Tumor-suppressor miRNA-27b-5p regulates the growth and metastatic behaviors of ovarian carcinoma cells by targeting CXCL1

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Abstract
MicroRNAs (miRNAs) play crucial functions in the progression of ovarian cancer. MiR-27b has been identified as cancer-associated miRNA. Nevertheless, the expression profile of microRNA-27b-5p (miR-27b-5p) and its functions in ovarian cancer are unexplored. Here, we demonstrated that miR-27b-5p was downregulated in ovarian carcinoma cells and clinical specimens. Higher expression of miR-27b-5p was allied to an unfavorable overall survival in ovarian cancer patient. Upregulation of miR-27b-5p decreased the viability, migration ability and invasion capacity of SKOV3 and A2780 cell. MiR-27b-5p also inhibited the growth of SKOV3 cell in nude mice. Additionally, we verified that C-X-C motif chemokine ligand 1 (CXCL1) was a target of miR-27b-5p in ovarian carcinoma cell. Restoring the expression of CXCL1 abolished the inhibitory impacts of miR-27b-5p in ovarian cancer carcinoma cell. This research revealed that miR-27b-5p restrained the progression of ovarian carcinoma possibly via targeting CXCL1.

Introduction
Ovarian carcinoma is still one of the most lethal gynecological malignancies. Most deaths from ovarian cancer are attributed to metastasis. Metastasis, which is a multistep process, allows tumor cell diffuse from the primary sites to distant tissues and includes migration, local invasion, intravasation, extravasation and malignant proliferation [1, 2]. Increasing investigations have demonstrated that the loss of cancer suppressors and the upregulation of oncogenes are associated with cancer cell metastasis [3, 4]. Hence, investigations the molecular mechanisms of cancer cell metastases may assist in the exploration of targeted therapies to improve the clinical outcomes of patients with ovarian cancer.

MicroRNAs (miRNAs) are a class of noncoding RNAs and modulate the expressions of target genes via binding to 3'-untranslated region (3'-UTR) of mRNAs [5]. Various miRNAs participate into the progression of malignant cancers [6-8]. For instance, deregulation of miR-194 contributes to colorectal carcinogenesis through targeting AKT2 pathway [9]. MiR-143-3p serves as a tumor suppressor via modulating the growth, invasion and epithelial-mesenchymal transition (EMT) by REGULATING QKI-5 in esophageal squamous cell carcinoma [10]. MicroRNA-7-5p inhibits cell growth,
induces cell cycle arrest and cell apoptosis via modulating Paired Box 2 (PAX2) in non-small cell lung cancer (NSCLC) [11]. In ovarian cancer, miRNA-141 enhances the anoikis resistance of metastatic ovarian carcinoma cell by regulating KLF12/Sp1/survivin axis [12].

Recently, a report has identified that miR-27b-3p serve as a cancer suppressor in breast carcinoma stem cell generation through inactivating ENPP1, to attenuate chemoresistance ability [13]. Furthermore, miR-27b-3p improves the sensitivity of liver and kidney cancer cell to anti-cancer drugs via activating p53-dependent apoptosis and reducing CYP1B1-mediated drug detoxication [14]. In ovarian cancer, miRNA-27b acts as an inhibitor of ovarian carcinoma-mediated vasculogenic mimicry via repressing the expression of VE-cadherin [15]. Nevertheless, the underlying role of miR-27b-5p in ovarian cancer has no reports and needs further research.

In our study, we evaluated the possible roles of miR-27b-5p-CXCL1 axis in the aggressive ability of ovarian carcinoma cell. We verified that the expression of miR-27-5p was distinctly lower in ovarian carcinoma samples and its low level was negatively associated with advanced stage and lymph node metastasis of ovarian cancer. Furthermore, upregulation of miR-27b-5p restrained ovarian cancer cell growth, colony formation capacity and aggressive phenotype in vitro, and tumorigenicity in vivo. In addition, CXCL1 was ascertained as a downstream of miR-27b-5p in ovarian cancer and restoring CXCL1 expression counteracts the suppressive effects of miR-27b-5p. Altogether, miR-27b-5p repressed the progression of ovarian carcinoma through regulating CXCL1.

