MicroRNA-20a mediates the cytotoxicity of natural killer cells in endometriosis via ERG/HLX/STAT4/perforin axis

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Li-Juan Chen
Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Bin Hu
The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology

Zhi-Qiang Han
Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology

Jian Ni
Brigham and Women’s Hospital, Harvard Medical School

Yong-Ming Zhou
The Affiliated Tianyou Hospital, Wuhan University of Science and Technology

Xue-Xing Chen
Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Hao Zhou sue_drHAO@126.com
Institute of Hematology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology

Corresponding Author

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Abstract
Background: Intriguingly, microRNA-20a (miR-20a) has been recently witnessed to be involved in the pathogenesis of endometriosis (EMS) but the molecular mechanism controlled by miR-20a is to be undefined. The present study is designed to probe into how miR-20a acts to regulate the cytotoxicity of natural killer (NK) cells.

Methods: Most of all, consistent with the clinical determination in EMS patients, miR-20a was determined to be down-regulated in NK cells isolated from nude mice. miR-20a could specifically bind to ERG and negatively regulates its expression in NK cells. Additionally, shRNA-mediated silencing of ERG decreased the expression of HLX. HLX up-regulated STAT4 by inducing proteasome degradation and inhibited NK cell cytotoxicity.

Results: Of great importance, forced expression of miR-20a consequently induced NK cell cytotoxicity in vitro by increasing perforin expression via enhancement of STAT4 that was caused by impairing the binding of ERG to HLX enhancer. The in vivo experiments further confirmed the promoting role of miR-20a in the cytotoxicity of NK cells isolated from EMS nude mice and subsequent protective role of miR-20a against EMS-induced endometrial injury.

Conclusion: The aforementioned data suggest that miR-20a potentiates the cytotoxicity of NK via up-regulating perforin mediated by ERG/HLX/STAT4, highlighting potential novel mechanisms associated with EMS progression.

Background
Endometriosis (EMS) is defined by the occurrence of endometrial tissues on the
outside of the uterus, mostly adhered to the pelvic peritoneum [1]. EMS may exist in different regions of the uterus, such as ovaries, fallopian tubes, vagina and etc. [2]. EMS is an estrogen-dependent chronic inflammatory disorder, which frequently occurs during childbearing years with pain and infertility, as well as symptoms observed including menorrhagia, deep dyspareunia, dyschezia, and misnicturition [3]. EMS affects 5% to 10% female population in their reproductive years worldwide [4], and has considerable social and psychological negative impacts on the quality of life in female sufferers, across several domains especially in pain and psychosocial functioning [5]. Although molecular mechanisms are not yet fully understood, immune cells are found to be key players in EMS, where natural killer (NK) cells not only kill off tumor and infected cells but also are key to successful pregnancy with critical functions in tissue remodeling in the uterus [6]. Our previous study has revealed that enhanced cytotoxicity of NK cells contribute to the dysfunctional placenta and associated pathologies [7].

NK cells are cytotoxic effector lymphocytes with the ability to lyse target cells, while NK cells also have roles in tissue remodeling in several organs including the uterus and are essential to placentation [6]. NK cell activity is first declared to be disrupted in the peripheral blood and peritoneal fluid of females with EMS in the research by Oosterlynck et al. [8], and subsequent investigators have identified the suppression of NK cell behaviors in the endometrium of patients with this disorder [9]. Mounting evidence has shown that peripheral blood NK cells and peritoneal NK cells have reduced cytotoxic function in EMS women, while the molecular clue for dysregulation of NK cells is still lacking [10]. Hence, identification of molecules that regulate NK cells might be in an urgent need to uncover the underlying mechanisms of EMS.
Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but not translated into proteins. Substantial numbers of ncRNAs including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) possess abilities to affect the development and persistence of EMS, unraveling that is of great importance and value to determine novel therapeutic targets and strategies [11]. The miRNAs are a group of short, single-stranded RNAs that control mRNA post-transcriptionally [12]. The members of miR-200 family, such as miR-143, 145, miR-20a and miR199a have been reported as the most commonly abnormally expressed miRNAs in EMS [13]. miR-20a is identified as a miRNA that could mediate endothelial and endometrial cell proliferation by inducing fibroblast growth factor-9 [14]. A recent study on malignancy associated miRNAs changes has revealed that miR-20a binds directly to the MICA/B mRNA, resulting in its degradation and reducing its membrane protein level, and thus indirectly suppress NK cell cytotoxicity [15]. We speculate that non-coding miR-20a might play a role in the functional dysregulation of NK cells in endometrial pathology.

