

Original Article

Long non coding RNA NRON inhibited breast cancer development through regulating miR-302b/SRSF2 axis

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Abstract: Aims: Long noncoding RNA NRON has been investigated in various tumors, such as hepatocellular carcinoma. However, the role of lncRNA NRON in breast cancer remains unclear. The aim of this study was to explore the function and mechanism of lncRNA NRON in breast cancer. Materials and methods: Overexpression and knockdown vectors were constructed. Proliferation and invasion were measured to evaluate the function of lncRNA NRON. A dual-luciferase reporter assay was utilized to analyze the potential binding target of lncRNA NRON. A rescue experiment was performed to verify the relationship between lncRNA NRON and SRSF2. Results: Our results showed that the expression of lncRNA NRON was significantly downregulated in breast cancer tissues. Overexpression of lncRNA NRON significantly inhibited proliferation and invasion in breast cancer cell lines. Knockdown of lncRNA NRON promoted breast cancer development. We also provided evidence that lncRNA NRON negatively regulated miR-302b. Moreover, we identified SRSF2 as a downstream target of miR-302b. Conclusion: Overall, we performed a comprehensive analysis to indicate that the lncRNA NRON/miR-302b/SRSF2 axis plays an important role in breast cancer. Our study is the first to prove that lncRNA NRON functions as a tumor suppressor in breast cancer.

Keywords: lncRNA, breast cancer, NRON, miR-302b

Introduction

Breast cancer is one of the most common malignant tumors in the world [1, 2]. The American Cancer Society reported that breast cancer accounted for 30% of new cases and 15% of deaths among all kinds of cancer in females in 2019 [3]. It has also been reported that although the incidence rates of other kinds of cancer are relatively stable, the incidence rates of breast cancer increase yearly [4]. There is no doubt that breast cancer is a major public health problem and poses a significant health care burden worldwide [5].

A great number of studies have been carried out to investigate the occurrence and development of breast cancer, but the underlying mechanism of how breast cancer begins and develops remains unknown [6]. Noncoding RNA has emerged as a new target for breast cancer intervention [7]. For example, Kim J and his colleagues demonstrated that the classic lncRNA MALAT1 could bind and inactivate the premetastatic transcription factor TEAD, pre-

venting TEAD from interacting with its coactivator YAP and target gene promoters and therefore inhibiting the progression and metastatic ability of breast cancer [8]. Gao YT and his colleagues found that lncRNA small nucleolar RNA host gene 7 (SNHG7) is significantly upregulated in breast cancer tissues and promotes cell proliferation by sponging miRNA-381, which has been found to play a protective role in various solid tumors [9].

Noncoding RNAs (ncRNAs) are defined as RNA molecules that cannot be translated into protein [10]. Long noncoding RNAs (lncRNAs) are a subtype of noncoding RNAs with lengths usually exceeding 200 nucleotides [11]. Long noncoding RNAs have been widely studied in various kinds of cancer and have been proven to take part in almost every step of the cell cycle and in basic biological cell processes [12-14]. It has been reported that the function of lncRNAs can be divided into four categories-signal, decoy, guide and scaffold. For example, lncRNAs can function as signaling molecules to regulate the transcription of downstream ge-

nes [15]. lncRNAs can also interact with proteins (usually transcription factors) to inhibit the function of these proteins or guide them to a specific locus of related genes [16]. In addition, lncRNAs can act as scaffolds to recruit proteins and RNAs to modify signaling. Among these possible mechanisms, the most widely researched is sponging and sequestering microRNAs and regulating the expression of target proteins [17, 18].

In the current research, we found that the expression of lncRNA NRON was downregulated in breast cancer cell lines and breast cancer tissues. The knockdown or overexpression of lncRNA NRON was associated with cell proliferation and invasion abilities. By bioinformatics analysis, we predicted that lncRNA NRON may sponge miR-302b by complementary pairing and that miR-302b may target the 3'-UTR of serine and arginine rich splicing factor 2 (SRSF2), which has been implicated as a regulator of apoptosis in cancer. By conducting these comprehensive experiments, we aimed to explore the underlying mechanism of how lncRNA NRON influences the progression of breast cancer and demonstrated that lncRNA NRON could play a protective role in inhibiting cell proliferation and invasion by sponging miR-302b, which can antagonize the miRNA effect on SRSF2. The current research may identify new targets for breast cancer intervention.

Materials and methods

Cell culture and samples

The breast cancer cell lines MDA-MB-231, MDA-MB-415, MDA-MB-468, and MCF-7 and the nontumor cell lines MCF10A and 293T were stored in our laboratory. Cells were cultured according to the culture methods described in the ATCC website. Breast cancer tissues were collected from patients who received surgical treatment for breast cancer, and normal tissues were collected from patients who received surgical treatment for benign lesions, such as fibroadenoma and breast cystic hyperplasia. These tissues were stored at -80°C for further research once resected. The study was approved by the Institute Research Ethics Committee of Affiliated Cancer Hospital of Zhengzhou University and every patient agreed to participate and signed the informed consent form.