Materials And Methods
Ovarian cancer tissues
Total 45 pairs of ovarian carcinoma tissues and adjacent samples (located > 3 cm from cancer tissue) were obtained from patients who received surgical resection in Jinan Central Hospital. Patients did not receive chemotherapy or radiotherapy before surgery. All samples were snap-frozen in liquid nitrogen and storage at -80 °C. This research was approved by the Ethics Committee of. Informed consent was obtained before this research.

Cell Transfection
Ovarian cancer cells (SKOV3, Caov-3, A2780 and OVCAR3) and normal ovarian cell line, HOSEpiC were obtained from Nanjing KeyGen Biotech (Nanjing, Jiangsu, China). Cell lines were maintained in RPMI-
1640 medium supplement with 10% FBS (Thermo Fisher Scientific), 100 µg/ml streptomycin and 100 µg/ml penicillin in 5% CO₂ at 37 °C. MiR-27b-5p mimic or miRNA negative control (NC) mimic was synthesized by GenePharma (Shanghai, China). MiR-27b-5p stable transfected cells were constructed by using over-expressing miR-27b-5p lentivirus carrier (Vigenebio). CXCL1 cDNA sequences were cloned into a pCDNA3.1 vector (Thermo Fisher Scientific). MiR-10a-5p combination with pCDNA3.1 vector carrying CXCL1 was transfected into SKOV3 or A2780 cell using Lipofectamine 3000 (Thermo Fisher Scientific).

Cell Proliferation

Cells (2 x 10⁴) were cultured into 96-well plates and incubated for 1 day, 2 days, 3 days, 4 days or 5 days. Then, cell counting kit-8 (CCK-8) solution (Beyotime Biotechnology) was added into 96-well plates. After 2 hours, the OD in each well was determined at 450 nm.

Colony Formation Assay

1 x 10³ cells was cultured in six-well plates and cultured for total two weeks. Then, the colonies in plate were fixed by 4% formaldehyde and dyed with 1% crystal violet. The number of cell colonies in plate was counted by using a microscope.

Soft Agar Colony Assay

1 x 10⁴ SKOV3 or A2780 cells were cultured in 0.35% agar in the middle of the agar. In 24 well plates, 0.5% agar was added into the bottom and 0.35% agar was added into the top. Cells in plates were maintained for two weeks. Finally, colonies were photographed and counted.

Immunoblotting

Total proteins were harvested using RIPA (Beyotime, China). Proteins were separated using 8% SDS-PAGE and the separated proteins were transferred onto PVDF membranes. PVDF membrane was incubated with CXCL1 or GAPDH at 4 °C for overnight. After that, the membranes were incubated with a secondary antibody (Beyotime Biotechnology). Finally, bands were measured by using enhanced chemiluminescence (ECL) detection system.

Qrt-pcr Assay

RNAs were abstracted by using TRlzol and First-strand cDNA was constructed with 1 µg RNA by using a Reverse Transcription kit (Takara Bio). The qRT-PCR was conducted on 7500 Real-Time PCR system
using a SYBR Green One kit (Takara Bio). U6 and GAPDH were served as endogenous controls. The primers were as following (sense and antisense): U6: GGAACGATACAGAGAGATTAGC and TGGAACGCTTCACGAATTTGCG; miR-27b-5p: CAAAUUCGGAUCUACAGGGUAUU and UACCCUGUAAGAUCGAUUUGUG; CXCL1: AACCGAAGTCATAGCCACAC and GTTGAGATTTTGTCACTGTCAGC; GAPDH: AAAGGTGGAGGAGTGGGT and GGGAAACTGTGGCGTGAT. The data were calculated using 2^{-\Delta\Delta Ct} method.

Luciferase Reporter Gene Analysis
The fragment of CXCL1 3'-UTR with wild-type miR-27b-5p binding sites (wt) or mutated binding sites (mut) was inserted into psiCHECK-2 vector (Promega). The vector combination with miR-27b-5p was transfected into SKOV3 or A2780 cells. Luciferase activities in SKOV3 and A2780 cells were assessed by using Luciferase assay kit (Promega).