Based on the miRNA-mRNA online prediction, ETS-related gene (ERG) was suggested as a putative target of miR-20a. Belonging to the ETS transcription factor family, ERG acts to hold endothelial homeostasis through controlling the transcription of endothelial function-associated genes and inhibiting the release of pro-inflammatory cytokines [16]. An online Multi Experiment Matrix (MEM) analysis from the previous study has revealed co-expression of ERG and HLX that participates in sprouting angiogenesis [17]. Strikingly, HLX can induce degradation of phosphorylated STAT4 in a dependent manner of dephosphorylation and proteasome [18]. Furthermore, STAT4 has been found to be vital for the effector functions of various immune cells, especially NK cells [19]. Regarding those findings, we speculate that miR-20a might
be implicated in the regulation of NK cell function via mediating the ERG/HLX/STAT4 axis. To test this hypothesis, we constructed a mouse model of EMS to identify the effect of miR-20a on NK cell in EMS, as well as in the ERG/HLX/STAT4 network in order to unravel the novel mechanisms in EMS development.

Materials and Methods

Study subjects

Volunteers were recruited from the Department of Obstetrics and Gynecology of Central Hospital of Wuhan and Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology from 2017 to 2019, who complained of severe dysmenorrhea, pelvic masses, and infertility. The recruited females aged 20–43 years were non-smokers, without signs or history of other inflammatory diseases, and none of them received hormonal therapy within at least 3 months prior to the recruitment. Patients were excluded if they met the following conditions including malignant tumors, benign ovarian cysts other than endometrial tumors, incidental finding during surgery for severe pelvic inflammatory disease, and known chronic, systemic, metabolic or endocrine disease, including polycystic ovary syndrome. The baseline clinical data were collected. The EMS patients included 60 patients with peritoneal and/or ovarian EMS diagnosed by laparoscopy and pathology, of which 18 patients were also diagnosed with infiltrating EMS. Meanwhile, 25 patients who were diagnosed with infertility caused by fallopian tube factors but confirmed without EMS by surgery were enrolled as controls. Blood specimens was collected from each participant on the days 1–3 before surgery and stored at -80°C. All operations were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology and followed the tenets of the Declaration of Helsinki.
Written Informed consent was obtained from all participants.

In silico analysis

EMS-associated miRNAs were selected based on literature retrieval, while the targets of miRNA candidates were selected using the established databases as follows: StarBase (CancerNum>8) (http://starbase.sysu.edu.cn), microRNA (mirsvr_score>0.75, energy<-20), RAID (score>0.7) (http://www.microrna.org/microrna/home.do), TargetScan (Total context++ score<-0.15; cumulative weighted context++ score<0) (http://www.targetscan.org/vert_72/) and miDIP (Integrated Score>0.45, Number of Sources>10) (http://ophid.utoronto.ca/mirDIP/). The intersected target genes from the above databases were subjected to protein-protein interaction (PPI) analysis using GeneMANIA database (http://genemania.org). The miRNA-gene binding sites were provided by TargetScan. The downstream gene of the target gene candidates was identified by co-expression analysis using the online tool MEM (https://biit.cs.ut.ee/mem/index.cgi), and the expression pattern of the downstream gene was retrieved from the GSE58178.

Cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples of EMS patients and controls. Human NK cells were isolated by magnetic beads (Biotec, Bergisch Gladbach, Germany) for the subsequent in vitro experiments [20]. Expression of CD3 and CD56 on the membrane was measured by flow cytometry using antibodies against CD3-FITC (2.5 μL; eBioscience, San Diego, CA, USA) and CD56-PE (2.5 μL; eBioscience, San Diego, CA, USA) to evaluate the purity of the cell population. Cells with more than 95% purity were selected. NK cells were
cultured in RPMI–1640 (HyClone, Logan, UT, USA). The immortalized human natural killer cell line NK–92 was cultured in MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL IL–2 (PeproTech, London, UK) and streptomycin/penicillin. Human erythroleukemia cell line K562 was cultured in RPMI–1640 medium containing 10% fetal bovine serum and streptomycin/penicillin. Mouse NK cells were isolated and cultured as described in previous literature [18].

**NK cell transfection**

NK cells in logarithmic growth phase were transfected with miR–20a mimic, shRNA targeting ERG (sh-ERG), shRNA targeting HLX (sh-HLX), shRNA targeting STAT4 (sh-STAT4), ERG overexpression plasmid (oe-ERG), HLX overexpression plasmid (oe-HLX), STAT4 overexpression plasmid (oe-STAT4) or corresponding negative controls (mimic-NC, sh-NC, oe-NC), which were constructed by Sangon Biotech Co., Ltd (Shanghai, China). The transfection was performed using lipofectamine 2000 (11668–019, Invitrogen) as instructed by the supplier.

**EMS mouse model**

Twenty-two clean inbred BALB/c female mice aged 6–8 weeks weighting 19–24 g were used for the animal experiments, while 10 of them were selected as normal control and the remaining mice were employed for modeling which was conducted as previously stated [21]. Based on the evaluation standard of a successful model [22], 10 mice were successfully modeled with a success rate of 83.33%. In brief, the endometrial tissues were collected with the use of a scalpel (about 6 hours after the withdrawal of progesterone). About 40 mg of endometrial tissues were suspended in 0.2 ml of PBS and injected into the peritoneal cavity of ovariectomized recipients.
The mice were then subcutaneously administered with 500 ng estradiol valerate (EV), every 3 days. Behavioral evaluation of EMS mice was conducted after 21 days after lesion formation. Adenovirus-packaged plasmid ($5 \times 10^9$) Ad-miR–20a agomir or Ad-agomir-NC was constructed and injected into the tail vein of EMS mice [23]. NK cytotoxicity was assessed after 4 weeks of treatment. The experiments involving animals were conducted with the approval of the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. All efforts have been made to minimize animal suffering.

