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LINC01096 knockdown inhibits progression of triple-negative breast cancer by increasing miR-3130-3p

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Abstract. – OBJECTIVE: Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer. Long noncoding RNAs (lncRNAs) have been reported to be involved in the development of TNBC. However, the role and mechanism of LINC01096 in TNBC are largely unclear. This work aims to investigate the effect of LINC01096 on cell viability, apoptosis, migration, and invasion of TNBC cells, as well as explore the interaction between LINC01096 and microRNA (miR)-3130-3p.

PATIENTS AND METHODS: Sixty TNBC patients were recruited. T47-D and BT-549 cells were cultured for experiments *in vitro*. The expression levels of LINC01096 and miR-3130-3p were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Cell viability, apoptosis, migration, and invasion were determined by MTT, flow cytometry, and trans-well assays. The target association between LINC01096 and miR-3130-3p was confirmed by the luciferase reporter assay.

RESULTS: The expression of LINC01096 was enhanced in TNBC tissues and cells. High expression of LINC01096 predicted poor outcomes of patients with TNBC. Silence of LINC01096 led to the suppression of cell viability, migration, and invasion, as well as the promotion of apoptosis in TNBC cells. MiR-3130-3p was targeted by LINC01096 and lowly expressed in TNBC. Overexpression of miR-3130-3p repressed cell viability, migration, and invasion, while it induced apoptosis. However, the knockdown of miR-3130-3p induced the opposite effect. This was weakened by inhibiting LINC01096.

CONCLUSIONS: Knockdown of LINC01096 inhibited cell viability, migration, and invasion; however, it promoted apoptosis in TNBC by up-regulating miR-3130-3p, indicating a novel target for the treatment of TNBC.

Key Words:
TNBC, LINC01096, MiR-3130-3p, Apoptosis, Migration, Invasion.

Abbreviations

TNBC, triple-negative breast cancer; TNM, tumor-node-metastasis.

Introduction

Breast cancer is a common malignancy with high incidence in women all over the world[1]. Triple-negative breast cancer (TNBC) is a common type of breast cancer with aggressive characteristics[2]. There is a great development in exploring therapeutic targets for breast cancer[3]. However, effective strategies remain limited in TNBC treatment. Therefore, novel targets are needed to be elucidated for understanding the mechanism of TNBC development and improving the therapeutic effect. Noncoding RNAs, including long noncoding RNAs (lncRNAs) and microRNAs (miRNAs), could serve as im-

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portant targets for therapeutic and diagnosis of diseases[4]. LncRNAs with over 200 nucleotides and no protein-coding potential play vital roles in cancer development[5]. Emerging evidence[6] suggested that multiple lncRNAs are dysregulated in TNBC. Furthermore, Rodriguez et al[7] showed that lncRNAs may be regarded as targets of diagnosis, prognosis, and therapeutics in TNBC. Several reports[8-10] described that lncRNAs could mediate TNBC progression by regulating proliferation, apoptosis, migration, and invasion. Previously Shen et al[11] demonstrated the abnormally expressed LINC01096 (ENST00000503938) in TNBC. Nevertheless, the exact role and mechanism of LINC01096 in TNBC remain dismal. Aberrantly expressed miRNAs have been reported[12] to play essential roles in angiogenesis and progression of breast cancer. Moreover, increasing efforts suggested that miRNAs are implicated in the prognosis and progression of TNBC[13,14]. For instance, several miRNAs such as miR-124, miR-4417, miR-374a-5p, and miR-890 have been revealed to participate in the development of TNBC by regulating cell proliferation, apoptosis, migration, and invasion[15-18]. To date, there is a little study on miR-3130-3p except for the work of Pan et al[19], who suggested the anti-role of miR-3130-3p in endometrial cancer. The database of DIANA tool online predicted the complementary sequences between LINC01096 and miR-3130-3p, suggesting LINC01096 as a potential sponge of miR-3130-3p. In this research, we focused on the function of LINC01096 on TNBC development *in vitro* and explored whether the underlying regulatory mechanism was associated with miR-3130-3p.

Patients and Methods

Patient Samples and Cell Culture

Cancer tissues and the corresponding adjacent normal samples were collected from 60 TNBC patients who have signed the informed consents from the Affiliated Hospital of Qingdao University. The clinical features of the patients were displayed in Table I and II. All tissues obtained by surgery were stored at –80°C. This investigation was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. The methods used were performed in accordance with relevant guidelines and regulations. Human TNBC cell lines (T47-D and BT-549) and normal human breast epithelial cell line MCF-10A were obtained from BeNa Culture Collection (Beijing, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO_2.

Cell Transfection

The full-length sequences of LINC01096 were used to generate LINC01096 overexpression vector (LINC01096) with pcDNA3.1 vector (Thermo Fisher Scientific, Waltham, MA, USA) as a negative control (NC). siRNA targeted LINC01096 (si-LINC01096), siRNA negative control (si-NC), miR-3130-3p mimic (miR-3130-3p), mimic negative control (miR-NC), miR-3130-3p inhibitor (anti-miR-3130-3p), and inhibitor negative control (anti-NC) were generated by GenePharm (Shanghai, China). These oligonucleotides with a final concentration of 30 nM were transfected into T47-D and BT-549 cells with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h.

Table I. Correlations between LINC01096 expression and clinical characteristics in TNBC patients.

Clinical parameters	High	Low	*p*-value
Age			>0.05
< 45 years	15	14	
≥ 45 years	17	14	
Tumor size			<0.05
≤ 2	14	17	
> 2	18	11	
Lymph node metastasis			<0.05
Yes	24	10	
No	8	18	
TNM stage			<0.05
I-II	9	16	
III-IV	23	12	

Table II. Correlations between miR-3130-3p expression and clinical characteristics in TNBC patients.

