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miR-30c-1* promotes natural killer cell cytotoxicity against human hepatoma cells by targeting the transcription factor HMBOX1

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Natural killer (NK) cells play a critical role in antitumor immunity, and the activation of NK cells is regulated by a series of NK cell receptors. Here, we show that crosslinking CD226, an important NK cell receptor, with the anti-CD226 mAb LeoA1 on NKL cells, regulated the expression of several microRNA and transmembrane tumor necrosis factor-α. Among them, miR-30c-1* was noticed because overexpression of miR-30c-1* triggered upregulation of transmembrane tumor necrosis factor-α expression and enhanced NK cell cytotoxicity against hepatoma cell lines SMMC-7721 and HepG2. Furthermore, we proved that the inhibitory transcription factor HMBOX1, which depressed the activation of NK cells, was the direct target gene of miR-30c-1*. In conclusion, our results revealed a novel regulatory mechanism: miR-30c-1* promoted NK cell cytotoxicity against hepatoma cells by targeting HMBOX1. (*Cancer Sci* 2012; 103: 645–652)

Hepatoma is a highly aggressive cancer associated with a high mortality rate (www.who.int). Previous studies show that proliferated natural killer (NK) cells are present in the hepatoma microenvironment and are essential in antitumor activity.[1,2] Thus, augmentation of NK cell responses to tumors might be a promising immunotherapy approach.[3] There are some approaches that can be taken to enhance the NK cells' cytotoxicity against tumors. The signals delivered through a variety of activating and inhibitory receptors, such as NK cell activating receptor natural-killer group 2, member D (NKG2D), CD226, natural cytotoxicity receptors and killer immunoglobulin-like receptors play important roles in determining the activation and cytotoxicity of NK cells.[4–6] Moreover, a number of transcription factors, such as activator protein 1 (AP-1),[7,8] E4BP4 (also known as nuclear factor interleukin-3 regulated [NFIL3])[9,10] and HMBOX1,[11,12] have been shown to contribute to the regulation of signal transduction in NK cells.

MicroRNA-mediated post-transcription gene regulation is involved in the diverse fundamental functional effects, such as cell proliferation, differentiation, apoptosis, tumor invasion and tumor metastasis.[13–15] In the immune system, microRNA appear to play key roles in the early differentiation and maturation of B cells;[16,17] in the induction, function and maintenance of the regulatory T-cell lineage;[18,19] and in regulating the differentiation of dendritic cells and macrophages via toll-like receptors.[20,21] As for the regulatory effects of microRNA on NK cells, recent studies have shown that Dicer-deficient and Dgcr8-deficient NK cells are significantly impaired in cell maturation and survival, and weaken the function of NKG2D, which indicates that miRNAs play a significant role in NK cell biology.[22] Here, we studied another microRNA molecule named miR-30c-1*, which was downregulated after CD226 crosslinking in NK cells, accompanied by decreased expression of transmembrane tumor necrosis factor-α (mTNF-α). In contrast, overexpression of miR-30c-1* led to increased mTNF-α expression and elevated NKL cells and peripheral blood NK cell cytotoxicity against human hepatoma cells by targeting the inhibitory transcription factor HMBOX1.

Methods and Materials

Cell culture and reagents. The NKL cell line (derived from an aggressive $CD3^{-}CD16^{+}CD56^{+}$ large granular lymphocyte leukemia),[23] which was generously provided by Professor Zhigang Tian (Institute of Immunology, University of Science and Technology of China), was cultured in α-MEM supplemented with 12.5% FBS, 12.5% horse serum, penicillin/streptomycin and 100 U/mL human recombinant IL-2 (Peprotech, Rocky Hill, NJ, USA). SMMC-7721,[24] HepG2 and 293T cell lines were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China) and were maintained in DMEM with 10% FBS and penicillin/streptomycin. Human peripheral blood NK cells were isolated using negative immunoselection Dynabeads Untouched Human NK Cells (Invitrogen, Carlsbad, CA, USA) from peripheral blood mononuclear cells of healthy donors (obtained from Xijing Hospital, Xi'an, China), which were isolated by density gradient centrifugation (Human lymphoprep, TBD, Tianjin, China). Informed consents were obtained from all the donors in the study. Human peripheral NK cells were cultured in RPMI1640 medium supplemented with 10% FBS, 2% human serum and 100 U/mL human recombinant IL-2. Chemosynthesis and carboxy fluorescein (FAM) labeled miRNA mimics (let-7c, miR-21, miR-30c, miR-30c-1*, miR-181d and miR-200a*; the sequences were from miRBase, http://www.mirbase.org), HMBOX1 siRNA (5′-UUUCAGAGACGUAACUCGUUCCAGG-3′), stable negative siRNA control, which has been tested in humans, mice and rat cells (the sequence is 5′-UUCUCCGAACGUGUCACGUTT-3′) and miR-30c-1* inhibitor (single strand complement to miR-30c-1*) were all synthesized by Genpharma (Shanghai, China).