Preparation of cytoplasmic and nuclear fractions

Cytoplasmic and nuclear fractionation experiments were carried out according to a previously described protocol. Briefly, after the cells were washed and centrifuged, 800 µL hypotonic buffer was added to each sample. Then, 10% Nonidet P-40 was added to a final concentration of 0.4% (35 µL). After centrifugation at 3,000 rpm for 7 min, the supernatants (450 µL; cytoplasmic fractions) were collected for processing, and the remaining supernatants were discarded. Samples were quickly spun again (3,000 for 30 s) to remove any remaining supernatant, and then the pellet (nuclear fraction) was gently resuspended in 500 µL hypotonic buffer and spun at 3,000 rpm for 2 min. After removing the buffer following the last spin, the samples were briefly spun once again for a few seconds to remove the remaining supernatant from the cells. Both the cytoplasmic and nuclear fractions were processed following the RNA STAT-60 (TEL-TEST) protocol as recommended by the manufacturer.

Transfection

SiRNAs against lincRNA NRON and the overexpression vector of lincRNA NRON were constructed by Gene Pharma (Shanghai, China). Vectors with wild-type (WT) or mutant (mut) binding sites for miR-302b were constructed by Gene Pharma (Shanghai, China). The 3'-untranslated region (UTR) of SRSF2, with wild-type (WT) or mutant (mut) binding sites for miR-302b, was amplified and cloned into the pGL3 vector (Promega, Madison, WI) to generate the vector pGL3-WT-SRSF2-3'-UTR or pGL3-mut-SRSF2-3'-UTR. The miR-302b mimic, miR-302b inhibitor, mimic NC, and inhibitor NC were purchased from Shanghai Gene Pharma (Shanghai, China). The breast cancer cell lines MCF-7 and MDA-MB-231 were transfected with vectors, miR-302b mimic, miR-302b inhibitor, mimic NC and inhibitor NC by Lipo3000 reagent (Invitrogen) according to the manufacturer's protocol. Cells were incubated for 48 h before further research.

Cell invasion assay

Transwell chambers (Corning Incorporated, Corning, NY, USA) with a pore size of 8 µm were used for the cell invasion assay. Briefly,

Role of lncRNA NRON in breast cancer

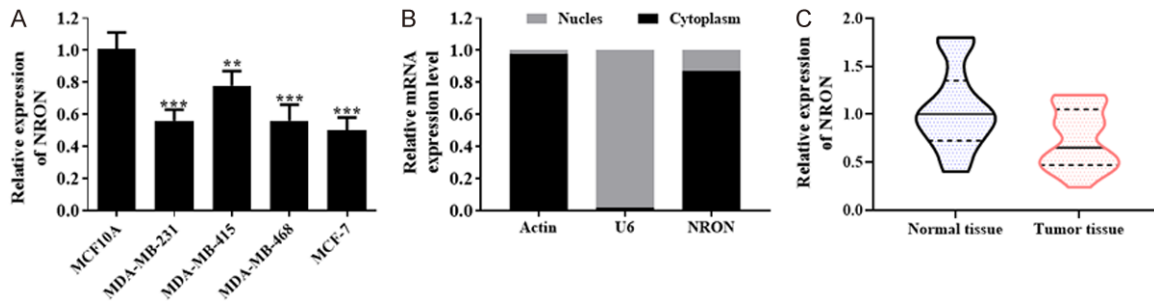


Figure 1. Biological information of lncRNA NRON. A. The expression of lncRNA NRON was significantly down regulated in breast cancer lines. B. lncRNA NRON was located in cytoplasm. C. The expression of lncRNA NRON was down regulated in tumor tissues compared with normal breast tissues.

4×10^4 cells in serum-free medium were directly added into the upper chamber. Medium containing 20% FBS was added to the lower chamber and served as a chemoattractant. After incubation in an incubator at 37°C and 5% CO_2 for 24 h, cotton swabs were used to gently wipe off the cells remaining on the membrane surface that had not passed through. The cells that migrated to the lower surface of the membrane were fixed with 4% paraformaldehyde, stained in 10% crystal violet and washed with PBS. The cells were counted in high-power fields microscopically.

CCK-8 assay

Cells were seeded into 96-well plates. At 0 h, 24 h, 48 h, 72 h and 96 h, 10 μL CCK-8 reagent was added to each well and incubated at 37°C for 2 h. The optical density was measured at 450 nm.

Dual-luciferase reporter assay

The Dual-Luciferase® Reporter Assay System (Promega) and 293T cells were used to conduct the dual-luciferase reporter assay. Vectors expressing lncRNA NRON and the SRSF2 mRNA 3'-UTR with the wild-type or mutant binding sites for miR-302b were cotransfected with miR-302b mimic, miR-302b inhibitor, mimic NC or inhibitor NC into 293T cells. Luciferase activity was analyzed using the dual-luciferase reporter system following the manufacturer's protocol. Firefly luciferase activity and Renilla luciferase activity were measured with Multiskan Spectrum (Thermo Fisher, USA).