Clinical parameters	High	Low	*p*-value
Age			>0.05
< 45 years	13	16	
≥ 45 years	15	16	
Tumor size	<0.05		
≤ 2	21	10	
> 2	7	22	
Lymph node metastasis			<0.05
Yes	14	20	
No	14	12	
TNM stage			<0.05
I-II	17	8	
III-IV	11	24	

MTT

After indicated transfection, T47-D and BT-549 cells (5 x 10^3/well) were seeded into 96-well plates in triplicates. After culture for 0, 24, 48 or 72 h, 10 µl MTT solution (Beyotime, Shanghai, China) was added to each well and the plates were maintained at 37°C for 4 h. Then, 100 µl dimethyl sulfoxide (DMSO) was added to each well to solubilize the formed formazan. Cell viability was determined by detecting the absorbance at 490 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry

Flow cytometry was used to determine cell apoptosis with an Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Transfected T47-D and BT-549 cells were cultured for 72 h and then were resuspended in a binding buffer. Subsequently, cells were stained with Annexin V-FITC and PI for 10 min in the dark, and the cell apoptosis was analyzed with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Transwell Assay

The transwell chambers with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were used for invasion assay, and those without Matrigel were used for migration assay. Transfected T47-D and BT-549 cells (1 x 10^5/ml) were resuspended in serum-free Dulbecco's Modified Eagle's Medium (DMEM), and 200 µl cell suspension was added in the upper chambers. The lower chambers were filled with 500 µl Dulbecco's Modified Eagle's Medium (DMEM) medium with 10% fetal bovine serum (FBS). After the incubation for 12 h, the non-traversed cells were wiped away, and traversed cells were stained with 0.1% crystal violet. The number of migrated or invasive cells was counted with three random fields using a 200x magnification microscope.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used for RNA isolation, and NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was applied to detect RNA concentration. 500 ng RNA was reversely transcribed to cDNA using All-in-One miRNA or mRNA First-Strand cDNA Synthesis Kit (Fulengen, Guangzhou, China) or Prime-Script RT reagent kit (TaKaRa, Dalian, China) following the manufacturer's instructions. The qRT-PCR was performed using SYBR mix (Fulengen, Guangzhou, China) on CFX96 Real-time PCR Systems (Bio-Rad, Hercules, CA, USA). The primer sequences were as follows: LINC01096 (Forward, 5'-ACGTGTGCAGCAGTTTACTG-3'; Reverse, 5'-TGCTAGGATTGGTAGTTTTGGTGT-3'); miR-3130-3p (Forward, 5'-ATTGCTGCACCGGAGACTG-3'; Reverse, universal adaptor PCR primers for miRNAs provided in kit); GAPDH (Forward, 5'-GAATGGGCAGCCGTTAGGAA-3'; Reverse, 5'- AAAAGCATCACCCGGAGGAG-3'); U6 (Forward, 5'-CTCGCTTCGGCAGCACA-3; Reverse, universal adaptor PCR primers for miRNAs provided in kit). The relative expression levels of LINC01096 and miR-3130-3p were normalized to GAPDH or U6, respectively, and calculated by the $2^{-\Delta\Delta Ct}$ method[20].

Luciferase Reporter Assay

The potential targets of LINC01096 were predicted by DIANA tools. The sequences of LINC01096 containing miR-3130-3p complementary sites were cloned into pGL3-control lucif-

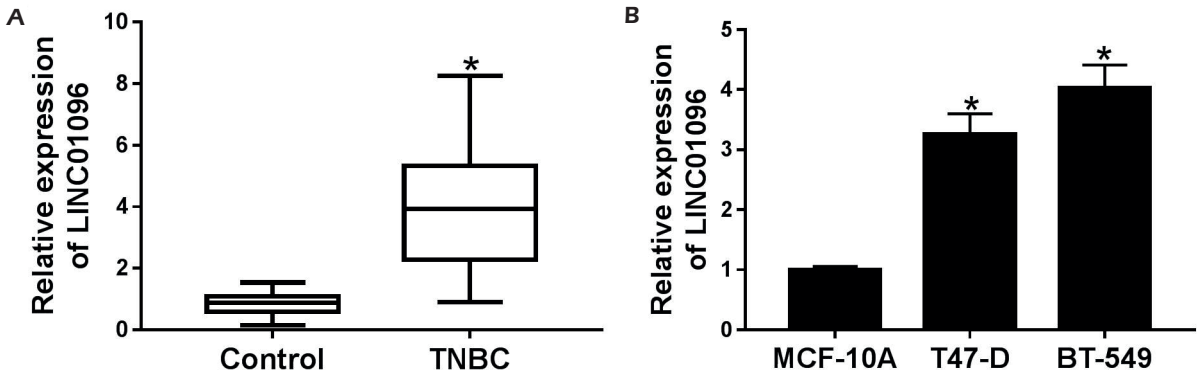

Figure 1. The expression of LINC01096 is increased in TNBC. ***A,*** QRT-PCR was performed to detect the expression of LINC01096 in TNBC tissues (n=60) and normal samples (n=60). ***B,*** Level of LINC01096 was measured in TNBC cells and control (MCF-10A) by qRT-PCR. *$p<0.05$ compared with control or MCF-10A group.

erase reporter vectors (Promega, Madison, WI, USA) and named as LINC01096-WT. The mutant vectors (LINC01096-MUT) were generated by mutating the seed sites (CGGUGCAG) to (AUUCGCAG) with a Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA, USA). T47-D and BT-549 cells were co-transfected with LINC01096-WT or LINC01096-MUT, together with miR-NC, miR-3130-3p, anti-miR-3130-3p or anti-NC. After 48 h post-transfection, the luciferase reporter system (Promega, Madison, WI, USA) was applied to assess the luciferase activity in T47-D and BT-549 cells.