Flow cytometric analysis and western blot. PE-conjugated anti-TNF-α, FITC-conjugated anti-IFN-γ, anti-TRAIL, anti-FasL and anti-CD107a were all from eBioscience. PE or FITC-conjugated mouse IgG1κ isotype control (BD Phamingen, San Diego, CA, USA) was used as the negative control. The expression of mTNF-α, TRAIL, FasL and CD107a were stained following the protocol described previously.[25] The expression of intracellular TNF-α and IFN-γ in NK cells were

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analyzed by flow cytometry (FCM) with BD Cytofix/Cytiperm Kits (BD Phamingen) following the instruction. A minimum of 20 000 gated events/samples were collected on a FACSCalibur (Elite ESP, Miami, FL, USA) and analyzed using CellQuest software. NKL cells of diverse groups were lysed in lysis buffer (BD Phamingen) and western blot analysis was performed as described before with rabbit anti-human HMBOX1 polyclonal antibody (Abcam, Cambridge, MA, USA).[26]

MicroRNA microarray analysis. The NKL cells were crosslinked with pre-coated LeoA1 (10 μg/mL) or control mAb (mouse IgG1κ isotype control) (10 μg/mL) for 2 h. Then, the total RNA was extracted using TRIzol (Invitrogen) and an RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. A miRCURY Array (v8.1) (Exiqon, Vedbaek, Denmark). was performed by KangChen Bio-technology in Shanghai.

Quantitative real time-PCR for miRNA. A QuantiMir RT Kit (Small RNA Quantitation System, System Biosciences, Mountain View, CA, USA) was used following the manufacturer's instructions to obtain the cDNA of NKL. The reverse primer was a 3′ adapter primer and the forward primer was designed based on the entire mature miRNA sequence. A U6 control was used for normalization. The following oligonucleotides were used. For U6 snRNA, 5′-GATGACACGCAAATTCGTGAA-3′; for let-7c, 5′-TGAGGTAGTAGGTTGTATGGTT-3′; for miR-21, 5′-TAGCTTATCAGACTGATGTTGA-3′; for miR-30c, 5′-TGTAAACATCCTACACTCTCAGC-3′. For miR-30c-1*, 5′-CTGGGAGAGGGTTGTTTACTCC-3′; for miR-181d, 5′-TTCATTCATTGTTGTCGGTGGGT-3′; and for miR-200a*, 5′-CATCTTACCGGACAGTGCTGGA-3′.

Relative quantitative real-time PCR analysis for miRNA was performed using SYBR green miRNA assays. All RT-PCR reactions, including no-template controls and RT-PCR minus controls, were run in triplicate in a GeneAmp PCR 9700 Thermo cycler (Applied Biosystems, Foster City, CA, USA). Gene expression levels were quantified using the ABI Prism 7900HT sequence detection system (Ambion, Austin, TX, USA). Relative expression was calculated using the comparative threshold cycle method.

Luciferase assay. The 3′UTR of *hmbox1* mRNA was cloned using specific primers (forward primer, 5′-GGGAATTCTGCCCTCAGCCTTTGCA-3′; backward primer, 5′-TATCTGCAGGAGAACTGAGGGCTATCT-3′) in the *EcoR* I and *Pst* I site of the reconstructed pGL3 Basic Vector (Promega, Fitchburg, WI, USA). 293T cells were transfected with 0.8 μg of luciferase reporter vector containing the target site and 100 nM miRNA mimics using Lipofectamine 2000. Assays were performed at 24 h after transfection using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity. The recombinant 3′UTR of HMBOX1-pGL3 mutations were generated using Takara MutanBEST Kit (Takara, Otsu, Japan) according to the manufaturer's protocols. The following oligonucleotides were used for mutation: 3′UTR of HMBOX1-pGL3 MUT1, 5′-ACCAACCAACCATTCTTCCCTCTCC-3′ and 5′-TGGTTATTATTTACTGGGAGTTAATACTGAGAGA-3′; and 3′UTR of HMBOX1-pGL3 MUT2, 5′-CAAACTTCCCTCTGGCAGCCCCCGAG-3′ and 5′-GTTGGTTGGTTGGTTATTATTTACTGGGAGTTAATACTGAGAGA-3′.

Specific killing activity of NKL cells to CFSE-labeled SMMC-7721 cells. 5,6-carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeled SMMC-7721 and HepG2 cells were plated in 24-well flat-bottom plates (Costar, Milpitas, CA, USA). NKL cells transfected with control miRNA, miR-30c-1* mimics or miR-30c-1* inhibitor, respectively, for 48 h were added into the plated at the ratio of 16:1, 8:1 or 4:1. The plates were centrifuged at 300*g*/min for 5 min and incubated at 37°C for 4 h. Cells were harvested and washed twice with PBS and stained with propidium iodide for 15 min at 37°C. A minimum of 100 000 gated events/samples were collected on a FACSCalibur and analyzed using CellQuest software (BD Phamingen) within 1 h.

Experimental animals. SCID/beige mice (6–8 week old, female) were purchased from Beijing Vitalriver Experimental Animal Center and housed at the Laboratory Animal Center of the Fourth Military Medical University, Xi'an, China under specific pathogen-free conditions. A total of 5×10^6 SMMC-7721 cells were engrafted s.c. in SCID/beige mice. On day 2, 9, 16 and 23 after tumor cell implantation, 100 μL normal sodium (NS) or 5×10^6 NKL cells with different treatments for 24 h in 100 μL NS (all NKL cells were exposured at 3000 rad to avoid NK cell tumorigenesis) respectively, injected into mice at the location where SMMC-7721 cells were injected. Tumor volumes were measured with a caliper rule on day 10, 17, 24 and 30 after tumor implantation and calculated according to the formula: ($[\pi/6] \times$ tumor length $\times$ tumor width^2). On the 30th day after tumor implantation, the tumors were measured and the animals were killed and photographed. All animal experiments were approved by our Institutional Animal Care and Use Committee.

