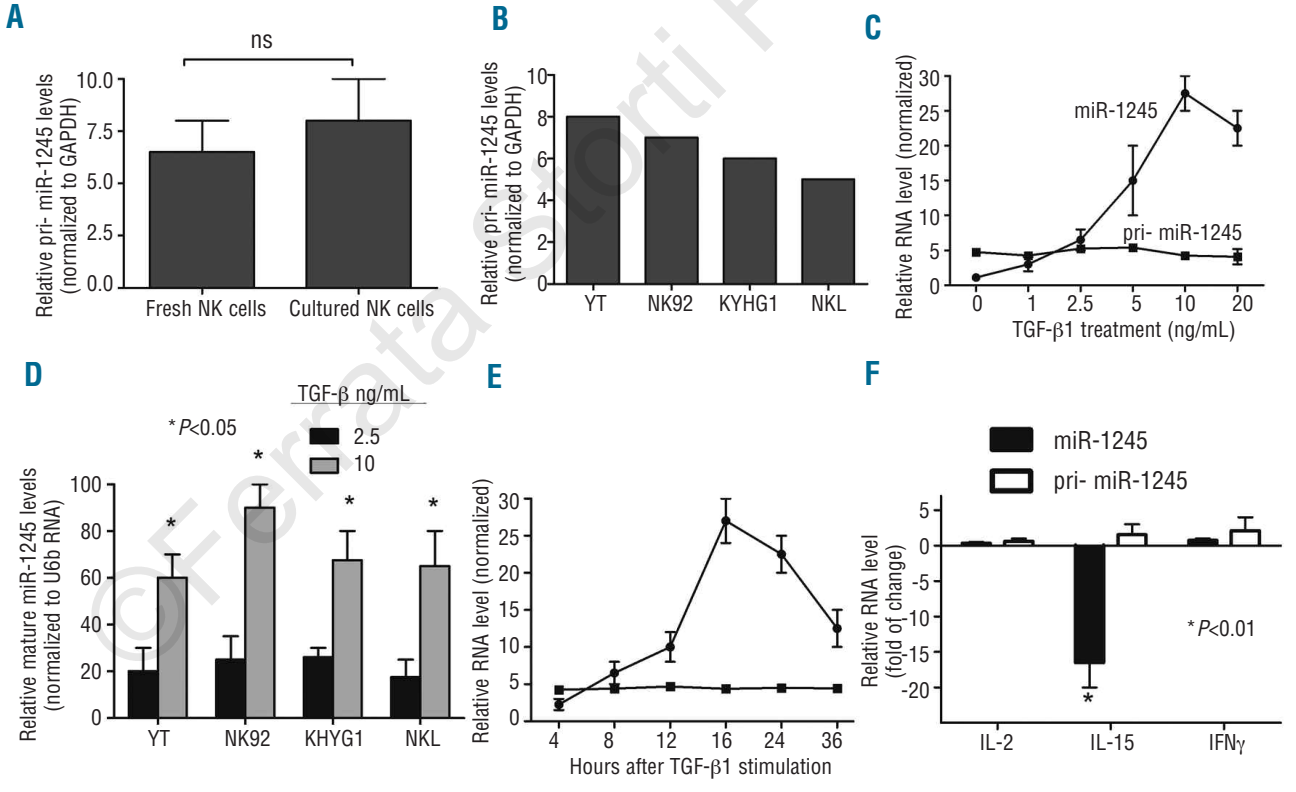

Figure 3. Post-transcriptional regulation of mature miR-1245 biogenesis in NK cells by TGF-β1. (**A**) The expression of the primary gene transcripts (pri-miR-1245) in fresh and cultured NK cells normalized to GAPDH. The data are the mean values ± S.E.M. from measurements in seven donors. (**B**) The expression of pri-miR-1245 normalized to GAPDH in the NK cell lines YT, NK92, KYHG-1, and NKL. (**C**) The expression of pri-miR-1245 and mature miR-1245 in primary NK cells treated with various concentrations of TGF-β1 for 24 h (n=7). (**D**) The expression of mature miR-1245 in NK cell lines after treatment with TGF-β1 (2.5 or 10 ng/mL) for 24 h. The error bars in (**C**) and (**D**) show the ± S.E.M. from three independent experiments, each measured in duplicate. (**E**) The time course of pri-miR-1245 and mature miR-1245 expression in primary NK cells after treatment with TGF-β1 (10 ng/mL) (n=7). The data are the means ± S.E.M. from three independent experiments, each measured in duplicate. (**F**) The expression of pri-miR-1245 (open bar) and mature miR-1245 (closed bar) in primary NK cells treated with interleukin (IL-2) (150 U/mL), interleukin-15 (IL-15) (50 ng/mL) or interferon-γ (IFN-γ) for 24 h. The error bars show the ± S.E.M. from three independent experiments, each measured in duplicate.

tion may, therefore, represent one of the downstream mechanisms by which TGF-β1 exerts such negative feedback to control NK cell activation.

NKG2D receptor plays a relevant role in tumor immunosurveillance and cancer cells use several mechanisms to escape NKG2D-mediated cytotoxicity including the down-regulation of NKG2D ligands on their surface, the release of soluble NKG2D ligands, to induce NKG2D silencing and the secretion of immunosuppressive cytokines. Indeed various cancer cells produce TGF-β1, which impairs NK cell functions in cancer patients, leading to stimulation of tumor growth[18,19,25] and TGF-β1 released by tumor-arising cells has been suggested to influence immunosurveillance; the tumor microenvironment represents an important source of TGF-β1 impairing NKG2D-mediated functions.[26] The present study suggests the involvement of miR-1245 in the mecha-