Statistical Analysis

The researches were repeated three times and data were presented as mean ± standard deviation (S.D). The statistical analysis was performed using GraphPad Prism 7 software (GraphPad Inc., La Jolla, CA, USA) and Student's *t* test or one-way ANOVA followed by Tukey's test was used to compare the difference between the different groups. The association between clinicopathologic features of TNBC patients and level of LINC01096 or miR-3130-3p was assessed by χ^2-test. The linear relation between levels of LINC01096 and miR-3130-3p in TNBC tissues was investigated by the Spearman's correlation coefficient. $p<0.05$ was considered statistically significant.

Results

The Expression of LINC01096 is Increased in TNBC

To measure the expression of lncRNA in TNBC, 60 TNBC tissues and matched normal samples were collected. As shown in Figure 1A, the expression of LINC01096 was significantly increased in TNBC tissues compared with that in control group. Moreover, the patients with TNBC were divided into high (n=32) or low (n=28) LINC01096 expression group. Table I summarized that high expression of LINC01096 was associated with tumor size, lymph node metastasis, and tumor-node-metastasis (TNM) stage of patients ($p<0.05$), but not with age ($p>0.05$). In addition, the level of LINC01096 was detected in TNBC cells, and results displayed that LINC01096 expression was evidently up-regulated in T47-D and BT-549 cells in comparison to that in MCF-10A cells (Figure 1B). These findings suggested that LINC01096 expression was enhanced in TNBC and a high expression of LINC01096 indicated the poor outcome of patients.

Knockdown of LINC01096 Suppresses the Progression of TNBC Cells

To investigate the function of LINC01096 on TNBC progression, T47-D and BT-549 cells were transfected with si-LINC01096 or si-NC. After the transfection, the abundance of LINC01096 was effectively reduced in T47-D and BT-549 cells with transfection of si-LINC01096 compared with that in si-NC group (Figure 2A). Moreover, the MTT assay revealed that knockdown of LINC01096 markedly decreased cell viability of T47-D and BT-549 cells at 72 h (Figure 2B). Meanwhile, the data of flow cytometry described higher apoptosis production in T47-D and BT-549 cells by silencing LINC01096 (Figure 2C). In addition, the transwell analysis displayed that interference of LINC01096 significantly repressed the abilities of migration and invasion in T47-D and BT-549 cells

(Figure 2D and 2E). These results revealed that LINC01096 silence inhibited TNBC progression.

MiR-3130-3p is a Target of LINC01096 in TNBC Cells

To explore the potential mechanism of LINC01096, its targeted miRNAs were searched by DIANA tools. As shown in Figure 3A, miR-3130-3p has complementary sequences of LINC01096, predicting the interaction between them. To confirm this prediction, luciferase reporter assay was performed in T47-D and BT-549 cells. As displayed in Figure 3B and 3C, the luciferase activity in the two cells was significantly reduced by miR-36130-3p overexpression and increased by the miR-3130-3p knockdown in LINC01096-WT group, while it was not affected in LINC01096-MUT group. Moreover, the expression of miR-3130-3p was measured in TNBC tissues. Results showed that miR-3130-3p expression was aberrantly decreased in TNBC tissues, and it was negatively correlated with LINC01096 level (Figure 4A and 4B). Meanwhile, the low expression of miR-3130-3p was associated with tumor size, lymph node metastasis, and TNM stage of patients (Table II). Additionally, the data of qRT-PCR showed that the expression of miR-3130-3p was remarkably down-regulated in T47-D and BT-549 cells compared with that in MCF-10A cells (Figure 4C). Also, the abundance of miR-3130-3p in T47-D and BT-549 cells was significantly decreased by LINC01096 overexpression (Figure 4D) and increased by LINC01096 knockdown (Figure 4E). These data showed that LINC01096 was a miR-3130-3p sponge in TNBC cells.

MiR-3130-3p is Involved in LINC01096-Mediated Regulation of TNBC Progression

The role of miR-3130-3p was investigated in TNBC cells by overexpressing its abundance, which was confirmed by qRT-PCR in Figure 5A. Furthermore, the overexpression of miR-3130-