**Westernblot analysis**

Cells were washed with PBS and lysed with cell lysis buffer (C0481, Sigma, USA) at 4°C for 30 min, and the cell lysate was centrifuged at 12000 g at 4°C for 5 min with the supernatant collected. The protein concentration was then determined by bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). After boiling for 5 min, 20 μg of the proteins were subjected to protein separation using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and subsequently electro-transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After being sealed with 5% skim milk for 1 h, the membrane was rinsed 3 times with Tris-buffered saline Tween-20 (TBST) and subsequently incubated with TBST-diluted rabbit primary antibodies to ERG (ab92513, 1:1000, Abcam, Cambridge, UK) and HLX (ab210808, 1:1000, Abcam), p65 (ab19870, 1:500, Abcam), PSTAT4 (Y693) (ab28815, 1:1000, Abcam), STAT4 (ab235946, 1:1000, Abcam), Perforin (ab47225, 1:100, Abcam) and GADPH (ab37168, 1:1000, Abcam) at 4°C overnight, followed by 3 TBST washes. The membrane was then immunoblotted with horseradish peroxidase (HRP)-labeled secondary antibody (HS101, goat anti-rabbit,
1:1000; TransGen Biotech, Beijing, China) at room temperature for 1 h. After being washed 3 times with TBST, the protein bands were developed with enhanced chemiluminescence (Baoman Biotechnology Co., Ltd., Shanghai, China). The gray value of each band was then analyzed by Image J software.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**

TRIzol kit (Invitrogen) was employed to extract total RNA from cells and tissues. The quality and concentration of RNA were measured using an ultraviolet-visible spectrophotometer (ND-1000, Nanodrop, Wilmington, DE, USA). Reverse transcription of 400 ng total RNA was performed using PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). With cDNA as a template, fluorescent quantitative PCR was carried out as instructed in the specifications of SYBR® Premix Ex TaqTM II (TliRNaseH Plus) kit (Takara). The reaction was carried out in triplicates by Thermal Cycler Dice Real-Time System (TP800, Takara). The primers were synthesized by Ribobio (Guangzhou, China) as shown in Table 1. 2^{-ΔΔCT} indicates the expression of gene or miRNA relative to GAPDH or U6. ΔΔCT = CT(target)-CT(ref).

**In vitro cytotoxicity assay**

K562 cells (1 x 10^6) labeled with 51Cr were incubated with Na_2^{51}CrO_4 (Amersham Biosciences, Piscataway, NJ, USA) at 37°C for 1 hour. The effector (E) cells were mixed with target (T) cells (1 x 10^4 cells/well) at the different ratios. Cell cytotoxicity was then assessed using Trypanblue exclusion test. Cytotoxicity in animals can be referred to a previous study [24].

**Chromatin Immunoprecipitation (ChIP)**

The NK cells were fixed with formaldehyde for 10 min to produce DNA-protein cross-
linking. The chromatin was sheared into 200–500 bp fragments with a sonicator (VC130, Sonics, USA) for 10 s, 10 s at intervals, 15 cycles. After the cells were fully lysed, 10% of the cell lysate was used as Input. The remaining cell lysates were then centrifuged at 1°C and 12000 g for 10 min. The supernatant was incubated overnight at 4°C with rabbit antibodies to ERG (ab92513, 1:100, Abcam), H3K27ac (ab203953, 1:200, Abcam), H3K4me1 (ab8895, 1:50, Abcam), H3K4me3 (ab8580, 1:100, Abcam) or rabbit IgG (ab171870, 1:300, Abcam) in the NC. The protein-DNA complex was precipitated with Pierce protein A/G Magnetic Beads (88803, Thermo Fisher Scientific Inc., Waltham, MA, USA). After centrifugation at 5000 g for 1 min, the supernatant was discarded and the non-specific binding was removed. After crosslinking was reversed at 65°C overnight, the DNA was purified by phenol/chloroform extraction. The fragment was then subjected to RT-qPCR to determine enrichment of ERG in the HLX enhancer region as well as STAT4 on the perforin promoter region. The primers for RT-qPCR are shown in Table 2.