Results

Crosslinking of CD226 with anti-CD226 mAb LeoA1 decreased mTNF-α expression in NKL cells. Anti-CD226 monoclonal antibody LeoA1 was first established by immunization with alloantigen-activated human T cells in 1985 and identified by its capacity to recognize CD226.[27] In the present study, NKL cells (a human NK cell line highly expressing CD226) were seeded into 24-well plates, which were pre-coated with LeoA1. Then, the expressions of CD107a, intracellular interferon-γ (inIFN-γ), FasL, TRAIL, inTNF-α and mTNF-α on NKL cells after crosslinking with LeoA1 or control mAb were measured by FCM (Fig. 1a). Only the expression level of mTNF-α was noticably decreased after CD226 crosslinking, with the levels of the other molecules almost unaltered. Further study showed that the expression of mTNF-α in NKL cells was suppressed after CD226 crosslinking with LeoA1 at 24 and 48 h (Fig. 1b, c). These results indicate that engagement of CD226 with LeoA1 in NKL cells triggers some mechanism(s) that downregulate the expression of mTNF-α.

Expressions of several microRNA decreased after CD226 crosslinking. Since microRNA have emerged as important regulatory factors in NK cells, we detected the miRNA expression profile of NKL cells after crosslinking with LeoA1. According to the microRNA microarray analysis results, the expression levels of a great deal of miRNA decreased in NKL cells when crosslinked with LeoA1 for 2 h (Table 1). Verification of six microRNA (let-7c, miR-21, miR-30c, miR-30c-1*, miR-181d and miR-200a*), which showed obvious variation by real-time quantitative PCR, coincided with the results of the miRNA array (Fig. 2).

Overexpressed miR-30c-1* promoted the cytotoxicity of natural killer cells against hepatoma cell lines SMMC-7721 and HepG2 *in vitro* through upregulation of mTNF-α expression. For a series of microRNA expression levels varied after CD226 crosslinking, we investigated the functions of these miRNA in NKL cells. By transfecting selected miRNA mimics (let-7c, miR-21, miR-30c, miR-30c-1*, miR-181d and miR-200a*) into NKL cells, it was found that exogenous miR-30c-1* mimics significantly elevated the expression of mTNF-α in NKL cells, whereas the inhibitor of miR-30c-1* showed the opposite effect (Fig. 3a,b). Meanwhile, when miR-30c-1* was transfected into peripheral blood NK cells, miR-30c-1* showed similar

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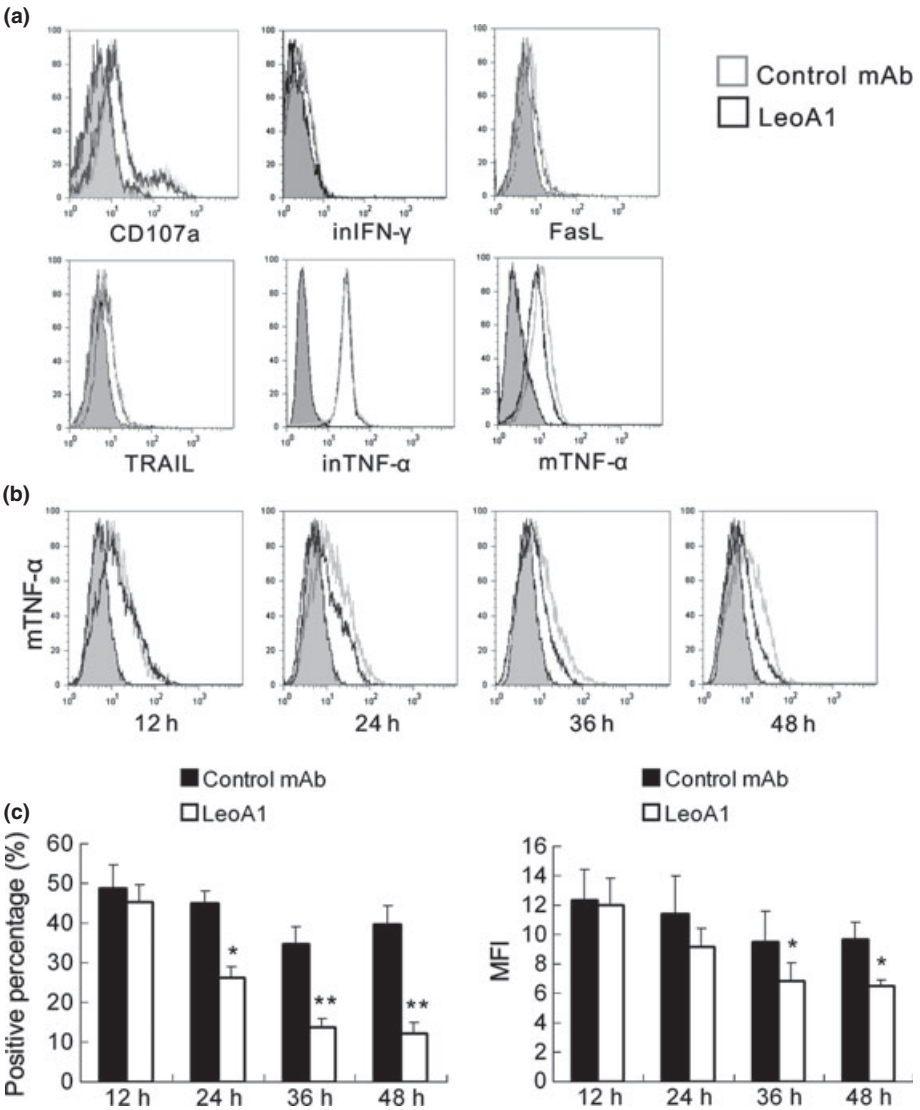