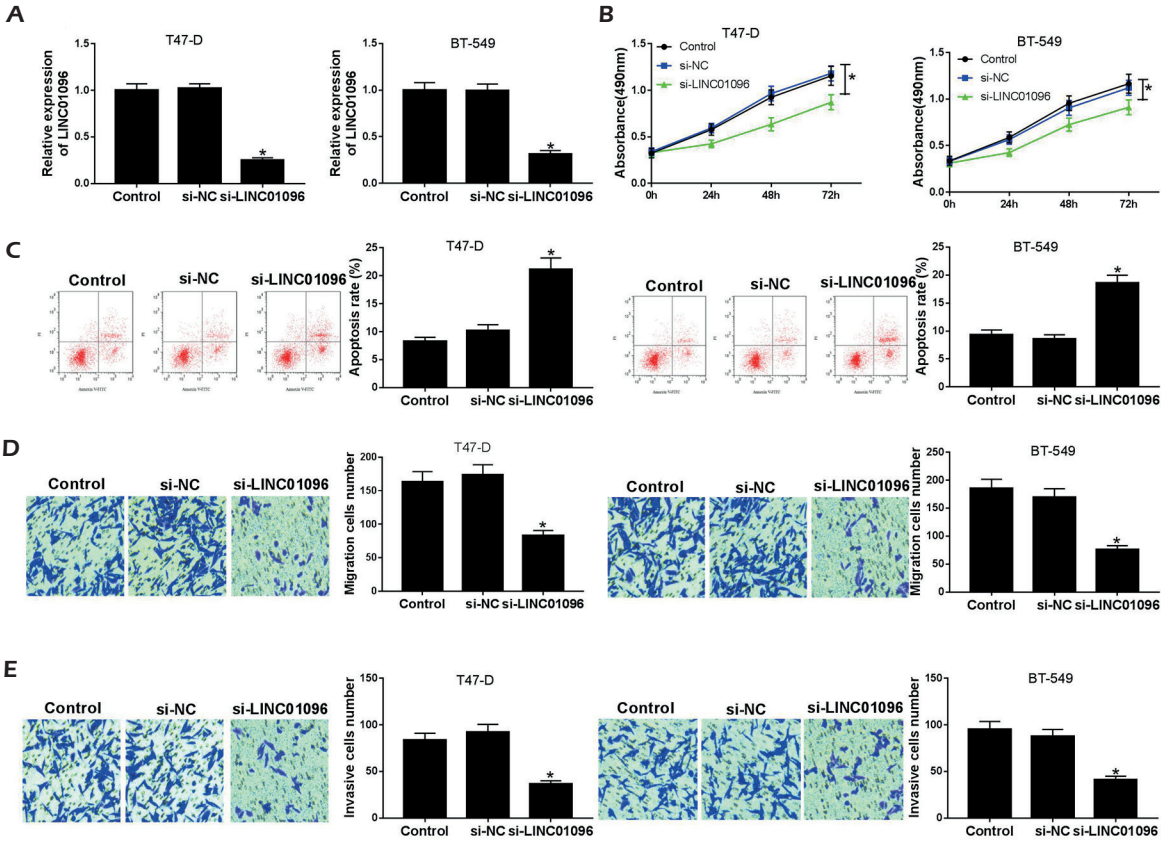

Figure 2. Knockdown of LINC01096 inhibits progression of TNBC cells. ***A,*** Abundance of LINC01096 in T47-D and BT-549 cells was measured after transfection of si- LINC01096 or si-NC. Cell viability (***B***), apoptosis (***C***), migration (100X) (***D***) and invasion (***E***) of T47-D and BT-549 cells transfected with si- LINC01096 or si-NC were determined by MTT, flow cytometry and trans-well assays, respectively (100X). Control is the non-transfected group. *$p<0.05$ compared with si-NC group.

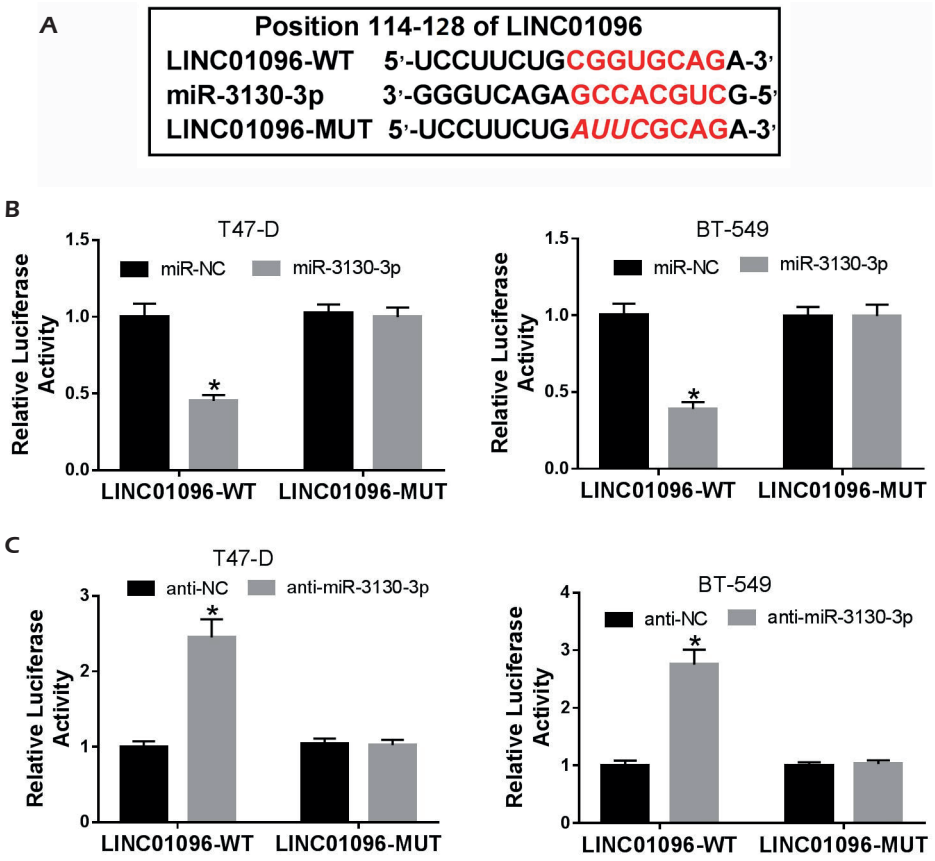

Figure 3. LINC01096 is a decoy of miR-3130-3p. ***A,*** Putative binding sites of LINC01096 and miR-3130-3p were predicted by DIANA tools. ***B,*** Luciferase activity was measured in T47-D and BT-549 cells co-transfected with LINC01096-WT or LINC01096-MUT and miR-3130-3p or miR-NC. ***C,*** Luciferase activity was measured in T47-D and BT-549 cells co-transfected with LINC01096-WT or LINC01096-MUT and anti-miR-3130-3p or anti-NC. *$p<0.05$ compared with miR-NC or anti-NC group.