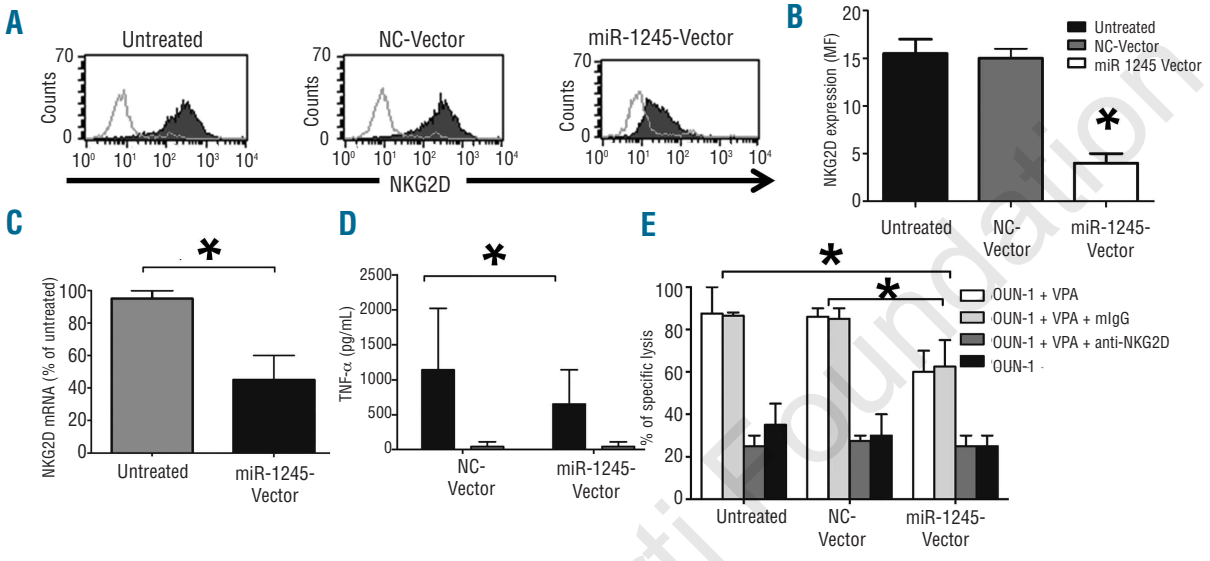

Figure 4. Exogenous miR-1245 decreases NKG2D expression in NK cells. (A) A representative result showing the cell surface expression of NKG2D on miR-1245-transduced primary NK cells. Cells stained with anti-NKG2D (filled histograms) or with the isotype antibody (open histogram). (B) The summarized data from 12 donors are shown. (C) Fresh NK cells were transduced as in the panels A and B, and the NKG2D mRNA levels were measured by quantitative RT-PCR 24 h after transduction. The relative results normalized to U6b RNA are expressed as the percentage of the mRNA compared to that for the control conditions (untreated cells). (D) Fresh NK cells transduced with the miR-1245-vector or with the NC-vector (n=7) and cultured in plates coated with a mixture of three recombinant NKG2D-Ls (MICA, ULBP-1 and ULBP-2) or control IgG. (E) Fresh NK cells treated as in panel D were left untreated (medium) and then assessed for their cytotoxicity against OUN-1 leukemia cells. The summarized data from seven donors (effector: target ratio, 5:1) are shown. The data are the means ± S.E.M. from three independent experiments. *$P<0.05$.