Transplanted Tumor Model
The stable over-expressed miR-27b-5p SKOV3 cells (5 \times 10^6) or control group were subcutaneously injected into BALB/C nude mice. Tumor volumes were measured and calculated. Tumor volume = 0.5 \times length \times width^2. After five weeks, mice were sacrificed. The tumor tissues were fixed and subjected for hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) assay using CXCL1 antibody. Animal experiment was approved by the Animal Care Committee and Use Committee of Jinan Central Hospital.

Statistical analysis
All statistical analyses were conducted using Graphpad prism. Results were presented as mean ± SD. The differences were analyzed by unpaired Student's t-test or one-way ANOVA followed by Tukey's post-hoc test. The relationship between the miR-27b-5p and CXCL1 was detected using Pearson’s correlation analysis. Survival estimation was analyzed using Kaplan-Meier method. P value less than 0.05 was statistically significant.

Results
MiR-27b-5p is downregulated in ovarian carcinoma
Firstly, we checked the expressions of miR-27b-5p in human ovarian carcinoma samples. The result of qRT-PCR analysis using 45 cases of ovarian carcinoma and adjacent normal tissues suggested that
miR-27b-5p was frequently downregulated in ovarian carcinoma compared with in adjacent tissues (Fig. 1A). Results also indicated that miR-27b-5p was drastically downregulated in ovarian carcinoma cells compared with in normal ovarian cell line, HOSEpiC (Fig. 1B). The relationship between the dysregulation of miR-27b-5p and the clinicopathological features ovarian cancer was future analyzed. As showed in Supplementary Table 1, the low level of miR-27b-5p was connected to the tumor stage and metastasis of ovarian cancer. Finally, survival analyses revealed that the decreased expression of miR-27b-5p was related to poor overall survival of patients with ovarian cancer (Fig. 1C). These findings indicated that miR-27b-5p potential exerted key roles in the tumorigenesis of ovarian cancer.

**Mir-27b-5p Inhibits Ovarian Carcinoma Cell Growth And Colony Formation**

To better illuminate the role of miR-27b-5p, SKOV3 and A2780 cell were transfected with miR-27b-5p. The qRT-PCR analysis shown that miR-27b-5p transfection raised miR-27b-5p level in both SKOV3 and A2780 cell (Fig. 2A). Results from CCK-8 assays indicated that introduction of miR-27b-5p remarkably reduced the cell viabilities of A2780 and SKOV3 cell (Figs. 2B). Moreover, miR-27b-5p distinctively suppressed the colony formation abilities of SKOV3 and A2780 cell in vitro (Fig. 2C). Consistently, transfection of miR-27b-5p suppressed colony formation of ovarian carcinoma cell in soft agar (Fig. 2D). Meanwhile, SKOV3 cells were treated with miR-27b-5p inhibitor to degrade the level of miR-27b-5p. As presented in Fig. 2E, miR-27b-5p inhibitor remarkably degraded the level of miR-27b-5p. As expected, miR-27b-5p silencing increased the cell viability and colony formation ability of SKOV3 cells in vitro (Fig. 2F-2G). These observations suggested that miR-27b-5p suppressed SKOV3 and A2780 cell growth in vitro.

**Mir-27b-5p Modulates Ovarian Cancer Cell Migration And Invasion**

We then conducted wound closure and Transwell invasion assay to analyze the action of miR-27b-5p in SKOV3 and A2780 cell mobility and invasiveness. We observed that transfection of miR-27b-5p markedly reduced the migrate and invasion abilities of SKOV3 and A2780 cell (Fig. 3A-3B). Nevertheless, downregulation of miR-27b-5p significantly impaired the migration and invasion abilities of SKOV3 cells (Fig. 3C-3D). Interestingly, the expression levels of MMP-2 and MMP-9 were decreased in miR-27b-5p overexpressing SKOV3 and A2780 cells as demonstrated by immunofluorescence.
staining assay (Fig. 3E). These data established miR-27b-5p as a regulator of ovarian cancer cell mobility and invasion.