Real-time PCR

Total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific). RNA reverse tran-

scription was performed using a PrimeScript™ RT reagent Kit with gDNA eraser (RR047A; Takara, Tokyo, Japan), and real-time PCR was performed using SYBR® Premix Ex Taq™ (RR420A; Takara, Tokyo, Japan). The data were normalized using GAPDH levels and further analyzed by the $2^{-\Delta\Delta\text{CT}}$ method.

Western blotting

Cells were harvested and lysed with RIPA lysis buffer containing proteinase inhibitor (Roche, USA). Total protein was quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples were resolved by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with primary antibodies against CCND1, CDK4, Bax, Bcl-2 and GAPDH at 4°C overnight, followed by incubation with a peroxidase-conjugated goat anti-rabbit (or mouse) IgG antibody. Immunopositive bands were analyzed using a FluorChem M system (ProteinSimple, San Jose, CA, USA).

Data analysis

We used SPSS 23.0 to calculate the values (means \pm standard error of the mean). Statistical analyses were analyzed using two-sided Student's t-test or one-way ANOVA. The level of statistical significance was $P < 0.05$.

Results

Biological characteristics of lncRNA NRON

To investigate the function of lncRNA NRON in breast cancer, we first detected the expression of lncRNA NRON in different cell lines. As shown in **Figure 1A**, the expression of lncRNA NRON was significantly downregulated in brea-

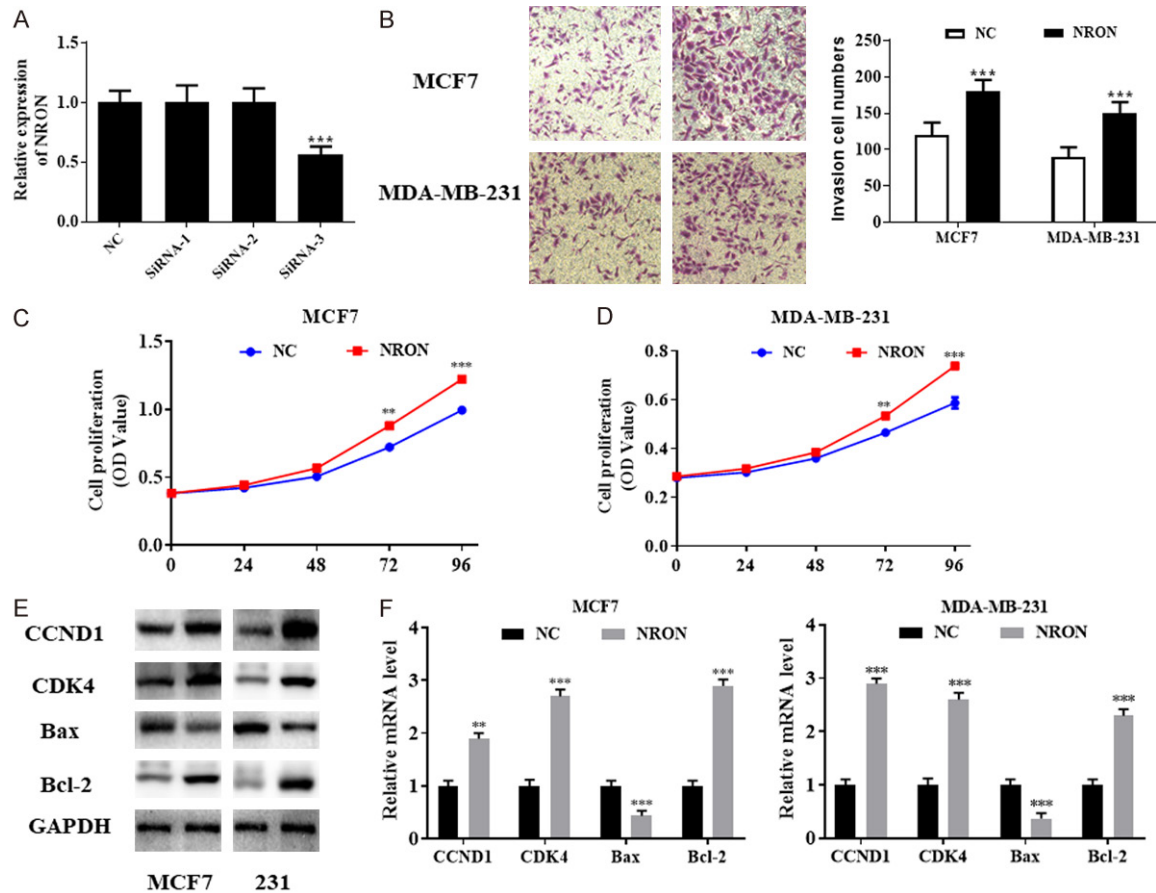


Figure 2. Knockdown of lncRNA NRON promoted the cell progress. A. The expression of lncRNA NRON was reduced via siRNAs. B. Knockdown of lncRNA NRON significantly promoted cell invasion in MCF7 and MDA-MB-231 cells. C. Knockdown of lncRNA NRON significantly promoted cell proliferation in MCF7 cells. D. Knockdown of lncRNA NRON significantly promoted cell proliferation in MDA-MB-231 cells. E. Proliferation markers and apoptosis markers were detected by western blot. Knockdown of lncRNA NRON significantly promoted cell proliferation in MCF7 and MDA-MB-231 cells. F. Knockdown of lncRNA NRON significantly increased the expression of CCND1 and CDK4 by real-time PCR.

st cancer cell lines. Next, we investigated the location of lncRNA NRON. We performed cytoplasmic and nuclear fractionation experiments and found that lncRNA NRON was located in the cytoplasmic fraction (Figure 1B). Moreover, we detected the expression of lncRNA NRON in tumor tissues, suggesting that lncRNA NRON was significantly downregulated in breast cancer tissues compared with normal tissues (Figure 1C).