Dual-luciferase reporter assay

The wild type (WT) sequence of ERG mRNA 3′-UTR and the mutant type (MUT) sequence after site-directed mutagenesis were synthesized. The sequences were inserted into pmiR-RB-REPORTTM vector (Ribobio) using restriction endonuclease cleavage. The empty plasmid was simultaneously transfected as a control. The constructed plasmids were co-transfected with mimic NC or miR–20a mimic, respectively into NK cells. After 48 h of transfection, the cells were lysed and centrifuged for 3 to 5 min with the supernatant collected. The luciferase activity was then detected using a dual-luciferase assay kit (RG005, Beyotime, Shanghai, China).
Statistical analysis

All data were processed using SPSS 21.0 statistical software (IBM Corporation, Armonk, NY, USA). Measurement data were expressed as mean ± standard deviation. The comparison between two groups was conducted using t-test, while that among multiple groups was performed using one-way analysis of variance. A value of $p<0.05$ was considered as statistically significant.

Results

miR–20a is lowly expressed in peripheral blood of patients with EMS and EMS mouse model

Ectopic expression of miR–20a is implicated in EMS [25]. However, its mechanism involved with EMS remains largely undefined. The cytotoxicity of NK cells is closely related to the occurrence of EMS [6, 26]. To further investigate the specific mechanism of NK cell cytotoxicity caused by EMS, the cytotoxicity of K562 cells in peripheral blood of patients with EMS and matched controls without EMS was assessed. The results displayed that NK cell cytotoxicity of patients with EMS was significantly lower than in the matched controls at E/T ratios of 6.25:1 and 3.15:1 (Fig. 1A). RT-qPCR was employed to determine the expression of miR–20a in peripheral blood of patients with EMS and those without EMS. The results showed that miR–20a was noticeably lower in peripheral blood patients with EMS than in those without EMS (Fig. 1B). To further explore the mechanism of miR–20a in EMS, an EMS mouse model was constructed. The cytotoxicity results in the mouse model were consistent with that in human peripheral blood samples (Fig. 1C). RT-qPCR determination exhibited that the expression of miR–20a in peripheral blood of EMS
mice was significantly lower than in that of normal mice (Fig. 1D). The above results suggested that reduced NK cell cytotoxicity was induced by EMS, and miR–20a was underexpressed in peripheral blood of EMS.

miR–20a targets and negatively regulates ERG expression

To further explore the regulation of miR–20a in NK cell cytotoxicity, mRNA-miRNA prediction was performed using StarBase, microRNA, RAID, TargetScan and miDIP databases. Based on the predicted results from mRNA-miRNA databases, 265, 92, 411, 1640 and 1548 target genes were obtained from databases StarBase, microRNA, RAID, TargetScan and miDIP, respectively. The Venn diagram constructed from those target genes revealed 5 overlapping genes, namely ATL3, CFL2, ERG, RAB5B and ZNFX1 (Fig. 2A). PPI analysis by GeneMANIA revealed that ERG and VFL2 had highest core levels (Table 2). ERG is a transcription factor and has not been reported to be associated with EMS. Hence, we selected ERG as the research object for the following experiments. The binding site of miR–20a in ERG was obtained from TargetScan (Fig. 2B). Hence, we speculated that miR–20a might regulate ERG to affect NK cell function. The results showed that the expression of miR–20a in the peripheral blood-derived NK cells from EMS patients was significantly lower than that in those from the control patients without EMS (Fig. 2C). Results from Western blot assay demonstrated that ERG was expressed at a higher level in peripheral blood-derived NK cells from EMS patients than in those from the patients without EMS (Fig. 2D). Furthermore, we constructed cell line overexpressing miR–20a by transfection with miR–20a mimic, and the miR–20a expression was determined by RT-qPCR. The expression of miR–20a in the cells transfected with miR–20a mimic was notably increased relative to that in cells transfected with mimic NC (Fig. 2E). In addition, the sequences of ERG 3’UTR-WT and ERG 3’UTR-MUT were constructed,
and the luciferase activity of ERG 3'UTR-WT in cells transfected with miR–20a mimic was noticeably lower than that in cells transfected with mimic NC (Fig. 2F), suggesting ERG to be a target of miR–20a. Results from RT-qPCR and Western blot assays revealed that mimic-mediated up-regulation of miR–20a notably inhibited the expression of ERG (Fig. 2G). The above results together demonstrated that miR–20a was highly expressed in endometriotic NK cells and targets ERG.

**miR–20a regulates HLX expression by down-regulating ERG**

The ERG-bound enhancer located at the upstream of HLX plays a critical role in endometrial epithelial-mesenchymal cell interactions [27, 28]. The co-expression analysis of ERG and HLX via online tool MEM revealed a significant co-expression relationship (Fig. 3A). To further investigate whether ERG and HLX are involved in NK cell cytotoxicity, the expression pattern of ERG and HLX in EMS was analyzed. Western blot analysis further validated the up-regulation of HLX protein expression in peripheral blood-derived NK cells from EMS patients as compared to the controls without EMS (Fig. 3B). Subsequently, ChIP assay was conducted to detect the enrichment of ERG, H3K27ac, H3K4me1 and H3K4me3 in the HLX enhancer regions. The results exhibited that ERG, K3K27ac and H3K4me1 were remarkably increased but H3K4me3 was reduced in HLX enhancer region (Fig. 3C). Next, mRNA and protein expression of ERG determined by RT-qPCR and Western blot assays was successfully decreased in NK cells after transfection with sh-ERG, and that was increased after transfection with oe-ERG (Fig. 3D). Furthermore, shRNA-mediated silencing of ERG showed a noticeable decrease in the expression of HLX (Fig. 3E).