3p led to significant reduction of cell viability in T47-D and BT-549 cells at 72 h (Figure 5B). In addition, the apoptosis of T47-D and BT-549 cells was notably induced by the addition of miR-3130-3p (Figure 5C). Moreover, as shown in Figure 5D and 5E, the up-regulation of miR-3130-3p in T47-D and BT-549 cells significantly repressed cell migration and invasion. To explore whether miR-3130-3p was required for LINC01096-mediated TNBC progression, T47-D and BT-549 cells were transfected with anti-NC, anti-miR-3130-3p, anti-miR-3130-3p, and si-NC or si-LINC01096. As shown in Figure 6A, the abundance of miR-3130-3p in the two cells was remarkably decreased by the transfection of anti-miR-3130-3p, which was restored by knocking down LINC01096. Besides, rescue experiments demonstrated that the deficiency of miR-3130-3p promoted theTNBC progression, revealed by the increase of viability, migration, and invasion, as well as reduction of apoptosis, which were attenuated via silencing LINC01096 (Figure 6B-6E). These results uncovered that LINC01096 regulated TNBC progression by sponging miR-3130-3p.

Discussion

We verified for the first time the carcinogenic role of LINC01096 in TNBC by detecting its effect on cell viability, apoptosis, migration, and invasion of TNBC cells. Moreover, we first confirmed the interaction between LINC01096 and

miR-3130-3p in TNBC cells. LncRNAs have been reported to exhibit pivotal roles in development, diagnosis, and treatment of TNBC[21,22]. Here we found that LINC01096 expression was enhanced in TNBC, which is also in agreement with the previous study[11]. However, the biological role of LINC01096 in TNBC remains poorly understood. Therefore, we postulated LINC01096 as a putative oncogenic lncRNA in TNBC. By loss-of-function studies, we showed that silencing LINC01096 suppressed TNBC progression, suggesting that LINC01096 could act as a promising target for TNBC treatment. LncRNAs could play essential roles in TNBC development by acting as sponge or competing endogenous RNA (ceRNA) of miRNAs[23]. For example, Zhang et al[24] reported that lncRNA human ovarian cancer-specific transcript 2 (HOST2) knockdown suppressed cell proliferation by regulating miR-let-7b and cyclin-dependent kinase 6 (CDK6) in TNBC. Fu et al[25] suggested that lncRNA MIR503HG repressed cell migration and invasion of TNBC by regulating olfactomedin 4 (OLFM4) via sponging miR-103. To figure out whether LINC01096 regulated TNBC progression by miRNAs, we used DIANA tools to search the potential targets and confirmed miR-3130-3p as a target of LINC01096 with luciferase reporter assay. The previous work demonstrated that miR-3130-3p could inhibit proliferation, migration, and invasion of endometrial cancer cells[19]. This indicated that miR-3130-3p might be a tumor suppressor in gynecological malignancies, but there is no report regarding miR-3130-3p in TNBC. We examined that miR-3130-3p was down-regulated in TNBC tissues and cells. By inhibiting cell viability, migration, and invasion and promoting apoptosis, miR-3130-3p might be a therapeutic target for TNBC treatment. Furthermore, rescue experiments uncovered that LINC01096 suppressed TBC progression by sponging miR-3130-3p. As far as we know, this study was the first to disclose this target association between LINC01096 and miR-3130-3p in TNBC. A target of miRNA wants to better understand the mechanism in this report. We used miRTarBase database, which predicted that TNFAIP2 might be a functional target of miR-3130-3p, and it has been reported as an oncogene in breast cancer[26]. Hence, we speculated that TNFAIP2 might be responsible for LINC01906-mediated TNBC progression by miR-3130-3p, which should be further confirmed. Moreover, previous researches[27,28] suggested that lncRNAs play important roles in regulating phenotype of cancer stem cells, which