Knockdown of lncRNA NRON promoted cell progression

To explore the function of lncRNA NRON, we constructed a knockdown vector with siRNAs. As shown in Figure 2A, the expression of lncRNA NRON was significantly decreased. We performed a comprehensive experiment to in-

vestigate the function of lncRNA NRON in breast cancer. Knockdown of lncRNA NRON significantly promoted cell invasion in two different cell lines (Figure 2B). CCK-8 assays showed that knockdown of lncRNA NRON markedly promoted the proliferation rates in MCF-7 and MDA-MB-231 cells (Figure 2C, 2D). We also detected cell proliferation and apoptosis markers using western blot and real-time PCR. The protein levels of CCND1 and CDK4 were significantly increased when lncRNA NRON was knocked down and Bax was decreased, as demonstrated by western blotting (Figure 2E). Real-time PCR produced similar results (Figure 2F). Thus, our results suggested that downregulation of lncRNA NRON could promote cell invasion and proliferation and inhibit cell apoptosis.

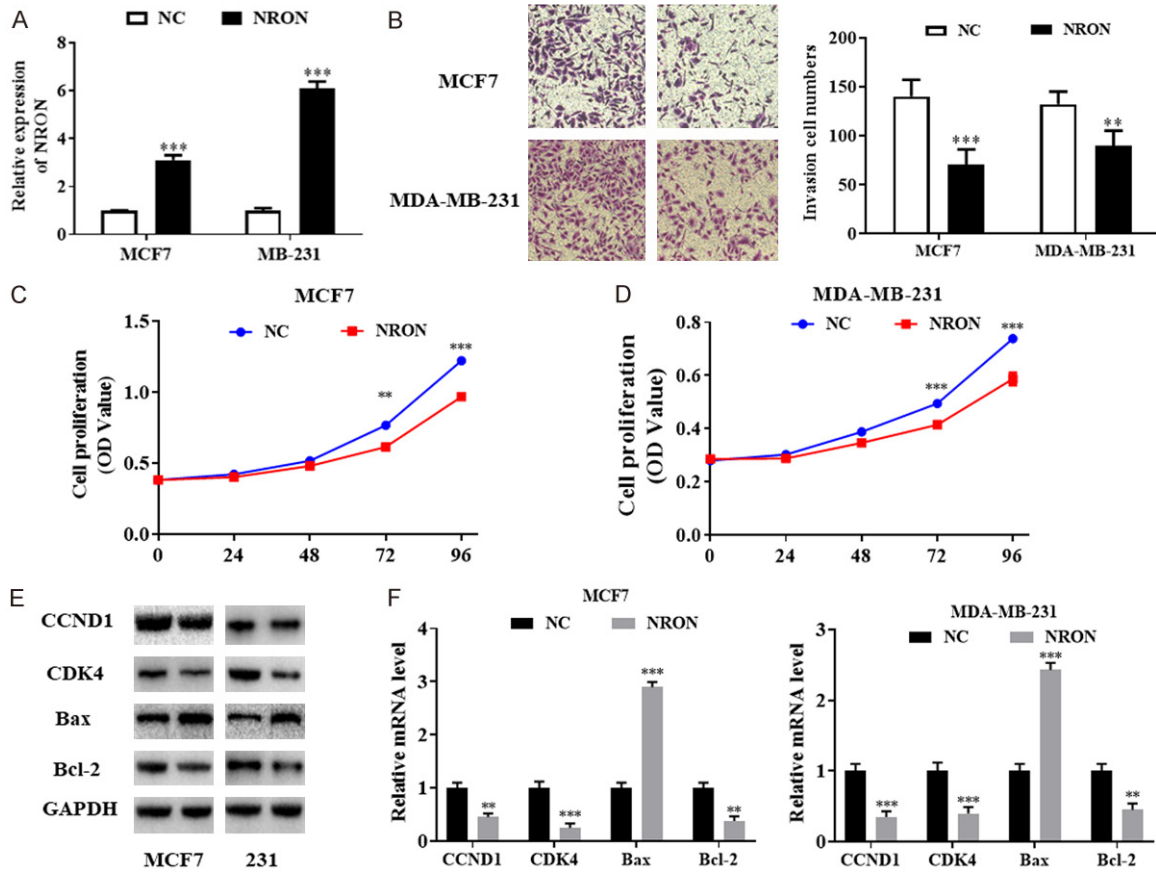


Figure 3. Overexpression of lncRNA NRON inhibited proliferation, invasion. A. The expression of lncRNA NRON was increased by plasmids. B. Overexpression of lncRNA NRON significantly inhibited cell invasion in MCF7 and MDA-MB-231 cells. C. Overexpression of lncRNA NRON significantly suppressed cell proliferation in MCF7 cells. D. Overexpression of lncRNA NRON significantly suppressed cell proliferation in MDA-MB-231 cells. E. Proliferation markers and apoptosis markers were detected by western blot. Overexpression of lncRNA NRON significantly inhibited cell proliferation in MCF7 and MDA-MB-231 cells. F. Overexpression of lncRNA NRON significantly decreased the expression of CCND1 and CDK4 by real-time PCR.