Fig. 1. The expression of mTNF-α on NKL cells was inhibited by crosslinking CD226 with LeoA1. (a) Flow cytometry analysis of expression of CD107a, intracellular IFN-γ, FasL, TRAIL, inTNF-α and mTNF-α in NKL cells after LeoA1 (black line) or control mAb (gray line) crosslinking for 24 h. Filled histogram represented isotype control mAb staining. (b,c) Flow cytometry analysis of mTNF-α expression on NKL cells after LeoA1 (black line, white bar) or control mAb (gray line, black bar) crosslinking for 12, 24, 36 and 48 h. Filled histogram represented isotype control mAb staining. Bars represent the positive percentage (left) or mean fluorescence intensity (right) (mean and SD). Data were representative of three independent experiments. $*P < 0.05$, $**P < 0.01$ (two-tailed unpaired Student's *t*-test).

regulatory effects on peripheral blood NK cells with the increased mTNF-α expression (Fig. 3b).

Furthermore, it was demonstrated that NKL cells transfected with miR-30c-1* mimics showed higher cytotoxicity against SMMC-7721 and HepG2 cells compared with those transfected with negative control miRNA mimics at different effector cell to target cell ratios, whereas the inhibitor of miR-30c-1* showed the opposite effect (Fig. 4a). When the neutralizing TNF-α mAb was added into the system, it was observed that the cytotoxicity of NKL cells transfected with miR-30c-1* decreased significantly. This suggested that the enhanced killing activity of NKL cells transfected with miR-30c-1* was at least in part due to the function of mTNF-α (Fig. 4b).

Exogenous miR-30c-1* enhanced the cytotoxicity of NKL cells *in vivo*. To test the cytotoxicity of miR-30c-1* transfected NKL cells *in vivo*, the SCID/Beige mice, which lack T cells, B cells and NK cells, were selected as the animal model. The tumor volumes and weights of the groups injected with NKL cells were apparently smaller than those injected with NS, which demonstrated the cytotoxicity of NKL cells to SMMC-7721 cells *in vivo*. Notably, it was found that the volumes and weights of the tumors in mice treated with miR-30c-1* modified NKL cells were smaller than those of the control group (Fig. 5a,b).

HMBOX1 was a target gene of miR-30c-1*. Because the function of miRNA relied on the inhibition of the target mRNA translation, we used the online prediction tool Microcosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl) to identify the putative target genes of miR-30c-1* (Table S1). Bioinformatics analysis indicated that HMBOX1 was one of the potential target genes of miR-30c-1*. To confirm that miR-30c-1* was able to directly bind to the 3′ UTR of HMBOX1 and inhibit HMBOX1 expression, a recombinant firefly luciferase reporter vector with a fragment of the 3′ UTR of HMBOX1 mRNA containing the putative miR-30c-1* binding sequence was cloned and co-transfected

Table 1. List of differentially expressed microRNA in LeoA1 crosslinked NKL cells

Upregulated		Downregulated	
Gene name	Fold change	Gene name	Fold change
hsa-miR-671	2.20458891	**hsa-miR-200a***	**0.017778943**
hsa-miR-367	1.505819406	hsa-miR-345	0.01968383
hsa-miR-409-3p	1.910346508	hsa-miR-139	0.020041717
hsa-miR-517*	1.552210559	hsa-miR-154*	0.020798009
hsa-miR-128b	1.616698534	hsa-miR-526c	0.021613617
hsa-miR-499	1.546684386	hsa-miR-371	0.023453074
hsa-miR-569	1.880384659	hsa-miR-211	0.025052147
		hsa-miR-422a	0.025634755
		hsa-miR-641	0.026245106
		hsa-miR-126*	0.029007749
		hsa-miR-590	0.029007749
		hsa-miR-412	0.031950564
		hsa-miR-497	0.032420425
		hsa-miR-448	0.032904312
		hsa-miR-523	0.034446702
		hsa-miR-628	0.036743149
		hsa-miR-520a*	0.038010154
		hsa-miR-621	0.038010154
		hsa-miR-149	0.038676998
		hsa-miR-130b	0.045928936
		hsa-miR-30c	**0.052490212**
		hsa-miR-485-5p	0.055114723
		hsa-miR-492	0.056048871
		hsa-miR-369-3p	0.056048871
		hsa-miR-454-3p	0.063593911
		hsa-miR-661	0.063593911
		hsa-miR-30c-1*	**0.063593911**
		hsa-miR-21	**0.063593911**
		hsa-miR-626	0.06484085
		hsa-miR-199a	0.066137667
		hsa-miR-302a	0.066805725
		hsa-miR-330	0.071888769
		has-let-7c	**0.076904264**
		hsa-miR-501	0.078735318
		hsa-miR-181d	**0.078735318**

The list includes 42 microRNA that shows a fold change >1.5 or <0.08 in comparison with control mAb crosslinked NKL cells, following normalization and data analysis. The 6 miRNA identified in bold are the miRNA that had been reported that were correlated with the development, differentiation and function of the immune system. *Denotes the less predominant miRNA form.