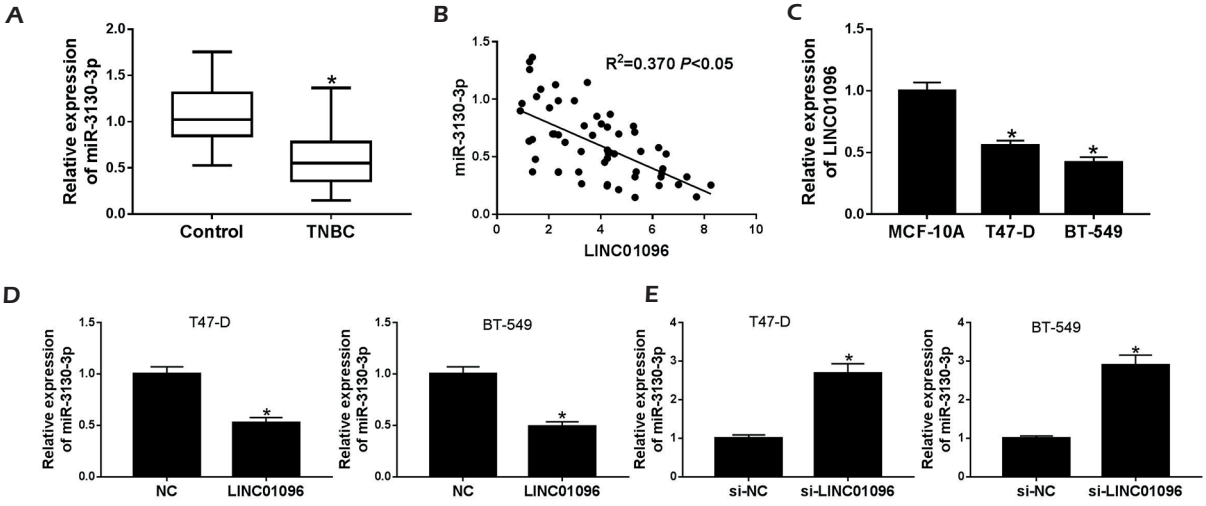

Figure 4. MiR-3130-3p expression is down-regulated and negatively regulated by LINC01096 in TNBC. ***A,*** Expression of miR-3130-3p was detected in TNBC tissues (n=60) and normal samples (n=53). ***B,*** Liner association between the levels of miR-3130-3p and LINC01096 in TNBC tissues was investigated. ***C,*** Level of miR-3130-3p was examined in TNBC cells and control (MCF-10A) by qRT-PCR. ***D,*** Expression of miR-3130-3p was measured in T47-D and BT-549 cells transfected with LINC01096 or NC. ***E,*** Level of miR-3130-3p was detected in T47-D and BT-549 cells transfected with si-LINC01096 or si-NC. *$p<0.05$ compared with matched control group.

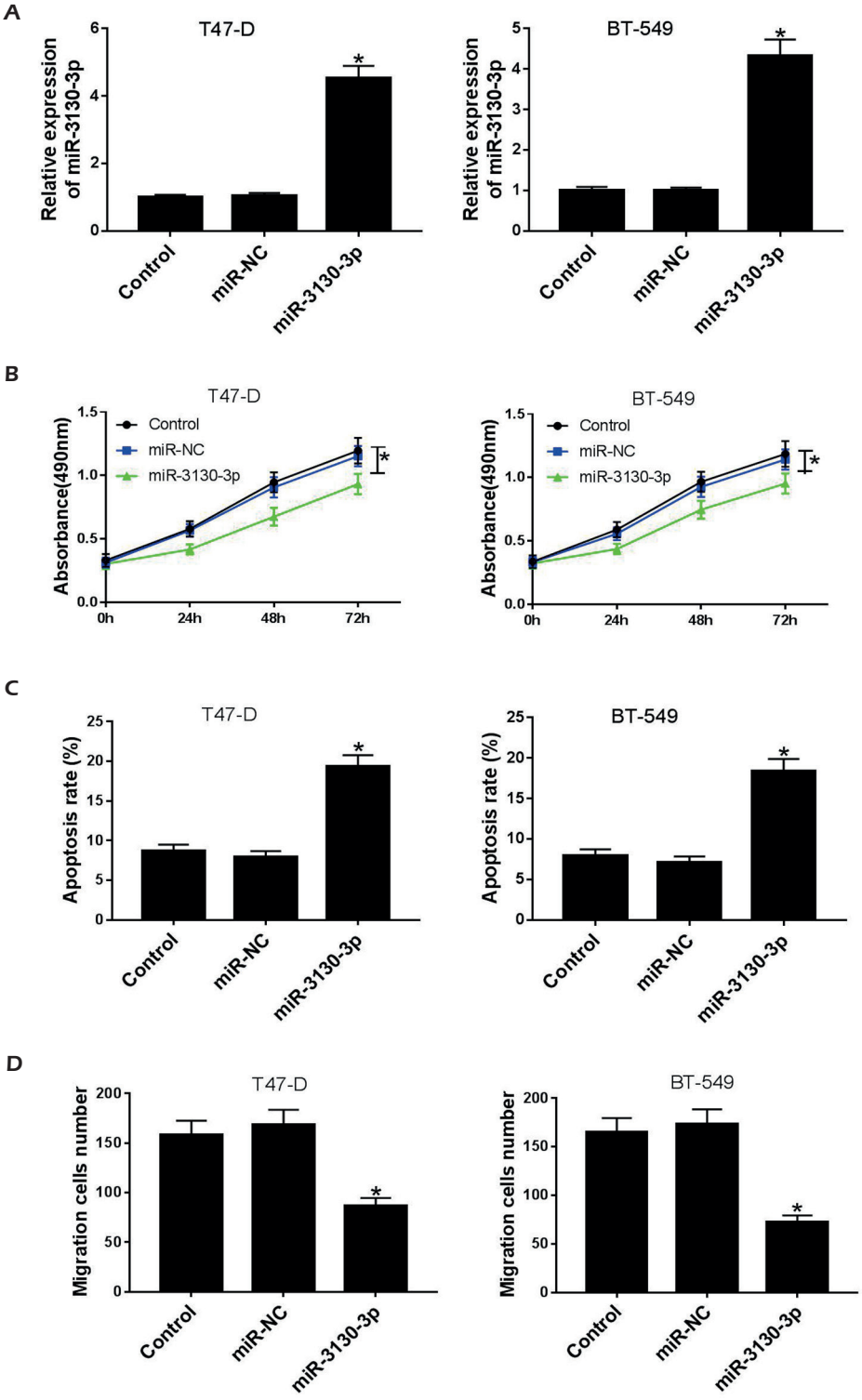

Figure 5. Overexpression of miR-3130-3p suppresses progression of TNBC cells. ***A,*** Abundance of miR-3130-3p in T47-D and BT-549 cells was detected after transfection of miR-3130-3p or miR-NC. Cell viability (***B***), apoptosis (***C***), migration (***D***) and invasion (***E***) of T47-D and BT-549 cells transfected with miR-3130-3p or miR-NC were analyzed by MTT, flow cytometry and trans-well assays, respectively. Control is the non-transfected group. *$p<0.05$ compared with miR-NC group.