Overexpression of lncRNA NRON inhibited proliferation and invasion

To further explore the function of lncRNA NRON in breast cancer, we constructed an overexpression vector of lncRNA NRON. As shown in **Figure 3A**, the expression of lncRNA NRON was increased in MCF-7 and MDA-MB-231 cells. Overexpression of lncRNA NRON significantly suppressed cell invasion and proliferation, as demonstrated by Transwell and CCK-8 assays in two different cell lines (**Figure 3B-D**). The protein levels of CCND1, CDK4 and Bcl-2 were significantly reduced after overexpression treatment; however, Bax was significantly increased (**Figure 3E**). Real-time PCR assays produced similar results in MCF-7 and MDA-MB-231 cells (**Figure 3F**). Thus, we concluded that overexpression of lncRNA NRON

can inhibit cell invasion and proliferation and promote cell apoptosis.

MiR-302b is the potential target of lncRNA NRON

To investigate the possible mechanism of lncRNA NRON, we first explored the potential binding miRNAs of lncRNA NRON. As shown in **Figure 4A**, we predicted that miR-302b may possess a potential binding sequence for miR-302b. Next, we performed a dual-luciferase reporter assay to examine this relationship. Luciferase activity was significantly reduced in the miR-302b mimic group but increased in the miR-302b inhibitor group. However, there was no significant difference between the mutant groups (**Figure 4B**). In addition, we proved that the mRNA level of lncRNA NRON was