Give the binding of miR–20a to ERG, whether miR–20a could affect the expression of HLX by regulating ERG was analyzed in the cells were transfected with miR–20a mimic, oe-ERG, or co-transfected with miR–20a mimic and oe-ERG. It was observed
that the levels of ERG, K3K27ac and H3K4me1 in the miR–20a mimic-transfected
cells were remarkably reduced, while that of H3K4me3 was significantly increased in
the HLX enhancer region. Meanwhile, the overexpression of ERG contributed to
increased enrichment of K3K27ac and H3K4me1 and reduced enrichment of
H3K4me3 in the HLX enhancer region. The reduced enrichment of ERG, K3K27ac and
H3K4me1 in the HLX enhancer region that induced by miR–20a mimic transfection
was restored by oe-ERG transfection, while the increased enrichment of H3K4me3
induced by miR–20a mimic transfection was diminished by oe-ERG transfection (Fig.
3F). As revealed by the data from RT-qPCR and Western blot assay, overexpression
of miR–20a notably inhibited the expression of HLX, while the overexpression of ERG
resulted in elevated expression of HLX. The expression of HLX inhibited by miR–20a
was rescued by overexpression of ERG (Fig. 3G). Collectively, these results provide
evidence that miR–20a could down-regulate the expression of HLX in NK cells
through the recruitment of ERG to the HLX enhancer region.

HLX promotes proteasomal degradation of STAT4 to inhibit NK cell
cytotoxicity

STAT4 is a critical mediator for signaling pathways in immune responses that
involve in the pathogenesis of EMS, and STAT4 polymorphism involves in the
development of this disease [29]. To further investigate the involvement of HLX in
the regulation of NK cell toxicity, we examined the mRNA and protein expression of
STAT4 by RT-qPCR and Western blot assay, which was was markedly lower in
peripheral blood-derived NK cells from EMS patients than in those from matched
controls without EMS (Fig. 4A). To further examine the effect of HLX on STAT4
expression, HLX was silenced or overexpressed in NK cells. The results of RT-qPCR
and Western blots displayed that shRNA-mediated inhibition of HLX resulted in increased protein expression of total STAT4 and pSTAT4 (Y693) which was reversely reduced by overexpression of HLX (Fig. 4B-C). In addition, NK cells were then treated with proteasome inhibitor MG132 in the presence of HLX to verify whether HLX inhibited the expression of STAT4 protein via the proteasome pathway. The results exhibited that short-term (3 - 6 h) treatment of MG132 could restore the decrease of pSTAT4 (Y693) caused by overexpression of HLX, but does not affect the expression of total STAT4 protein (Fig. 4D). Furthermore, the NK cytotoxicity at different E:T ratios (ET5 and ET1.5) was examined by Cr release assay. Results demonstrated that silencing of HLX noticeably promoted NK cell cytotoxicity, while the overexpression of HLX inhibited NK cell cytotoxicity (Fig. 4E). These results suggested that HLX could regulate STAT4 proteasome degradation and NK cell cytotoxicity.

miR–20a regulates perforin expression to affect NK cell cytotoxicity via ERG/HLX/STAT4 signaling pathway

It has been demonstrated that IL–12 could induce NK cell proliferation and activation by regulating STAT4-binding perforin [30]. Results from Western blot analysis suggested that perforin protein expression was noticeably lower in peripheral blood-derived NK cells from EMS patients than in those from matched controls without EMS (Fig. 5A). Perforin is a key protein associated with the regulation of human NK cell cytotoxicity [31–33]. In addition, Western blot assay revealed that the protein expression of perforin was notably elevated by the overexpression of STAT4 (Fig. 5B). The promoter of perforin was amplified and ChIP was conducted to detect the binding of STAT4 to the perforin promoter. It was
observed that overexpression of STAT4 promoted the recruitment of STAT4 in the promoter region of perforin (Fig. 5C). The 51Cr release assay provided data showing that the overexpression of STAT4 prominently promoted NK cell cytotoxicity (Fig. 5D). These results suggested that STAT4 could target and regulate perforin protein to enhance NK cell cytotoxicity. Further investigation was conducted to determine whether miR–20a could affect NK cell cytotoxicity through regulation of perforin expression via the ERG/HLX/STAT4 signaling pathway. Results revealed that forced expression of miR–20a that induced by miR–20amimic or shRNA-mediated silencing of HLX resulted in elevated expression of STAT4 and perforin protein. On the contrary, overexpression of ERG noticeably reduced the expression of STAT4 and perforin protein, which was restored by co-transfection with miR–20a mimic or sh-HLX (Fig. 5E). At the same time, the NK cell cytotoxicity detected by Cr release assay was induced by overexpression of miR–20a or silencing of HLX, but inhibited by overexpression of ERG. The inhibited NK cell cytotoxicity mediated by ERG was rescued by miR–20aoverexpression or HLX knockdown (Fig. 5F). As a result, miR–20a could induce NK cell cytotoxicity by increasing perforin expression via the ERG/HLX/STAT4 axis.

