Sp1-activated Long Noncoding RNA NCK1-AS1 Facilitates Cell Growth of Breast Cancer via Sponging miR-361-5p

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Abstract

Background: Breast cancer (BC) is one of the most lethal and malignant tumors in the world. Accumulating documents have illuminated the vital roles of long noncoding RNAs (lncRNAs) which are closely related to the progression of human cancers, including BC. LncRNA NCK1-AS1 was reported to be an oncogene in some cancers; nevertheless, its functionality is not well elucidated in BC.

Methods: NCK1-AS1 expression status was detected in human breast cancer tissues and cell lines by the means of RT-qPCR. The impacts of NCK1-AS1 deficiency on breast cancer cell stemness, viability, proliferation, migration, invasion, and apoptosis were measured in vitro via sphere formation assay, western blot analysis, CCK-8, colony formation, transwell, TUNEL and flow cytometry analysis. Furthermore, luciferase reporter assay, RIP and ChIP were used to verify the mutual effects between molecules.

Results: In this study, lncRNA NCK1-AS1 was verified to be elevated in BC tissues and cells. In addition, NCK1-AS1 silencing hampered BC cell growth by inhibiting cell stemness, viability, proliferation, migration and invasion as well as promoting cell apoptosis. Moreover, NCK1-AS1 functioned as a molecular sponge for miR-361-5p and modulated SP1 expression. Rescue experiments signified that SP1 overexpression restored NCK1-AS1 silencing-mediated suppression on BC cell growth. Importantly, SP1 was uncovered to bind with NCK1-AS1 promoter, suggesting a positive feedback loop of NCK1-AS1/miR-361-5p/SP1 in BC cells.

Conclusion: Taken together, these findings showed for the first time that NCK1-AS1 served as a carcinogenic gene in BC cells via combining with miR-361-5p to upregulate SP1.

Introduction

Breast cancer (BC) is a kind of heterogeneous neoplasm, and both genetic and epigenetic variations could conduce to its occurrence and development (1). It is also one of the commonly diagnosed aggressive malignancies in female worldwide. According to the Global Cancer Statistics 2018, over 2,088,849 new cases of BC were diagnosed and ultimately generated approximately 626,679 deaths throughout world (2). Clinically, tremendous advances have been achieved in the diagnosis and treatment of BC for recent decades, but the relapse and metastasis remain the leading causes of death in patients (3). It is known to all that the potential molecular mechanisms of BC are complicated, which may contain the alterations of particular genomic regions (4). Therefore, it has become a crucial issue and challenge to illuminate the molecular regulatory mechanisms underlying BC development and progression to distinguish molecular biomarkers for BC patients.

Long non-coding RNAs (lncRNAs) are a class of RNAs with more than 200 nucleotides and could not translate into proteins (5). Previously, lncRNAs were deemed as useless RNAs and consequently received little attention. Nowadays, emerging evidence have indicated the pivotal functions of lncRNAs in the biological processes of human tumors, such as cell differentiation (6), proliferation (7), cell cycle

distribution (8, 9) and apoptosis (10). In addition, an increasing magnitude of literatures have reported the dysregulated lncRNAs in BC, especially BDNF-AS, DSCAM-AS1, LOC645166 and HOST2. Based on different circumstances, lncRNA plays the roles of tumor suppressor or carcinogene in BC tumorigenesis and development. For example, linc-ROR accelerates the proliferative capacity of BC cells via modulating miR-194-3p-mediated MECP2 upregulation (11). In addition, silenced lncRNA SBF2-AS1 represses tumorigenesis of BC by decoying miR-143 and inhibiting RRS1 (12). Similarly, it is reported that YY1-activited LINC00673 enhances cell proliferation of BC through mediating miR-515-5p/MARK4/Hippo signaling (13). All these discoveries suggested that some lncRNAs are expressed individually in BC, implicating in the initiation and development of BC through competitively combining with miRNAs and affect downstream target molecules. Hence, identifying the specific lncRNAs related to BC for advancing therapeutic treatment is essential. Recently, lncRNA NCK1-AS1 is highlighted for its contributing effects on the progression of several human cancers. Knockdown of lncRNA NCK1-AS1 suppresses nasopharyngeal carcinoma cell metastasis via elevating miR-135a expression (14). Through NCK1-AS1/miR-6857/CDK1 pathway, NCK1-AS1 induces cell cycle progression and facilitates cell proliferation in cervical cancer (15). Nevertheless, the function and molecular regulatory mechanism of lncRNA NCK1-AS1 in BC were largely obscure.

In this investigation, we sought to study the functional role and potential mechanism of NCK1-AS1 in BC. Our results manifested that NCK1-AS1 accelerates BC cell growth by modulating miR-361-5p/SP1 axis.