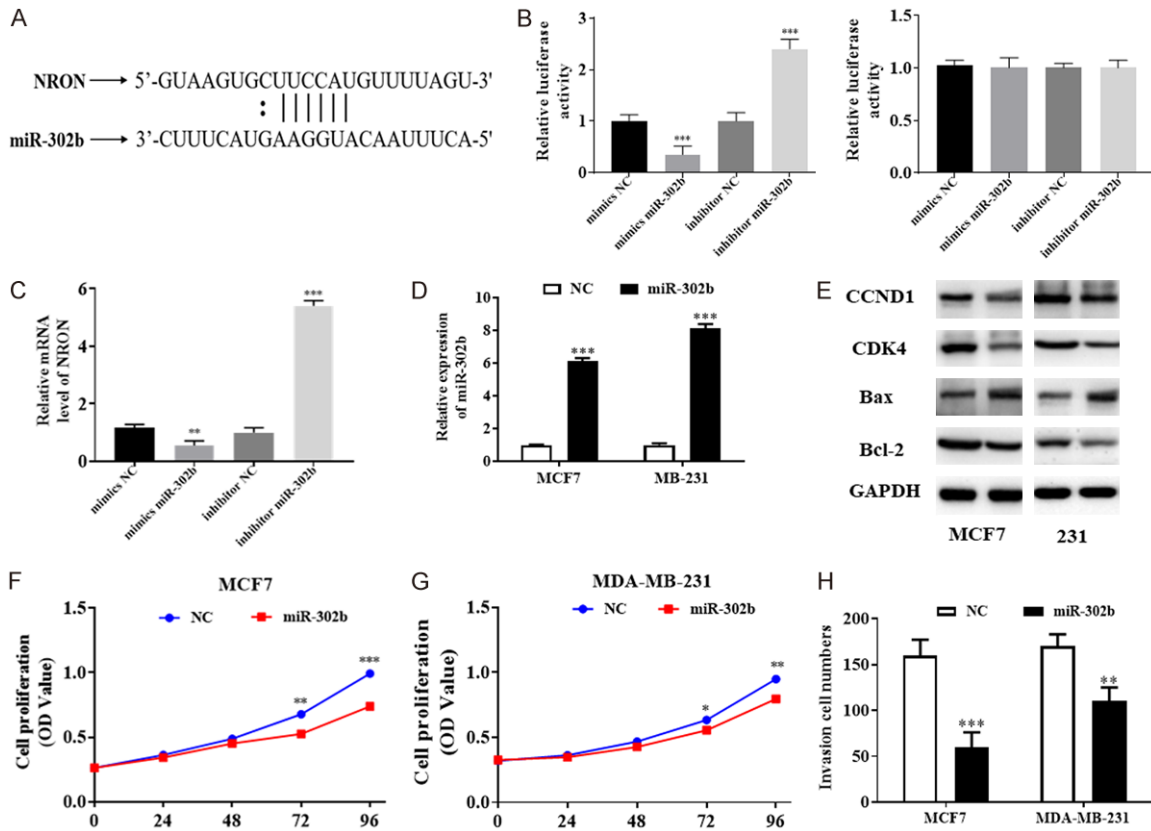


Figure 4. miR-302b is the potential target of lncRNA NRON. A. The possible binding sequence was showed. B. The relative luciferase reporter activity was reduced in the group of miR-302b overexpression and increased in the inhibitor group. C. Overexpression of miR-302b distinctly reduced the mRNA level of lncRNA NRON and vice versa. D. The expression of miR-302b was confirmed by miR-302b mimics. E. Overexpression of miR-302b significantly reduced the protein level of CCND1, CDK4 and Bcl-2 and increased Bax in two different cell lines. F. Overexpression of miR-302b significantly inhibited cell proliferation in MCF7 cells. Proliferation was measured by CCK-8 assay. G. Overexpression of miR-302b significantly suppressed cell proliferation in MDA-MB-231 cells. H. Overexpression of miR-302b significantly inhibited cell invasion in MCF7 and MDA-MB-231 cells.

markedly downregulated in the miR-302b overexpression group, while the opposite was observed in the miR-302b inhibitor group (Figure 4C). To date, there has been no report about miR-302b in breast cancer. Thus, we performed a comprehensive experiment to assess the function of miR-302b in breast cancer. First, we constructed an overexpression vector of miR-302b. The expression of miR-302b was significantly increased in MCF-7 and MDA-MB-231 cells (Figure 4D). Overexpression of miR-302b significantly decreased the protein levels of the cell proliferation markers CCND1 and CDK4, as measured by western blot analysis. The protein level of Bax was significantly increased in the miR-302b mimic group (Figure 4E). We also assessed the effect of miR-302b on proliferation via the CCK-8 assay, which demonstrated that overexpression of lncRNA NRON significantly inhibited cell proliferation

(Figure 4F, 4G). Finally, we performed a Transwell assay to assess the effect on invasion. Overexpression of miR-302b significantly inhibited cell invasion (Figure 4H). Thus, we concluded that miR-302b is the target of lncRNA NRON and plays an oncogenic role in breast cancer.

SRSF2 is a direct target of miR-302b

To further explore the downstream target of miR-302b, we used bioinformatics analysis to predict the possible target of miR-302b. As shown in Figure 5A, SRSF2 possessed 3 binding sites of miR-302b. Next, we performed a dual-luciferase reporter assay to examine this relationship. Luciferase activity was significantly reduced in the WT group, whereas no difference was detected in the mutant group, suggesting that SRSF2 is the direct target of