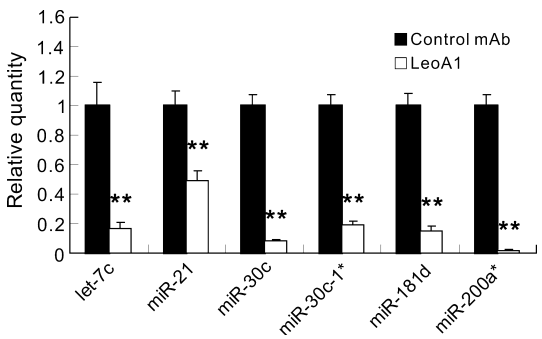

Fig. 2. The expression levels of let-7c, miR-21, miR-30c, miR-30c-1*, miR-181d and miR-200a* were decreased when NKL cells were crosslinked with LeoA1 by RT-PCR. Bars represent the relative quantity of miRNA expression in NKL cells (mean and SD). Data were representative of three independent experiments. $^{**}P < 0.01$ (two-tailed unpaired Student's *t*-test).

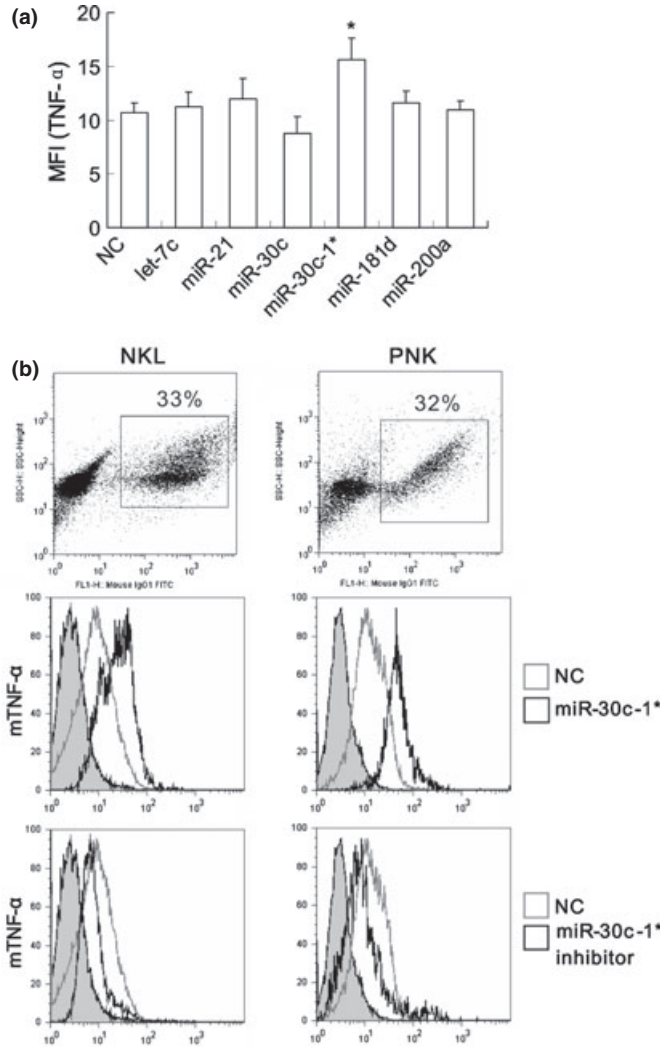

Fig. 3. Exogenous miR-30c-1* increased mTNF-α expression in NK cells. (a) The mTNF-α expression level on NKL cells transfected with miRNA mimics for 24 h. Bars represent mean fluorescence intensity (MFI) of mTNF-α expression level in transfected NKL cells. (b) Variation of mTNF-α expression on NKL cells and peripheral blood NK cells (PNK) transfected with FAM labeled negative control miRNA (gray line), miR-30c-1* (black line) or miR-30c-1* inhibitor (black line), respectively, for 24 h. The FAM-positive cells were gated to analyze the expression of mTNF-α. Filled histogram represented isotype control mAb staining.

into 293T cells with either control miRNA mimics, miR-30c-1* mimics or unrelated miRNA mimics (miR-181d), respectively. As expected, only miR-30c-1* inhibited the luciferase activity (Fig. 6b). To demonstrate further that the downregulation of HMBOX1 by miR-30c-1* was mediated through the predicted binding site, two kinds of double substitution mutations in the 3′ UTR of HMBOX1 that disrupted the complementation with the 5′-seed-matched sites (Fig. 6a, 3′ UTR of HMBOX1-MUT1) or 3′ supplementary pairing sites (Fig. 6a, 3′ UTR of HMBOX1-MUT2) of miR-30c-1* were generated, and it was found that both mutations abolished the repression effect mediated by miR-30c-1* on HMBOX1 (Fig. 6c). Furthermore, to confirm that exogenous miR-30c-1* could decrease the protein level of HMBOX1 in NKL cells, we transfected miR-30c-1* mimics into NKL cells and detected the protein expression level of HMBOX1 (Fig. 6d). The results