Materials And Methods

Clinical specimens

Thirty paired BC tissues and adjacent normal breast tissues were procured from patients undergoing surgical excision at The First Hospital of Jilin University (Jilin, China). All patients did not receive any preoperative treatments for cancer. The collected tissue samples were promptly frozen with liquid nitrogen and preserved at -80°C for the following experiments. Written informed consents were signed by all enrolled patients. This research was approved by the Ethics Committee of The First Hospital of Jilin University (Jilin, China).

Cell lines

BC cell lines (HCC1937, BT-20, MCF-7, and MDB-MB-436) and a human normal breast cell line (MCF-10A) were commercially obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% FBS at 37°C in the presence of 5% CO_2.

Cell transfection

Particular shRNAs against NCK1-AS1 (sh-NCK1-AS1#1 and sh-NCK1-AS1#2) or SP1 (sh-SP1#1 and sh-SP1#2) and their corresponding negative controls (sh-NC) were acquired from GenePharma (Shanghai,

China). In addition, GenePharma produced miR-361-5p mimics, NC mimics, and NC inhibitor. Above plasmids were respectively transfected into BT-20 or MCF-7 cells for 48 h. Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, USA).

RT-qPCR analysis

Trizol reagent (Invitrogen, USA) was utilized to isolate total RNA from tissues or cells according to the manufacturer's instructions. PrimeScript™RT Reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was adopted to conduct reverse transcription. The expressions of NCK-AS1, miR-361-5p and SP1 were examined by RT-qPCR with SYBR Green (Takara Bio, Inc., Dalian, China). GAPDH functioned as an internal reference. The relative expressions were estimated by the $2^{-\Delta\Delta Ct}$ method.

Sphere formation assay

BT-20 and MCF-7 CSCs (2×10^3/well) were incubated in ultra-low adhesion plates (Corning Incorporated, USA) with serum-free medium including 100 μg/mL human bFGF, 20 μg/mL insulin and 10 ng/mL human EGF. Two weeks later, the spheres were stained and counted by an inverted microscope (Nikon TE2000-U).

Western blot analysis

Total protein was extracted from cells utilizating a Total Protein Extraction kit (Nanjing KeyGen Biotech Co., Ltd., China). Protein BCA kit was conducted to measure the concentration of proteins. Then, the proteins were electrophoresed on SDS-PAGE and transferred onto PVDF membranes (Millipore). Afterwards, the membranes were sealed in 5% non-fat milk, and incubated with primary antibodies at 4°C all night. The next day, appropriate secondary antibodies were added and incubated for 1 hour at room temperature (RT). Protein bands were visualized with ECL detection system (Thermo Fisher, Rockford, IL, USA). The GAPDH antibody was used as the internal reference. The primary antibodies were presented as follows: Sox-2 (ab97959); Oct-4 (ab19857); Nanog (ab80892); GAPDH (ab8245).

Cell counting kit-8 (CCK-8) assay

CCK-8 assay was performed to check cell viability in line with the supplier's instructions. Briefly, transfected BT-20 or MCF-7 cells were planted into 96-well plates (3×10^3 cells/well) and cultivated for 0, 24, 48 and 72 h. Each well was added with CCK-8 reagent and incubated for 2 h at 37°C. Finally, the absorbance (OD) at 450 nm was confirmed.

Colony formation assay

For colony formation assay, BT-20, or MCF-7 cells were harvested and planted into 6-well plates (1×10^3 cells/well). Two weeks later, the cells were immobilized, and dyed with 0.1% crystal violet. The colonies were counted by a gel documentation system (Bio-Rad).

Transswell assays

Cell migratory and invasive abilities were examined by transwell assays. For invasion assay, BC cells (3×10^4 cells/well) were seeded in the upper chamber coated with Matrigel (Corning Incorporated). The lower chamber was added with 600 μl DMEM comprising 10% FBS. 48 hours later, the invaded cells were immobilized using ethanol, stained in crystal violet, and counted under a microscope. The migration assay was conducted the same steps without coating the membranes with Matrigel.

Fluorescence in situ hybridization (FISH) assay

FISH assay was applied to confirm the location of NCK1-AS1 in BT-20 or MCF-7 cells. The NCK1-AS1 probe was obtained from RiboBio, Co., Ltd. (Guangzhou, China). BT-20 or MCF-7 cells were cultured with NCK1-AS1 probe overnight at 37 °C, and then blocked by 3% BSA. The nucleus was stained using DAPI. The fluorescence microscope (Olympus Optical Co., Ltd., Japan) was used to capture the images under 5 different visual fields.