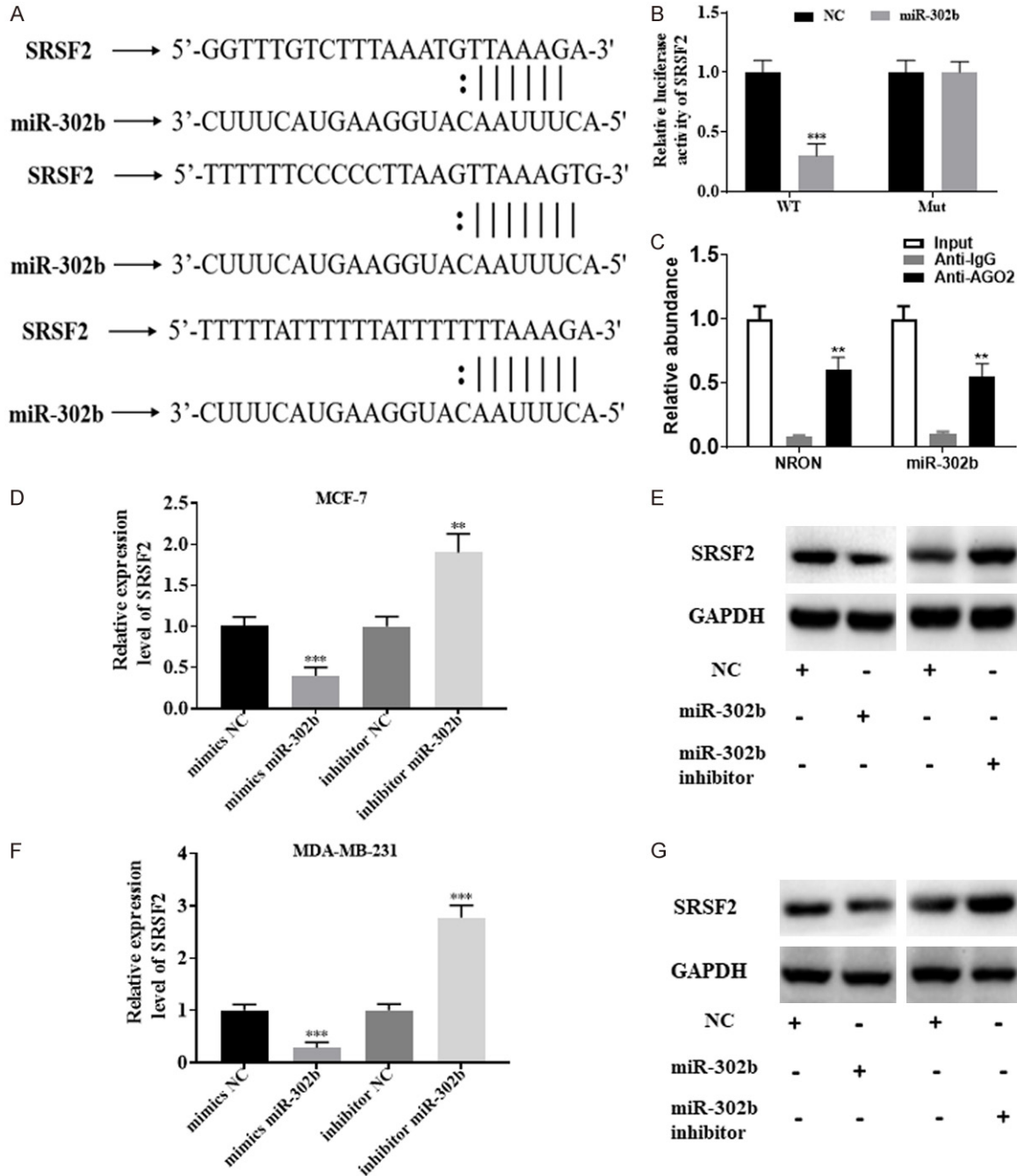


Figure 5. SRSF2 is the direct target of miR-302b. A. The potential binding sequences were showed. B. The luciferase activity was downregulated in wt group whereas no difference was detected in mutant group. C. RIP experiment confirmed the binding between miR-302b and NRON. D. Overexpression of miR-302b distinctly reduced the mRNA level of SRSF2 and vice versa in MCF7 cells. E. Overexpression of miR-302b distinctly reduced the protein level of SRSF2 and vice versa in MCF7 cells. F. Overexpression of miR-302b distinctly reduced the mRNA level of SRSF2 and vice versa in MDA-MB-231 cells. G. Overexpression of miR-302b distinctly reduced the protein level of SRSF2 and vice versa in MDA-MB-231 cells.

miR-302b (Figure 5B). Overexpression of miR-302b in MCF-7 cells clearly reduced the mRNA and protein levels of SRSF2 while the opposite was observed when miR-302b was inhibited

(Figure 5C, 5D). Similar results were produced in MDA-MB-231 cells (Figure 5E, 5F). Thus, we concluded that SRSF2 is a direct target of miR-302b and negatively regulates SRSF2.

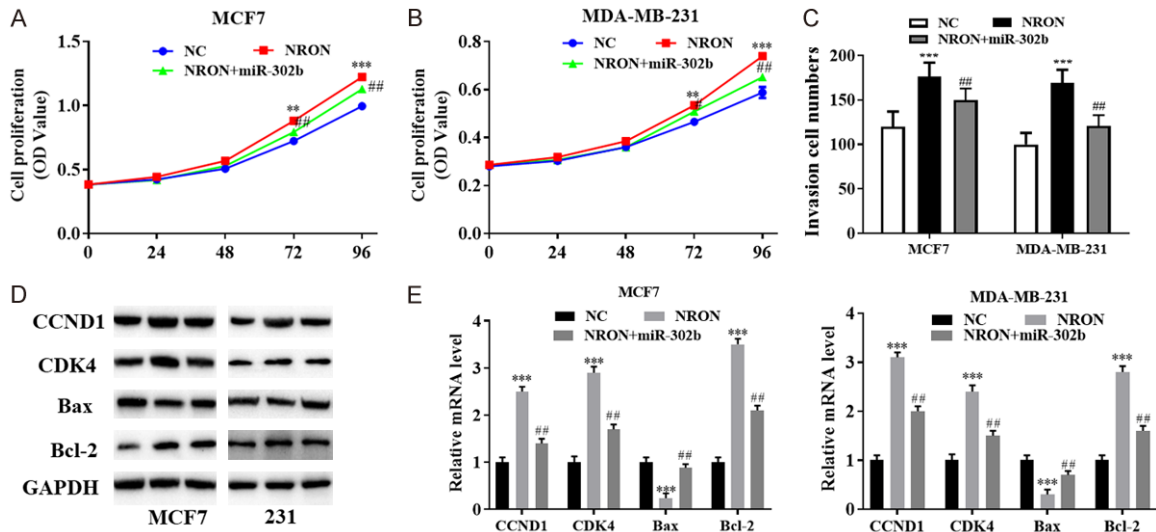


Figure 6. lncRNA NRON functioned through regulating miR-302b. A. Overexpression of miR-302b could restore the effect of lncRNA NRON. B. Overexpression of miR-302b could reduce the proliferation rate compared with the lncRNA NRON group by CCK-8. C. Co-transfection of miR-302b and lncRNA NRON significantly decreased cell invasion numbers compared with knockdown lncRNA NRON group. D. The protein level of CCND1, CDK4 and Bcl-2 were downregulated in co-transfection group of miR-302b and NRON. E. The mRNA level of CCND1, CDK4 and Bcl-2 were downregulated in co-transfection group of miR-302b and NRON by real-time PCR.