Overexpression of miR–20a enhances NK cell cytotoxicity in mice with EMS

To further analyze the regulatory effects of miR–20a on the ERG/HLX/STAT4/perforin axis and affects the NK cell cytotoxicity in EMS mice in vivo, Ad-miR–20a agomir or Ad-agomir NC (5 × 10^9) was injected into the tail vein of EMS mice. After 4 weeks of treatment, NK cells in peripheral blood of mice were isolated and purified, and cytotoxicity of NK cells was examined. First of all, results from RT-qPCR showed that
miR–20a expression was noticeably elevated in peripheral blood and its derived NK cells of EMS mice injected with Ad-miR–20a agomir, as shown in Fig. 6A. The Cr release assay revealed that adenovirus-mediated up-regulation of miR–20a resulted in promoted NK cell cytotoxicity (Fig. 6B). The results of RT-qPCR and Western blot showed that the mRNA levels of ERG and HLX were markedly decreased, and that of STAT4 and perforin was increased by adenovirus-mediated up-regulation of miR–20a (Fig. 6C-D). Next, the degree of pathological damage in the mouse ectopic endometrium was detected by HE staining. The results showed that the EMS mice infected with Ad-agomir NC showed endometrioid-like glands and interstitial cells, cylinder-shaped glandular epithelial cells, and infiltration of glandular epithelial cells and interstitial cells in the surrounding tissues, while overexpression of miR–20a obviously ameliorated the above-mentioned pathological damages (Fig. 6E). These results together suggested that miR–20a could enhance the cytotoxicity of mouse NK cells in EMS by regulating the ERG/HLX/STAT4/perforin pathway in vivo.

Discussion

EMS is an inflammatory disorder that affects ~5–10% of female population in their reproductive age [4]. EMS is risky for several chronic diseases and is considered to be relatively harmful to long-term health of females [34]. miRNAs involve in the regulation of dysregulated genes in EMS and exert promise as indicators and therapeutic tools for EMS [35]. Inhibiting the cytotoxicity of NK cells induces the immune escape of ectopic endometrium, ultimately facilitating the occurrence and advancement of EMS [36]. This study mainly focused the role of miR–20a in regulating NK cell cytotoxicity and the detailed molecular mechanisms (Fig. 7). Altogether, our data suggested that miR–20a-induced inhibition of ERG up-regulated
perforin and STAT4 to increase NK cell cytotoxicity by reducing HLX expression. First of all, we confirmed the down-regulated expression of miR–20a in peripheral blood of EMS patients, which was in line with the previous report of a microarray-based miRNA expression analysis in matched ectopic and eutopic endometrial tissues [37]. The EMS patients and recurred cases exhibited a low expression of miR–20a relative to healthy subjects [38]. Partially consistent with our findings, plasma miR–20a expression is down-regulated in female with EMS as compared to matched controls [39]. Subsequent experiments in this paper identified that miR–20a was lowly expressed in endometriotic NK cells and targeted ERG. Through ChIP assay, recruitment of ERG to the HLX enhancer region was identified. Overexpression of ERG increases the levels of H3K27ac and H3K4me1 and reduces that of H3K4me3 in the HLX enhancer region. Enhancers are crucially involved in cellular development through their spatiotemporal regulation of gene expression. Histone H3acetylation at lysine 27 (H3K27ac) and mono-methylation at lysine 4 (H3K4me1) are the predominant histone modifications that can be found at nucleosomes around enhancer elements. Co-occurrence of H3K27ac and H3K4me1 has been recognized as an indicator to identify active enhancers [40, 41]. Transcriptional regulator ERG is a nuclear protein that binds to purine-rich sequences of DNA. It is required for hematopoiesis maintenance and down-regulated in the process of early T lymphopoiesis. ERG can behave as an oncogene and accelerate the progression of T-acute lymphoblastic leukemia, and is transcriptionally mediated by ERG +85 stem cell enhancer in leukemic cells [42, 43]. Our findings further strengthen that ERG participates in transcriptional regulation through epigenetic modulation in the cellular context of NK cells. And this was in accordance with the report of ERG-mediated recruitment of histone
methyltransferase SETDB1 (also known as ESET) and subsequent modification of local chromatin structure [44]. Meanwhile, loss of ERG is associated with decreased level of H3K4me1 and H3K27ac in enhancer regions [45]. On the contrary, ERG represses histone H3 lysine 9 trimethylation (H3K9me3) at the YAP1 gene promoter by interacting with histone demethylase KDM4A [46]. A highly conserved ERG-bound HLX enhancer is responsible for its VEGF-induced neo-vascularization [27]. It was further corroborated that miR–20a down-regulated the expression of HLX in NK cells through recruitment of ERG to the HLX enhancer region.