Figure continued

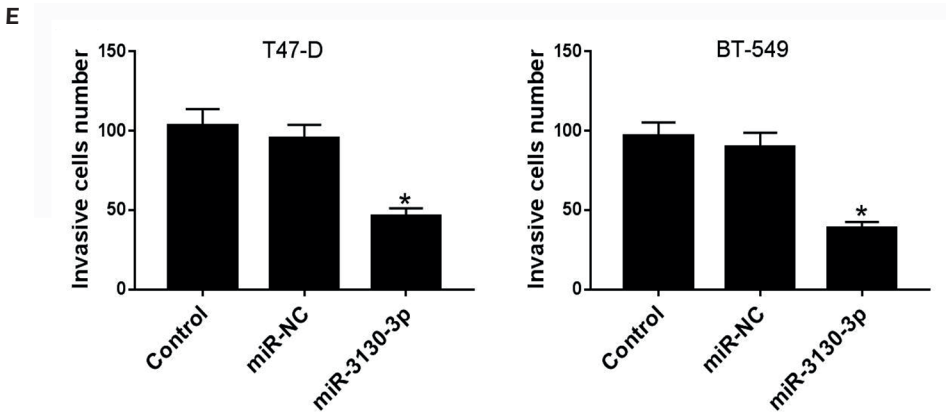

Figure 5. *(Continued).* (***E***) of T47-D and BT-549 cells transfected with miR-3130-3p or miR-NC were analyzed by MTT, flow cytometry and trans-well assays, respectively. Control is the non-transfected group. **p*<0.05 compared with miR-NC group.

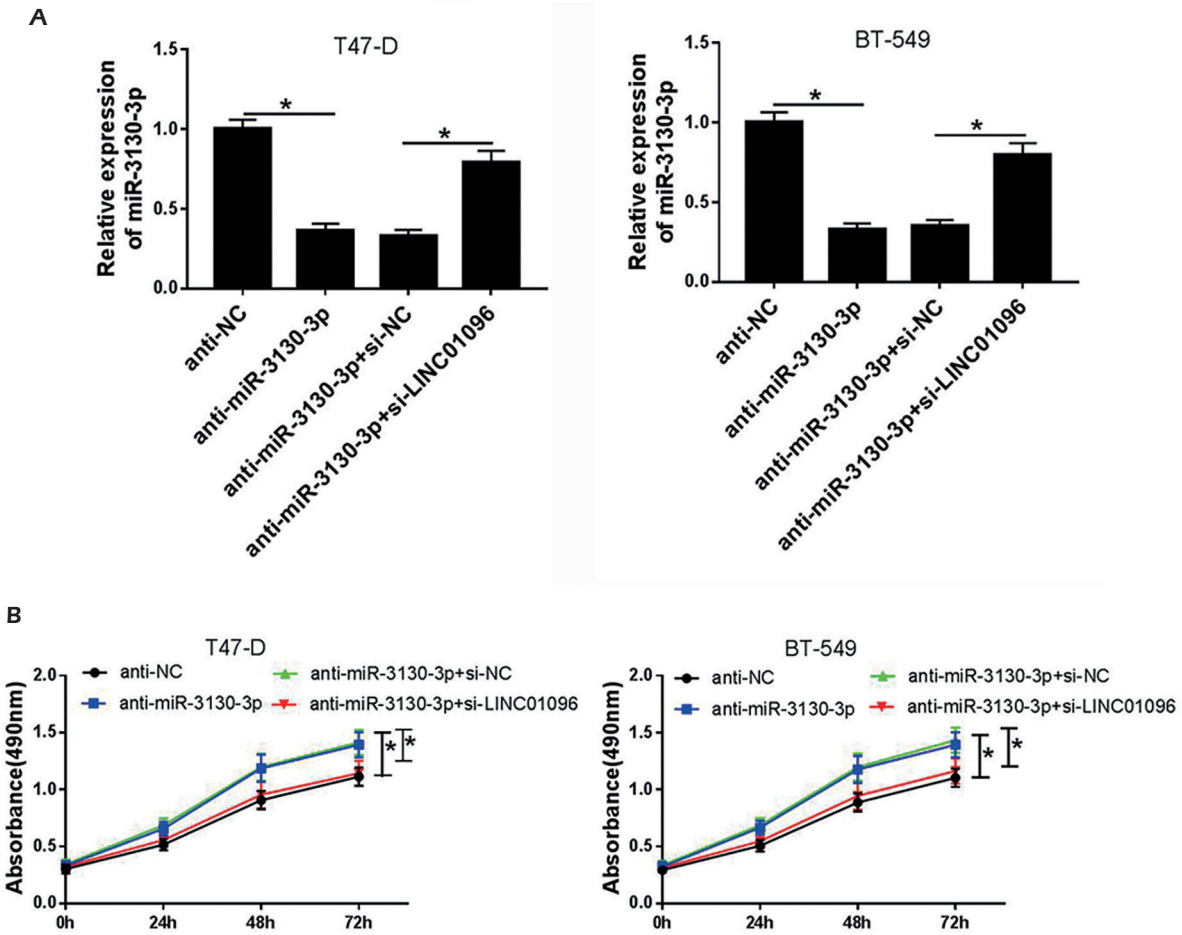

Figure 6. LINC01096 mediates progression of TNBC cells by regulating miR-3130-3p. ***A,*** Abundance of miR-3130-3p in T47-D and BT-549 cells was examined after transfection of anti-NC, anti-miR-3130-3p, anti-miR-3130-3p and si-NC or si-LINC01096. Cell viability (***B***), apoptosis (***C***), migration (***D***) and invasion (***E***) of T47-D and BT-549 cells transfected with anti-NC, anti-miR-3130-3p, anti-miR-3130-3p and si-NC or si-LINC01096 were assessed by MTT, flow cytometry and transwell assays, respectively. **p*<0.05 compared with matched group.