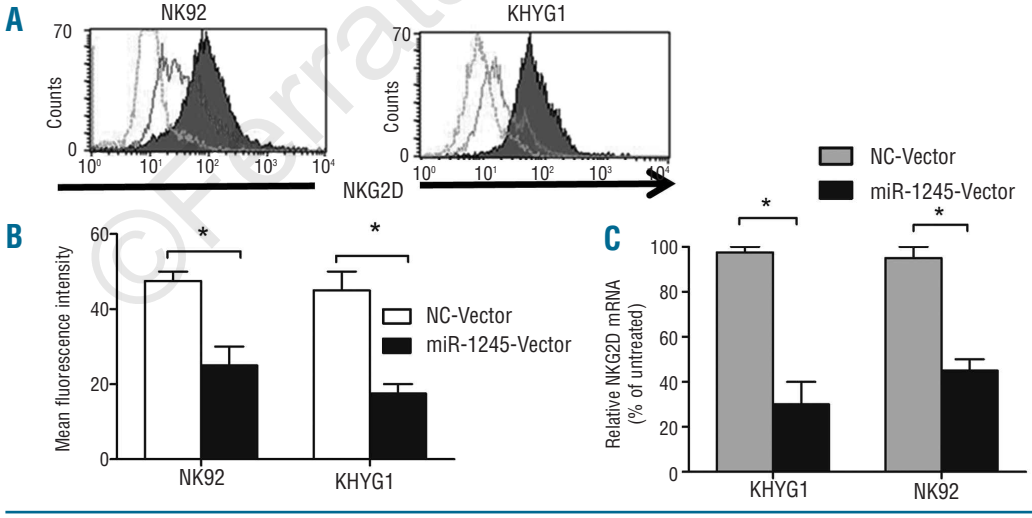

Figure 5. Down-regulation of the NKG2D receptor in NK cell lines stably overexpressing miR-1245. NK cell lines, NK92 andKHYG1 overexpressing miR-1245 were established by lentiviral delivery of a vector containing the miR-1245 precursor. (A) miR-1245 overexpressing cells or those transduced with a negative control vector were examined for NKG2D expression by flow cytometry. The filled histograms represent cells transduced with negative control vector, empty histograms represent cells transduced with the miR-1245-vector, and dashed line histograms represent cells stained with the isotype antibody. A representative figure showing the results of three independent experiments is shown. (B) Summarized results of three independent experiments for each cell line. (C) Total RNA was extracted from the above-described cells, and the normalized levels of the NKG2D transcript were measured by quantitative RT-PCR.