The next important finding was that HLX induced proteasomal degradation of STAT4 to inhibit NK cell cytotoxicity. Endometriosis is related to defective NK cell function in the ability to eliminate endometrial cells in ectopic sites. Aberrant NK cell tolerance is associated with impaired STAT4 phosphorylation [47]. Hence, STAT4 activation is highlighted as a potential therapeutic target triggering NK cell tolerance [48]. Such STAT4 activation is existed prevalingly in immune cells such as T cells, NKT cells, macrophages, Kupffer cells and etc. [49]. Upregulation of HLX is required for re-expression of IFN-γ in imprinted helper T (Th) cells [50], while the Th1 development can be impaired by suppression of IFN-γ/STAT1 and repression of HLX [51]. The implications of STAT4 and HLX are also defined in Th1/Th2 cell differentiation [52]. However, their relationship was freshly confirmed in this study that HLX elevated the level of phosphorylated STAT4 (Y693) by inducing proteasomal degradation of STAT4. Interestingly, phosphorylated STAT1 and STAT3 levels are detected to be closely associated with perforin expression [53]. This was also determined in our study that STAT4 was enriched in the perforin promoter. Perforin is a glycoprotein responsible for pore formation in cell membranes of target cells where perforin is able to polymerize and form a channel. Perforin has been
previously reported to mediate cytotoxicity of human intra-epithelial lymphocytes and serine esterase release [54]. Impaired perforin production causes disrupted NK cell cytotoxicity [55], whereas re-expression of perforin is witnessed as a contributor to the cytotoxic activity of NK cells [56]. This perforin-mediated cytotoxicity is capable of impeding the growth of malignant cells in host tissues and making them dormant for a short duration about at least one month [57]. More importantly, STAT4 phosphorylation induces the release of perforin in CD4+ T cells to strengthen its cytotoxic activity, ultimately contributing to inhibition of tumor growth [58]. In our study, we found that forced expression of miR–20a down-regulated HLX expression by recruiting ERG to the HLX enhancer region, and subsequently up-regulated the expression of perforin and STAT4, whereby inducing NK cell cytotoxicity. Further validated in vivo by developing a mouse model of EMS, elevation of miR–20a alleviated EMS-induced damages through strengthening mouse NK cells.

Conclusion
To conclude, our study demonstrates that elevation of miR–20a acts to reverse the EMS-induced abnormal NK cell activity via mediating the ERG/HLX/STAT4/perforin axis. This contributes to understanding of novel immune mechanisms and will pave the way for treating EMS with miRNA-related biomedical applications. However, there remains big challenges to achieve clinical practical use of miRNA-targeted therapies, which stimulates that more attentions should be paid on this.

Abbreviation
EMS, Endometriosis; NK, natural killer; ncRNAs, Non-coding RNAs; miRNAs,
microRNAs; IncRNAs, long non-coding RNAs; ERG, ETS-related gene; MEM, Multi Experiment Matrix; PBMCs, Peripheral blood mononuclear cells; EV, estradiol valerate; BCA, bicinchoninic acid; TBST, Tris-buffered saline Tween-20; HRP, horseradish peroxidase; RT-qPCR, Reverse transcription quantitative polymerase chain reaction; ChIP, Chromatin Immunoprecipitation; WT, wild type; H3K27ac, Histone H3 acetylation at lysine 27

Declaration

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Li-Juan Chen, Bin Hu, Zhi-Qiang Han, Yong-Ming Zhou, Hao Zhou and Jian Ni wrote the paper and conceived and designed the experiments; Li-Juan Chen, Zhi-Qiang Han, Yong-Ming Zhou, Xue-Xing Chen and Bin Hu analyzed the data; Jian Ni, Hao Zhou and Xue-Xing Chen collected and provided the sample for this study. All authors have read and approved the final submitted manuscript.
Consent for publication

Consent for publication was obtained from the participants.

Availability of data and material

The datasets generated/analysed during the current study are available.

Ethics approval and consent for publication

All operations were approved by the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology and followed the tenets of the Declaration of Helsinki. Written Informed consent was obtained from all participants. The experiments involving animals were conducted with the approval of the Animal Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. All efforts have been made to minimize animal suffering.

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Tables
### Table 1 Primer sequences for RT-qPCR