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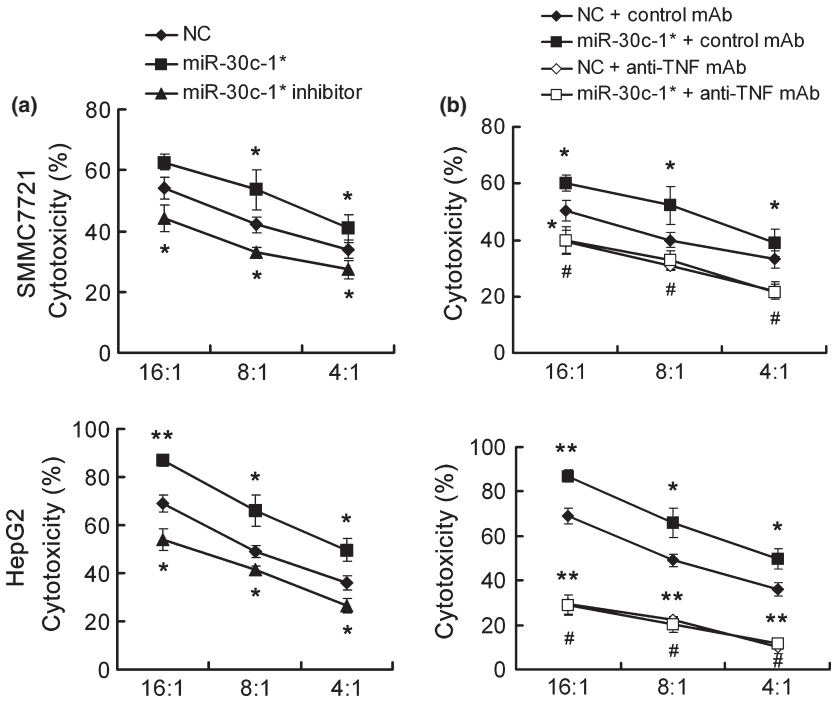

Fig. 4. Exogenous miR-30c-1* up-regulated the cytotoxicity of natural killer (NK) cells *in vitro*. (a) Specific killing activity of NKL cells after transfection with control miRNA, miR-30c-1* mimics or miR-30c-1* inhibitor respectively for 48 h. SMMC-7721 and HepG2 cells were used as target cells, respectively. $*P < 0.05$, $**P < 0.01$ compared with the group transfected with negative control (NC) (two-tailed unpaired Student's *t*-test). (b) Blockage of NKL cells specific killing against SMMC-7721 and HepG2 cells by anti-TNF-α mAb. Data were representative of three independent experiments. $*P < 0.05$, $**P < 0.01$ compared with the group transfected with NC and blocked with control mAb; $\#P < 0.01$ compared with the group transfected with miR-30c-1* and blocked with control mAb (two-tailed unpaired Student's *t*-test).

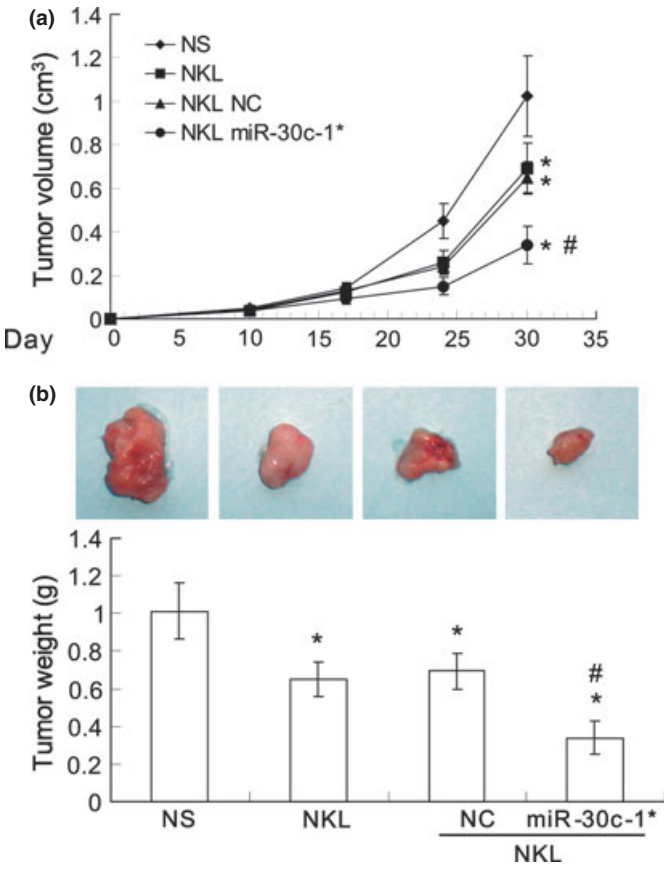

Fig. 5. Overexpression of miR-30c-1* enhanced the cytotoxicity of NKL cell *in vivo*. (a) Measurement of tumor volumes in SCID/Beige mice. Tumor volume measurements were taken every week beginning with the 10th day and before sacrifice. (b) Quantitation of tumor weight. The tumors were surgically removed, weighed and photographed. Data were means ± SD of five animals in each group. $*P < 0.05$ compared with the group injected with NS; $\#P < 0.05$ compared with the group injected with NKL cells transfected with NC.

implied that HMBOX1 protein expression was inhibited by miR-30c-1* mimics.