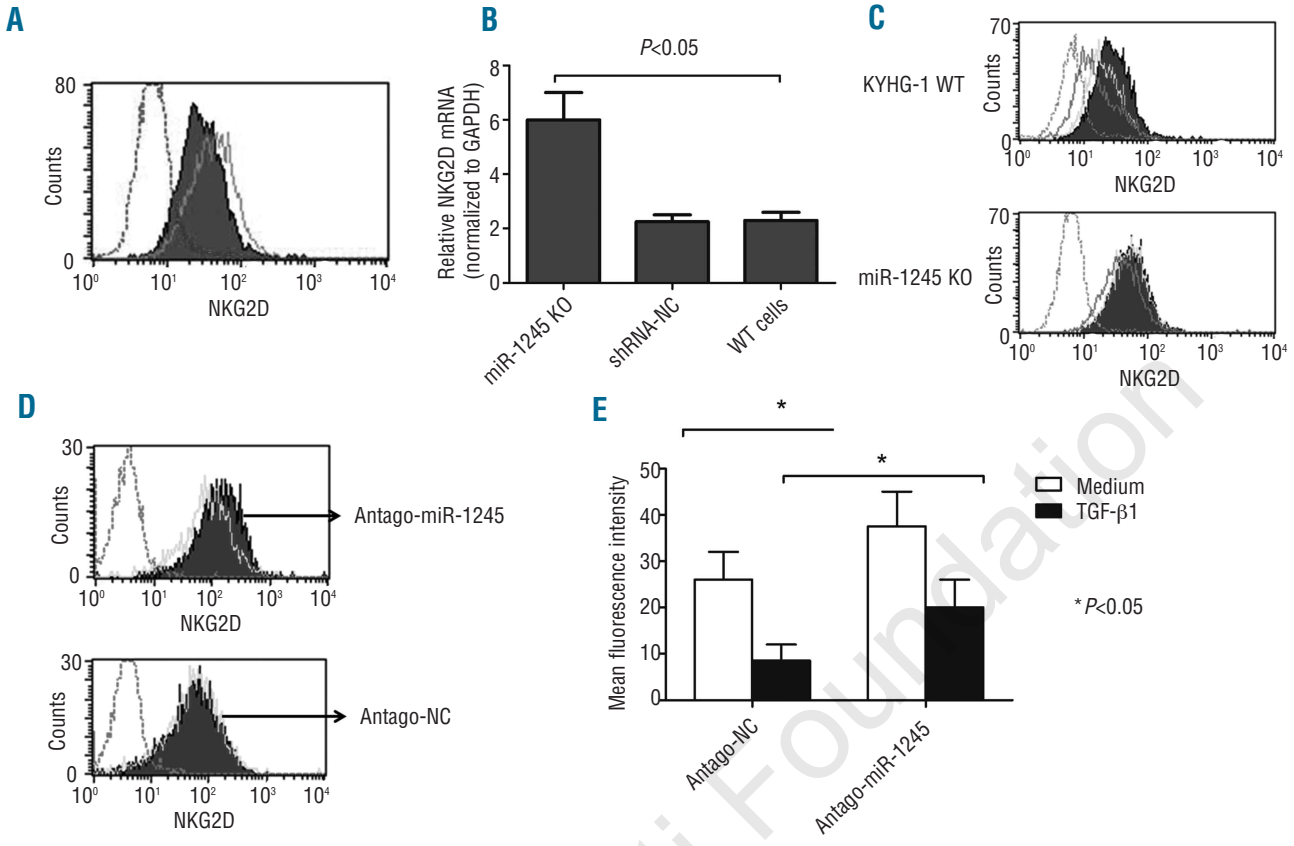

Figure 6. Knockdown of miR-1245 increases the expression of NKG2D in NK cells, and renders the cells less sensitive to TGF-β1. (A) KYHG-1 cells were examined by flow cytometry for NKG2D expression. The filled histogram represents shRNA-NC-transduced KHYG-1 cells stained with anti-NKG2D antibody. In the open histogram, the solid line shows the data from the miR-1245 5KO KHYG-1 cells stained with the anti-NKG2D antibody, and in the open histogram, the dotted line indicates cells stained with the isotype antibody. A representative figure from three independent experiments is shown. (B) Total RNA was extracted, and the levels of *NKG2D* mRNA normalized to the levels of *GAPDH* mRNA were measured by RT-PCR. The figure shows the means ± S.E.M. from three independent experiments. (C) miR-1245 5KO KHYG-1 cells or wild-type (WT) KHYG-1 cells were cultured for 48 h in the presence or absence of TGF-β1, and their NKG2D expression levels were examined by flow cytometry. The filled histogram represents untreated cells, the green histograms represent cells treated with TGF-β1 (5 ng/mL), the red histograms represent cells treated with TGF-β1 (10 ng/mL) and the blue histograms with the dotted line represent cells stained with the isotype antibody. A representative figure from the data obtained from three independent experiments is shown. (D) Cultured NK cells were left untreated or were transfected with antago-miR-1245 or antago-NC, and 48 h later, their NKG2D expression levels were examined by flow cytometry. The filled histogram represents cells transfected with antago-miR-1245 or antago-NC, the open histogram with solid lines represents untreated cells, and the open histograms with the dotted line represent cells stained with the isotype antibody. A representative figure from the data obtained from three independent experiments is shown. (E) Primary NK cells were transfected as in the panel (D). Twenty-four hours later, they were treated with TGF-β1 (10 ng/mL) and cultured for another 24 h, and their NKG2D expression levels were examined by flow cytometry. Summarized data for the NK cells from five donors are shown. The data are the means ± S.E.M. from three independent experiments.