Mir-27b-5p Targets Cxcl1 In Ovarian Cancer Cell

Bioinformatics analysis tool (http://www.targetscan.org/vert_71/) was selected to find the target gene of miR-27b-5p. Among these candidates, CXCL1 (Fig. 4) was predicted as a downstream gene of miR-27b-5p, owing to CXCL1 has been implicated in the malignant phenotypes of ovarian cancer[16, 17]. Then, the result of luciferase reporter experiment implied that transfection of miR-27b-5p impaired the luciferase activities in A2780 and SKOV3 cell transfected with reporter plasmid containing wt-CXCL1. Notably, the luciferase activities in cell transfected with reporter plasmid containing mut-CXCL1 were not inhibited by miR-27b-5p, which indicating that miR-27b-5p bound to the 3’-UTR of CXCL1 (Fig. 4B). In addition, the results of qRT-PCR and immunoblotting analysis suggested that the mRNA (Fig. 4C) and protein (Fig. 4D) levels of CXCL1 were significantly reduced in miR-27b-5p overexpressing A2780 and SKOV3 cell. To elucidate the correlation between miR-27b-5p and CXCL1, the level of CXCL1 in ovarian carcinoma tissue was measured, and we found that the mRNA level of CXCL1 was upregulated in ovarian carcinoma tissue when compared to in adjacent sample (Fig. 4E). Finally, an inversely correlation between miR-27b-5p and CXCL1 in ovarian cancer tissue was confirmed through Spearman's correlation analysis (Fig. 4F). Altogether, these findings implied that miR-27b-5p regulated the level of CXCL1 in ovarian cancer cell.

Restoring Cxcl1 Expression Counteracts The Suppressive Effects Of Mir-27b-5p

Then, several rescue experiments were carried out to ensure that CXCL1 is essential for the functions of miR-27b-5p in ovarian cancer. Firstly, pc-CXCL1 or control pcDNA3.1 vector was transfected into A2780 and SKOV3 cell. Immunoblotting analysis and qRT-PCR assay verified that transfection of pc-CXCL1 rescued the expression of CXCL1 in ovarian cancer cell in the presence miR-27b-5p (Fig. 5A-5B). Upregulation of miR-27b-5p inhibited SKOV3 and A2780 cell viability (Fig. 5C) and colony formation ability (Fig. 5D) in vitro, whereas reintroduction of CXCL1 abolished these effects. Meanwhile, miR-27b-5p hindered the migration and invasion of SKOV3 and A2780 cell, whereas reintroduction of CXCL1 abrogated these effects (Fig. 5E-5F). Altogether, miR-27b-5p served
suppressive roles in ovarian cancer progression through decreasing CXCL1 expression.

Mir-27b-5p Inhibits Ovarian Cancer Cell Growth In Mice
To assess whether overexpression of miR-27b-5p affects ovarian cancer cell progression in vivo, nude mice was subcutaneously inoculated with miR-27b-5p stable transfected SKOV3 cell. Tumor volume in each group was detected each week, and nude mice were sacrificed after five weeks. As shown in Fig. 6A, the tumor volume of tumor tissue formed by miR-27b-5p stably transfected SKOV3 cells was markedly smaller than tumor tissue derived from miR-NC transfected cell. Consistently, the tumor weight in mice inoculated with miR-27b-5p stably transfected SKOV3 cell was markedly smaller than tumors in the miR-NC group (Fig. 6B). qRT-PCR assay revealed that the level of miR-27b-5p was markedly higher in miR-27b-5p group than that in miR-NC group (Fig. 6C). More importantly, IHC staining confirmed that the expression of CXCL1 was significantly inhibited in miR-27b-5p transfected group (Fig. 6D). Therefore, miR-27b-5p exerted cancer inhibiting property in SKOV3 cell growth in vivo.