HMBOX1 inhibits mTNF-α expression in NKL cells. As an inhibitory transcription factor, it has been reported that HMBOX1 could depress the secretion of cytokines, such as TNF-α and IFN-γ in NK cells[12,28]. To further confirm this, HMBOX1 was knocked down in NKL cells with specific siRNA (Fig. 7a). It was found that mTNF-α expression increased after transfection of HMBOX1 siRNA into NKL cells for 48 h (Fig. 7b). Thus, these results further confirm the inhibitory function of HMBOX1 in NK cells.

Discussion

The potential of NK cells in cancer immunotherapy has become more apparent as the molecular mechanisms governing NK cell activity have been elucidated in recent years. It has been proven that NK cell activity is modulated by the complex interplay of multiple activating, inhibitory and costimulatory receptors and cytokines. Among them, CD226 is known as a NK cell activating receptor because the anti-CD226 mAb LeoA1 can upregulate the NK cell cytotoxicity in redirected cytotoxicity assay.[29–31] LeoA1 has also been found to inhibit the maturation and cytotoxicity of NK cells and CTL in mixed lymphocyte culture,[25,27] (our group obtained similar results: data not shown). In this study, when NKL cells were incubated with the pre-coated LeoA1, the expression level of mTNF-α on NKL cells decreased. As NKL cells also express CD112 and CD155, which are known as the ligands of CD226 (Fig. S1),[32] in our study, LeoA1 probably blocked the interaction of CD226 with CD112 and CD155, and intercepted the activating signal of CD226, which resulted in the inhibition of mTNF-α expression. Meanwhile, we also found that the expression level of CD226 decreased when NKL cells were cultured with the pre-coated LeoA1 (Fig. S2), so the decreased expression of mTNF-α might be due to the downregulated CD226 expression on NKL cells. However, the mechanisms need further investigation.

Recently, it has been reported that miRNA have important modulatory functions in the immune system, including in NK

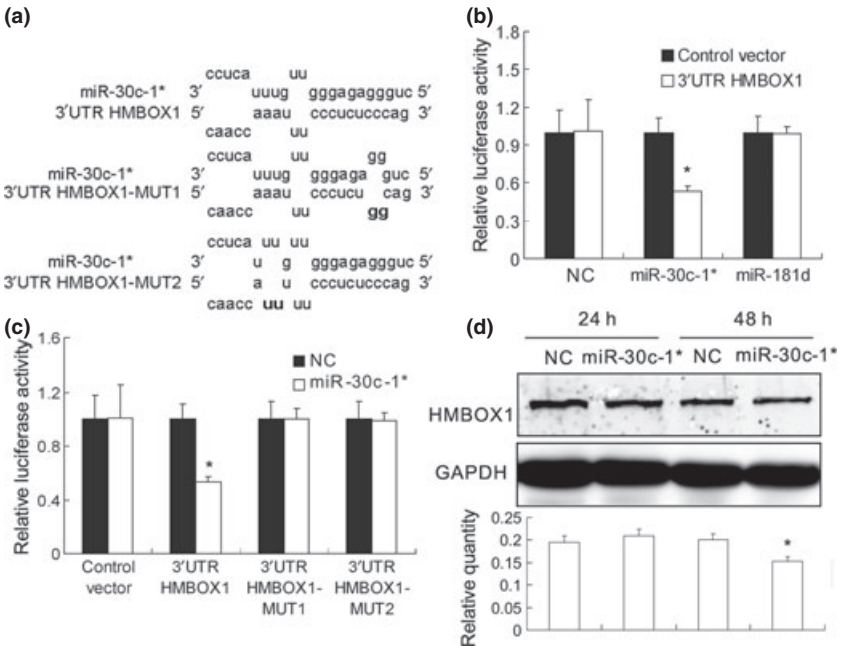

Fig. 6. HMBOX1 was directly targeted by miR-30c-1*. (a) The complementary sites of miR-30c-1* and 3′UTR of HMBOX1. The mutated positions were shown in bold. (b) Luciferase assay confirmed that HMBOX1 was one of the target genes of miR-30c-1*. Firefly luciferase activity was normalized to the average luciferace activity of cells transfected with control reporter vector. (c) Luciferase assay when the miR-30c-1* binding sites in the 3′ UTR of HMBOX1 were mutated. Firefly luciferase activity was normalized to the average luciferace activity of cells transfected with control miRNA. (d) Western blot of the expression of HMBOX1 in NKL cells after transfection with control miRNA or miR-30c-1*. Bars represent the ratio of integrated destiny value of HMBOX1 relative to that of GAPDH. Data were representative of three independent experiments. $^*P < 0.05$ (two-tailed unpaired Student's *t*-test).