nism(s) whereby the TGF-β1 produced in cancer patients may modulate NK cell functions. It is also conceivable that tumor cells might secrete miR-1245 and use this as a way to down-regulate NKG2D expression in order to escape NK cell-mediated killing. Although it is highly speculative, the release of microRNA through exosomes could provide a route by which tumor cells may deliver functional miR-1245 to impair NKG2D expression. In fact, tumor-derived exosomes have been reported to down-regulate NKG2D expression in NK cells.[19] Our finding that miR-1245 was detectable in exosomes of some patients with non-Hodgkin's lymphoma as well as in patients with acute myelogenous leukemia adds support to this hypothesis, although we were unable to investigate the possible correlation between miR-1245 expression in exosomes and NKG2D levels, since no NK cells from those patients were available for this study. Thus, further studies on the functional relevance of exosome derived miR-1245 are warranted.

The mature miR-1245 over-expression induced by TGF-β1 in NK cells occurs at the post-transcriptional level which is consistent with the mechanism by which TGF-β1 induces mature miR-21 in human vascular smooth muscle cells.[10,20] The regulation of miR-21 biogenesis involves the recruitment of TGF-β1–ligand specific SMAD signal transducer to members of the DROSHA microprocessor complex which facilitate the processing of primary transcript into pre-microRNA.[27]

The over-expression of miR-1245 reduced NKG2D expression at both the protein (Figure 4B) and mRNA (Figure 4C) levels in primary NK cells, thus suggesting that miR-1245 suppresses the expression of NKG2D through mRNA degradation, which is consistent with a recent study showing that decreasing target mRNA is the main mechanism by which mammalian microRNA regulate gene expression.[28]

However, the reduction of the expression of NKG2D appears to be greater at the protein level than at the mRNA level. Accordingly, in addition to mRNA degradation, miR-1245 could cause translational repression of mRNA; this possibility is supported by previous reports that microRNA reduces translation initiation[29] and increases ribosome drop-off.[30]

The present study did not investigate miR-1245 expression in different NK cell subsets such as $CD56^{bright}$ and $CD56^{dim}$. However, NK cells isolated from healthy individuals were more than 95% $CD16^{pos}$, suggesting that the vast majority of the NK cells were $CD56^{dim}$ and responsible for cytolytic activity and target killing.[31] Elucidating the functional roles of miR1245 in $CD56^{bright}$ $CD16^{neg}$ NK cells, which are the main source of cytokine production, will be an interesting challenge.

In conclusion, the current study has identified miR-1245 as a novel regulator of NKG2D expression by targeting the 3'UTR region of the *NKG2D* gene. TGF-β1, which is a potent NKG2D suppressor in NK cells, post-transcriptionally enhanced the expression of mature miR-1245, leading to down-regulation of the NKG2D receptor. There is a large amount of evidence to support the role of NKG2D in tumor immunosurveillance and progression. Although several clinical trials of TGF-β1 antagonists for cancer treatment are underway,[32] the main concern is that TGF-β1 antagonists could enhance the proliferation of tumor cells, which may counterbalance their potential therapeutic benefits on immunomodulation.[33] A recent study has shown that the processing of pri-microRNA could be selectively blocked.[34] miR-1245 could, therefore, be a promising target for tumor immunotherapy to improve NK cell activity.

Authorship and Disclosures

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