| Gene            | Primer sequence (5’-3’)                      |
|-----------------|---------------------------------------------|
| miR-20a (Homo)  | F: 5’- TACGATAAAGTGCTTATAGTGAGGTAAG -3’     |
|                 | R: 5’- GTCTTTGTCGAGCGAGTG -3’               |
| miR-20a (Murine)| F: 5’- GCGAATTCATTGTGTAGTGAGGGCCCT -3’     |
|                 | R: 5’- CGCTCGAGAATCCCATAGACCAGTGCTCA -3’   |
| ERG             | F: 5’- AATTTAAAGCCTAGGCGAGC -3’             |
|                 | R: 5’- CTGCCGAATTCGCTTAGCCATG -3’           |
| HLX             | F: 5’- AGCCGAACATACGAGTCTCTCA -3’           |
|                 | R: 5’- GATTAAGATGCCTTACCTCTCTCA -3’         |
| STAT4           | F: 5’- ATAGGCATCAAACATGAATCTAGT -3’         |
|                 | R: 5’- TAAATAGATCGTGTGAGCAGT -3’            |
| U6 (Homo)       | F: 5’- ATGGGAACGATAAGAGATT -3’              |
|                 | R: 5’- GTCTTTGTAGGCGAGTG -3’                |
| U6 (Murine)     | F: 5’- CGCAGAGCTTGGTGGGGAG -3’              |
|                 | R: 5’- CACAATTACCTTTAGAGT -3’               |
| GAPDH           | F: 5’- CACCATCTCTGAGGAGGAGC -3’             |
|                 | R: 5’- TCACGCCACAGTTTCCGGA -3’              |

**Note:** RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, forward; R, reverse; miR-20a, microRNA-20a; ERG, ETS-related gene; HLX, H2.0 like homeobox; STAT4, signal transducer and activator of transcription 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### Table 2 PPI analysis of keys genes from online database GeneMAIA

| Gene  | Degree |
|-------|--------|
| ERG   | 21     |
| CFL2  | 21     |
| RAB5B | 8      |
| ATL3  | 3      |
| ZNFX1 | 2      |

**Note:** PPI, protein-protein interaction; ERG, ETS-related gene; CFL2, cofilin 2; RAB5B, a member RAS oncogene family; ATL3, Atlastin GTPase 3; ZNFX1, zinc finger NFX1-type containing 1.

**Figures**
Figure 1

NK cell cytotoxicity was reduced by EMS and miR-20a was lowly expressed in periphery.
Figure 1

NK cell cytotoxicity was reduced by EMS and miR-20a was lowly expressed in peri
NK cell cytotoxicity was reduced by EMS and miR-20a was lowly expressed in peripheral blood of patients with EMS. A two-group comparison was conducted using independent sample t-test. The cell experiment was performed in triplicates.

Figure 1

miR-20a targets ERG and negatively regulates its expression in NK cells of EMS. A
miR-20a targets ERG and negatively regulates its expression in NK cells of EMS. A

Figure 2

miR-20a targets ERG and negatively regulates its expression in NK cells of EMS. A
Figure 3

miR-20a recruits ERG in the HLX enhancer region to negatively control the expression of HLX.
miR-20a recruits ERG in the HLX enhancer region to negatively control the expres...
miR-20a recruits ERG in the HLX enhancer region to negatively control the expression of HLX.

Figure 3

HLX promotes proteasomal degradation of STAT4 to inhibit NK cytotoxicity. A, RT-

Figure 4
HLX promotes proteasomal degradation of STAT4 to inhibit NK cytotoxicity. A, RT-qPCR and Western blot analysis show that HLX treatment significantly decreases STAT4 expression. B, RT-qPCR and Western blot analysis of EMS-treated cells show a decrease in STAT4 expression compared to control. C, Western blot analysis of phosphorylated STAT4 (p-STAT4) in different groups. D, Western blot analysis showing the time course of p-STAT4 expression after HLX treatment. E, Graphs showing the percentage of cytotoxicity in different groups.

**Figure 4**

HLX promotes proteasomal degradation of STAT4 to inhibit NK cytotoxicity. A, RT-qPCR and Western blot analysis show that HLX treatment significantly decreases STAT4 expression. B, RT-qPCR and Western blot analysis of EMS-treated cells show a decrease in STAT4 expression compared to control. C, Western blot analysis of phosphorylated STAT4 (p-STAT4) in different groups. D, Western blot analysis showing the time course of p-STAT4 expression after HLX treatment. E, Graphs showing the percentage of cytotoxicity in different groups.
miR-20a regulates perforin expression to affect NK cytotoxicity via the ERG/HLX/STAT4 pathway.

Figure 5

miR-20a regulates perforin expression to affect NK cytotoxicity via the ERG/HLX/STAT4 pathway.
miR-20a regulates perforin expression to affect NK cytotoxicity via the ERG/HLX/STAT4 pathway. A, Western blots were performed using one-way ANOVA with Tukey’s post-hoc test. The cell experiment was repeated three times.
miR-20a controls NK cell cytotoxicity in endometriotic mice via the ERG/HLX/STAT axis.
miR-20a controls NK cell cytotoxicity in endometriotic mice via the ERG/HLX/STAT4 axis in vivo.

Figure 6
miR-20a controls NK cell cytotoxicity in endometriotic mice via the ERG/HLX/STAT
Figure 7

The mechanism map of miR-20a-mediated ERG/HLX/STAT4/perforin axis in EMS. a
The mechanism map of miR-20a-mediated ERG/HLX/STAT4/perforin axis in EMS. 

(a) Endometriosis

(b) Reduced cytotoxicity of peritoneal NK cells

Figure 7
The mechanism map of miR-20a-mediated ERG/HLX/STAT4/perforin axis in EMS. a