Discussion
The dysregulations of miRNAs have been proved as crucial drivers in ovarian cancer metastasis and development. But, the level of miR-27b-5p is yet unknown in human ovarian carcinoma. In the study, we observed that miR-27b-5p was downregulated in clinical ovarian carcinoma tissue. Moreover, miR-27b-5p was lowly expressed in ovarian cancer cells compared with in control normal cell line, HOSEpiC. We also found that miR-27b-5p low level was connected to the advanced stage and metastasis of ovarian cancer. The survival analyses suggested that patients who had low level of miR-27b-5p exhibited poorer overall survival. All these observations indicated that miR-27b-5p exerted a very critical action in the carcinogenesis of ovarian cancer.
Increasing researches have demonstrated that miRNAs exert critical functions in various biological process associated with cancer, including cell proliferation, apoptosis, metastasis and chemotherapy-resistant [18–21]. Substantive investigations reveal that the aberrant levels of miRNAs are strongly linked to the growth and metastasis of ovarian cancer. For instance, miR-138 represses ovarian carcinoma cell metastasis via modulating the expressions of SRY-Box Transcription Factor 4 (SOX4)
and Hypoxia Inducible Factor 1 Subunit Alpha (HIF-1α) [22]. MiRNA-375 represses the growth, drug sensitivity and metastasis of ovarian carcinoma cell by targeting PAX2 [23]. Previous investigation has reported that miR-27b suppresses NSCLC cell growth and invasion through regulating LIM Domain Kinase 1 (LIMK1) [24]. MiR-27b represses the growth and progression in neuroblastoma cell by targeting PPARγ [25]. In our study, we detected that transfection of miR-27b-5p inhibited ovarian cancer cell viability and colony formation of cells in vitro. Meanwhile, upregulation of miR-27b-5p remarkably reduced the migration ability and invasiveness of ovarian carcinoma cell.

The classic pattern of miRNAs-regulating their target genes are to bind with the 3’-UTR of genes. The 3’-UTR prediction tool (http://www.targetscan.org/vert_71/) displayed that the binding between miR-27b-5p and BAP31 3’-UTR regions was identified. The result of luciferase reporter gene analysis proved that miR-451a bound to the 3’-UTR of CXCL1. Furthermore, the expression of CXCL1 was significantly impaired by miR-27b-5p in SKOV3 and A2780 cell. Previous report has indicated that CXCL1 induces the proliferation of ovarian carcinoma cell through transactivation of epidermal growth factor receptor (EGFR) [16]. Serum CXCL1 is a novel circulating tumor marker for the differential diagnosis between benign ovarian masses and ovarian cancer [26]. In the current study, restoring CXCL1 expression counteracts the suppressive effects of miR-27b-5p in ovarian cancer cell. Finally, miR-27b-5p inhibited ovarian carcinoma cell growth in vivo and decreased the expression of CXCL1 in tumor tissue.

Our study revealed that miR-27b-5p was down-expressed in ovarian carcinoma and its low level was connected to the advance stage and unfavorable prognosis of patients. Mechanistically, miR-27b-5p repressed the tumor growth and metastatic behaviors of ovarian cancer cell possibly via suppressing CXCL1. Our observations might bring new insights into ovarian cancer progression and revealed a novel mechanism by which miR-27b-5p regulated proliferation and malignant metastatic phenotypes of ovarian carcinoma cell via targeting CXCL1.

Declarations
Availability of data and materials
The datasets during and/or analyzed during the current study available from the corresponding author
on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Shan Yong Qin and Min Wei Liu designed this study, performed the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Figures
MiR-27b-5p is downregulated in ovarian cancer. A. qRT-PCR for determining relative levels of miR-27b-5p in ovarian cancer and adjacent tissues (n=45). **P<0.05 vs. adjacent. B. qRT-PCR detection of miR-27b-5p expression in ovarian carcinoma cells and normal ovarian cell line, HOSEpiC. **P<0.05 vs. HOSEpiC. C. Kaplan-Meier analysis of relationship between expression of miR-27b-5p and ovarian cancer patients’ overall survival.
MiR-27b-5p inhibits the proliferation of ovarian cancer cells in vitro. A. SKOV3 and A2780 cells were transfected with miR-NC or miR-27b-5p mimics and the level of miR-27b-5p was analyzed by qRT-PCR assay. B. CCK-88 assay of SKOV3 and A2780 transfected with miR-NC or miR-27b-5p. C. Colony formation assay was conducted to evaluated cell proliferative ability in miR-27b-5p overexpressing SKOV3 and A2780 cells. D. Soft colony formation assay was conducted to evaluated cell proliferative ability in miR-27b-5p overexpressing SKOV3 and A2780 cells. **P<0.05 vs. miR-NC. E. SKOV3 cells were transfected with miR-NC inhibitor or miR-27b-5p inhibitor and the level of miR-27b-5p was analyzed by qRT-PCR assay. F. CK-88 assay of SKOV3 cell transfected with miR-NC inhibitor or miR-27b-5p inhibitor. G. Colony formation assay was carried out using SKOV3 transfected with miR-NC inhibitor or miR-27b-5p inhibitor. **P<0.05 vs. miR-NC inhibitor.
Figure 3