lncRNA NRON functions by regulating miR-302b

We performed a rescue experiment to verify the relationship between lncRNA NRON and miR-302b. First, we cotransfected the knock-down vector of lncRNA NRON and miR-302b mimics in two different cell lines. Proliferation assays showed that the rates were significantly reduced in the cotransfection group compared with the lncRNA NRON downregulation group, as demonstrated by CCK-8 assay (**Figure 6A, 6B**). The numbers of invaded cells were markedly decreased in the cotransfection group (**Figure 6C**). In addition, overexpression of miR-302b could restore the pro-proliferative effect of lncRNA NRON. The protein levels of CCND1, CDK4 and Bcl-2 were significantly decreased in the cotransfection group compared with the lncRNA NRON group (**Figure 6D**). Similar results could be obtained by real-time PCR (**Figure 6E**). Thus, we concluded that lncRNA NRON functioned by sponging miR-302b to influence the progression of breast cancer.

Discussion

Increasing reports have demonstrated that lncRNAs are important regulators of breast cancer progression and metastasis [1]. In the

current research, we investigated lncRNA NRON, which has been found to play a protective role in various kinds of tumors [19-21]. To reveal its role in breast cancer, we detected the expression of lncRNA NRON in different breast cancer cell lines, namely, MDA-MB-231, MDA-MB-415, MDA-MB-468, MCF-7 and the nontumor cell line MCF10A. The results showed that the expression of lncRNA NRON was significantly reduced in breast cancer cell lines compared with nontumor cells. In addition, we detected its expression in breast tumors and normal tissues, and the result was similar to the cell experiment. It has been reported that lncRNAs can be located both in the nucleus and the cytoplasm, and the functions of lncRNAs vary with their location [14]. For example, nuclear lncRNAs can establish and maintain the conformation of chromosomes, organize the architecture of nuclei and regulate alternative splicing [22], while in the cytoplasm, lncRNAs can modulate mRNA stability and translation by sequestering microRNAs and therefore enhancing the expression of target mRNAs [23]. To reveal the function of lncRNA NRON, we conducted cytoplasmic and nuclear fractionation experiments to detect the expression of lncRNA NRON. The results revealed that lncRNA NRON was mainly located in the cytoplasm.

To further confirm that lncRNA NRON participates in the development of breast cancer, we knocked down and overexpressed lncRNA NRON in MCF-7 and MDA-MB-231 cells by transfecting the cells with siRNAs or an overexpression vector of lncRNA NRON and found that cell proliferation and invasion were significantly increased in lncRNA NRON knockdown MCF-7 and MDA-MB-231 cells. The expression of cell proliferation markers such as CCND1, CDK4 and Bcl-2 was upregulated with the downregulation of the apoptotic marker Bax, which indicated that cell apoptosis is inhibited in lncRNA NRON knockdown cells. In lncRNA NRON-upregulated cells, cell proliferation and invasion were significantly decreased. These results are similar to previous studies that demonstrated that lncRNA NRON plays a protective role in tumor progression.

As we mentioned above, cytoplasmic lncRNAs can modulate mRNA stability and translation by sequestering microRNAs [24]. We hypothesized that lncRNA NRON can regulate apoptosis signaling by decoying and sponging microRNAs and further stabilizing microRNA-targeted mRNAs. To investigate the possible mechanism of lncRNA NRON, we conducted bioinformatic analysis and found that miR-302b may be a direct target of lncRNA NRON. In addition, arginine rich splicing factor 2 (SRSF2), whose mutation has been proven to be associated with the development of hematologic tumors [25], may be a direct target of miR-302b with 3 binding sites identified in the 3'-untranslated region (UTR) of its mRNA. Dual-luciferase reporter assays were used to further verify the association between lncRNA NRON and miR-302b, miR-302b and the 3'-UTR of SRSF2 mRNA. The luciferase activity was significantly reduced in 293T cells cotransfected with miR-302b mimics and lncRNA NRON and miR-302b mimics and the 3'-UTR of SRSF2 mRNA compared with the corresponding mutation group. The results indicated the direct interactions of the two pairs. The results of real-time PCR and western blot further proved this hypothesis.

Many researchers have found that lncRNAs could be good candidates for tumor biomarkers and possessed high specificity, high sensitivity, and noninvasive characteristics. Furthermore, lncRNAs in body fluids may serve as promising biomarkers for cancers diagnosis

and prognosis. Thus, further study about the dysregulated lncRNAs in body fluids via deep sequencing or single cell RNA-sequence may provide new clues for better understanding the potential mechanism.

Finally, we performed a rescue experiment to verify the presence of the lncRNA NRON/miR-302b/SRSF2 axis. We cotransfected the lncRNA NRON knockdown vector and miR-302b mimics in the two cell lines, and we found cell proliferation and invasion were significantly reduced in the cotransfection group compared with the lncRNA NRON downregulation group. The expression levels of CCND1, CDK4 and Bcl-2 were also significantly decreased in the cotransfection group compared with the lncRNA NRON group. The results indicated that overexpression of miR-302b could restore the pro-proliferative effect of lncRNA NRON.

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Disclosure of conflict of interest

None.

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