Figure continued

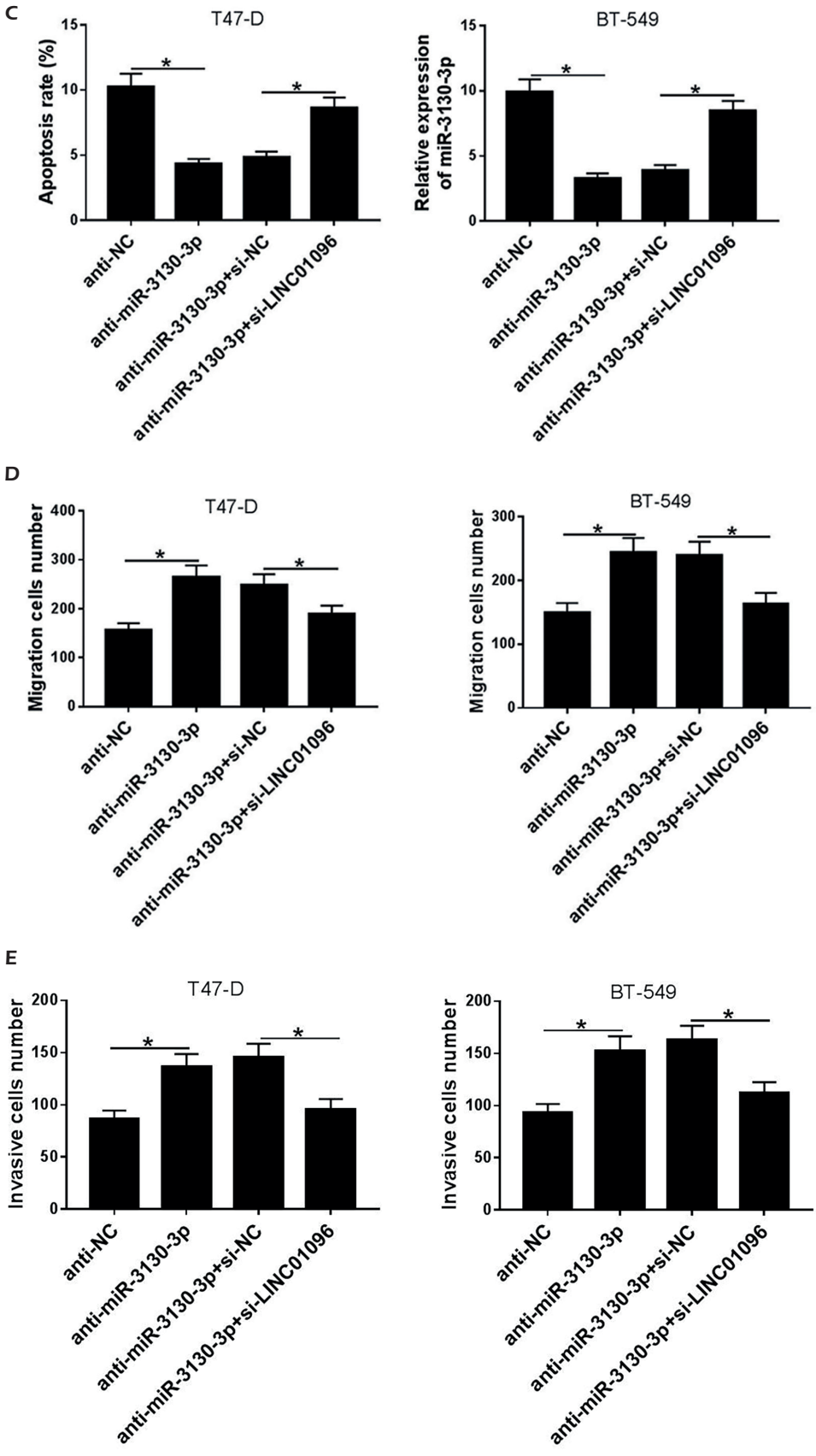

Figure 6. *(Continued).* Apoptosis (***C***), migration (***D***) and invasion (***E***) of T47-D and BT-549 cells transfected with anti-NC, anti-miR-3130-3p, anti-miR-3130-3p and si-NC or si-LINC01096 were assessed by MTT, flow cytometry and transwell assays, respectively. *$p<0.05$ compared with matched group.

contribute to malignancies of cancers, including TNBC. Hence, in the next future, it is necessary to investigate whether LINC01096 could regulate stemness in TNBC.

Conclusions

Silencing LINC01096 suppressed the progression of TNBC *in vitro*, possibly by increasing miR-3130-3p. This research provided a new mechanism for the pathogenesis of TNBC and indicated a promising target for TNBC treatment.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Declarations Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University. The methods used in this study were performed in accordance with relevant guidelines and regulations.

Consent for Publication

All authors of this work consent to the publication of this manuscript.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

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