MiR-27b-5p inhibits ovarian cancer cell migration and invasion. A. Wound heal assay showing migration in the miR-NC and miR-27b-5p mimics transfected SKOV3 and A2780 cells. B. Transwell assay showing invasion in the miR-NC and miR-27b-5p mimics transfected SKOV3 and A2780 cells. **P<0.05 vs. miR-NC. C. Wound heal assay showing migration in the miR-NC inhibitor and miR-27b-5p inhibitor transfected SKOV3 cells. D. Transwell assay showing invasion in the miR-NC inhibitor and miR-27b-5p inhibitor transfected SKOV3 cells. **P<0.05 vs. miR-NC inhibitor. E. Immunofluorescence analysis of
MMP-2/9 in SKOV3 and A2780 cells transfected with miR-NC or miR-27b-5p.

A

| Predicted consequential pairing of target region (top) and miRNA (bottom) |
|-------------------|-----------------|
| 5’...CCAGAAAGGGAGGAGGAGGACUCAC... | wt CXCL1 |
| 3’ CAAGUGUUAGUCGAUUCGAGA | |

5’...CCAGAAGGGAGGAGGAGGCCAAGUGC... mut CXCL1

B

Figure 4
Identification of CXCL1 as a target of miR-27b-5p. A. miR-27b-5p and its wild-type (wt)
binding sites in the 3′-UTR of CXCL1. The mutant binding sites (mut) were produced in the complementary site for the seed region of miR-27b-5p. B. psiCHECK-2 carrying CXCL1-3′-UTR wt or psiCHECK-2 carrying CXCL1-3′-UTR mut, along with miR-27b-5p mimics or miR-NC, were cotransfected into SKOV3 and A2780 cell. The luciferase activity was detected using a luciferase reporter assay system. **P<0.05 vs. miR-NC. C. The mRNA levels of CXCL1 in miR-27b-5p overexpressing SKOV3 and A2780 cells were examined using qRT-PCR. D. The protein levels of CXCL1 in miR-27b-5p overexpressing SKOV3 and A2780 cells were examined using western blot analysis. E. The level of CXCL1 in ovarian cancer tissues and adjacent non-tumorous tissues were assessed using qRT-PCR. **P<0.01 vs. non-tumorous tissues. F. Spearman's correlation analysis was utilized to examine the expression correlation between miR-27b-5p and CXCL1 mRNA in ovarian cancer tissues.
Overexpression of CXCL1 reverses the effects of miR-27-5p. A. SKOV3 and A2780 cells were co-transfected with miR-27b-5p mimics and pc-CXCL1 or pcDNA3.1. The level of CXCL1 was detected by qRT-PCR. B. Western blot analysis was utilized for the detection of CXCL1 protein expression in SKOV3 and A2780 cells following pcDNA3.1 or pc-CXCL1 transfection. C. SKOV3 and A2780 cells were co-transfected with miR-27b-5p mimics and pc-CXCL1 or pcDNA3.1. Following transfection, the proliferation of SKOV3 and A2780 cells treated as aforementioned were examined using CCK-8 assays. D. The colony formation of SKOV3 and A2780 cells treated as aforementioned were examined. E. The migration of SKOV3 and A2780 cells treated as aforementioned were examined using wound healing assay. F. The invasion of SKOV3 and A2780 cells treated as aforementioned were examined using Transwell invasion assay. **P<0.05 vs. miR-NC, ###P<0.05 vs. miR-27b-5p+pc-CXCL1.
miR-27b-5p inhibits tumor growth of SKOV3 cell in vivo. A. Overexpressed miR-27b-5p suppressed tumor growth. B. Analysis of tumor weight after xenograft tumor removed. C. The level of miR-27b-5p in tumor tissue was detected using qRT-PCR assay. D. IHC staining for CXCL1 in xenograft tumor and CXCL1 positive staining was shown using histogram.

**P<0.05 vs. miR-NC.

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