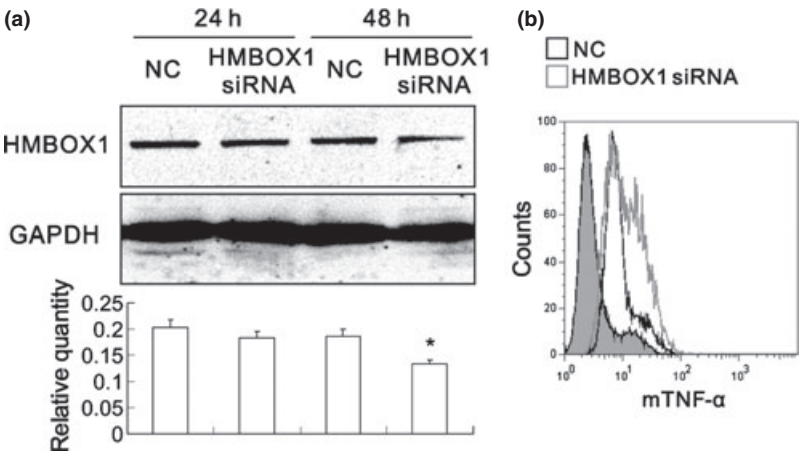

Fig. 7. HMBOX1 inhibited mTNF-α expression on NKL cells. (a) Western blot of the expression of HMBOX1 in NKL cells after transfection with control siRNA or HMBOX1 siRNA. Bars represent the ratio of integrated destiny value of HMBOX1 relative to that of GAPDH. (b) The expression level of mTNF-α on NKL cells transfected with control siRNA (Black line) or HMBOX1 siRNA (gray line) for 48 h. Filled histogram represented isotype control mAb staining. Data were representative of three independent experiments. $^*P < 0.05$ (two-tailed unpaired Student's *t*-test).

cells.[13,33,34] Fehniger identified that miR-223 was downregulated upon NK cells activated with IL-15 and its target was granzyme B, indicating that this miRNA might contribute to regulation of NK cell cytotoxicity.[35] To further explain why the mTNF-α expression level decreased after NKL cells were incubated with the pre-coated LeoA1, the microRNA expression profile of NKL cells was detected by microRNA array after NKL cells were incubated with the pre-coated LeoA1 for 2 h. The expression levels of some miRNA molecules varied. Among the varied miRNA molecules, six miRNA molecules, let-7c, miR-21, miR-30c, miR-30c-1*, miR-181d and miR-200a*, which showed apparent variation by microRNA array, were further confirmed by RT-PCR, and miR-30c-1* was noticed for its effect on the expression of mTNF-α in NK cells.

Previous research has shown that there is constitutive expression of membrane TNF-α on peripheral blood NK cells and NK cell lines.[36] Meanwhile, mTNF-α has vital anti-tumor effects on tumor cells and exhibits a specific and tumoricidal spectrum in a cell-to-cell contact-dependent manner compared to the systemic activity of soluble TNF-α.[37,38] On the other hand, TNF-α could sensitize NK cells to activating stimulation, such as IL-2, and promote the cytotoxicity of NK cells.[39] So, we believe that upregulation of mTNF-α induced by miR-30c-1* probably affected the NK cell cytotoxicity. It was also demonstrated that miR-30c-1* mimics increased NK cell cytotoxicity *in vitro* and *in vivo*. Moreover, according to the anti-TNF-α mAb blocking experiment, the elevated cytotoxicity of NKL cells transfected with miR-30c-1* was at least partly due to the upregulation of the mTNF-α expression level.

Because the functions of miRNAs rely on the identification of their target mRNAs, we tried to search for the target genes of miR-30c-1* in NKL cells. We found that HMBOX1 was a potential target gene of miR-30c-1* by bioinformatics analysis. Furthermore, experiments including luciferase assay and western blot proved that HMBOX1 was one of the target genes of miR-30c-1*. HMBOX1 was first isolated and identified in 2006 and showed transcription repressor activity in the transcriptional reporter assays.[11,12] Recently, it has been found that overexpression of HMBOX1 significantly inhibits NK cell cytotoxicity.[25] In this study, we also demonstrated that downregulating HMBOX1 with miR-30c-1* or specific siRNA in NKL

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cells resulted in the elevation of mTNF-α expression. However, in $CD8^+$ T cells, which also have strong cytotoxic activity and highly express CD226, the mRNA level of HMBOX1 was much lower than that in NK cells. Furthermore, miR-30c-1* showed no apparent effects on $CD8^+$ T cells (Fig. S3).

In conclusion, first we found that the enhanced expression of miR-30c-1* in NKL cells could lead to increased cytotoxicity of NKL cells against hepatoma cell lines SMMC-7721 and HepG2 through upregulation of mTNF-α. Second, we found that at least in part the increased cytotoxicity of NK cells transfected with miR-30c-1* was due to the targeting of the inhibitory transcription factor HMBOX1, which has a suppression function in NK cells. Our results may provide a potential therapeutic strategy for hepatoma treatment by regulating NK cell cytotoxicity via microRNA modulating.

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Disclosure Statement

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Flow cytometry analysis of CD112 and CD155 expression on NKL cell.

Fig. S2. CD226 expression was down-regulated on NKL cells after crosslinking with LeoA1.

Fig. S3. The cytotoxicity of $CD8^{+}$T cells was not affected by miR-30c-1*.

Table S1. List of predictive miRNA targeting HMBOX1 gene from MicroCosm Target.

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