RNA pull-down assay

GenePharma constructed NCK1-AS1 biotin probe and NCK1-AS1 no-biotin probe. Then the probes were cultivated with streptavidin magnetic beads (BioMag, Shanghai, China) for 1 h at RT. Cell lysates of BT-20 and MCF-7 cells were cultured with probe-coated beads at 4°C. Finally, the pulled-down RNAs were eluted and the expressions of microRNAs (miRNAs) were detected using RT-qPCR.

Luciferase reporter assay

To construct the pmirGLO-NCK1-AS1 WT/Mut and pmirGLO-SP1 WT/Mut vector, the sequences comprising the wild-type or mutant binding sites targeting miR-361-5p of NCK1-AS1 and SP1 were subcloned into the pmirGLO luciferase vector (Promega, USA). NCK1-AS1 WT/Mut vector or SP1 WT/Mut were transfected with miR-361-5p mimics, or respective controls (NC mimics) in BT-20 and MCF-7 cells. Moreover, the sequences containing NCK1-AS1 promoter region was cloned into the pGL3 luciferase vector (Promega, USA). The vector was respectively transfected into BT-20 and MCF-7 cells with sh-SP1#1, sh-SP1#2, or sh-NC. Luciferase activity was measured using the Luciferase Reporter Assay System (Promega, USA) 48 hours later.

RNA immunoprecipitation (RIP)

The EZ-Magna RIP kit (Millipore, USA) was used to conduct RIP assay. After centrifugation and collection, BT-20 and MCF-7 cells were lysed in RIP lysis buffer (Thermo Fisher Scientific). Antibody against Ago2 (Millipore) or negative control IgG (Millipore) was conjugated with magnetic beads. The enrichment in immunoprecipitation was detected finally.

Chromatin immunoprecipitation (ChIP)

The combination between SP1 and NCK1-AS1 promoter was examined using ChIP assay. The EZ ChIP Chromatin Immunoprecipitation Kit (Millipore, USA) was adopted for ChIP assay as per the manufacturer's directions. Briefly speaking, anti-SP1 antibody (Millipore, USA) was applied to immunoprecipitate the chromatin, and IgG served as negative control. Finally, coprecipitated DNA was purified and the expressions of target genes were examined using RT-qPCR.

Statistical analysis

SPSS 20.0 Software (SPSS Inc., Chicago, IL) was used to analyze data. All results are displayed as the mean ± SD by at least three independent assays. Data were calculated under Student's t test (two groups) or one-way ANOVA (multiple groups). Statistical significance was considered when p-value was < 0.05.

Results

LncRNA NCK1-AS1 expression is elevated in breast cancer tissues and cells

To confirm the expression profile of NCK1-AS1 in BC tissues and adjacent normal tissues, we adopted RT-qPCR analysis and the results presented that NCK1-AS1 expression was highly expressed in BC tumor tissues (n=30) compared with the corresponding normal tissues (n=30) (Figure 1A). Additionally, compared with human normal breast epithelial cell line (MCF-10A), NCK1-AS1 expression was detected to be elevated in BC cell lines (HCC1937, BT-20, MCF-7, and MDB-MB-436), especially in BT-20 and MCF-7 cells (Figure 1B). Consequently, BT-20 and MCF-7 cells were chosen for further experiments. Overall, NCK1-AS1 expression was highly expressed in BC tissues and cells, suggesting that NCK1-AS1 may take a part in BC cell development.

NCK1-AS1 silencing inhibits BC cell growth

The function of NCK1-AS1 was further explored and shRNAs targeted NCK1-AS1 (sh-NCK1-AS1#1 and sh-NCK1-AS1#2) were transfected into BT-20 and MCF-7 cells to knock down NCK1-AS1 expression. As shown in Figure 2A, sh-NCK1-AS1#1/2 successfully decreased NCK1-AS1 expression in BC cells. Then, a series of loss-of-functional assays were conducted. Sphere formation assay depicted that the number of spheroids derived from BT-20 and MCF-7 cells was lessened upon NCK1-AS1 silencing (Figure 2B). Results from western blot analysis demonstrated that NCK1-AS1 deficiency reduced the expressions of proteins (Sox-2, Oct-4 and Nanog) related to stemness (Figure 2C). The influence of NCK1-AS1 depletion on the viability and proliferation of BC cells were subsequently checked by CCK-8 and colony formation assays. As a result, silenced NCK1-AS1 significantly hindered the viability of BC cells (Figure 2D). We also observed the reduced number of colonies upon NCK1-AS1 deficiency (Figure 2E). Transwell assays were then carried out to examine the migratory and invasive abilities of BC cells, and the results illuminated that BC cell migration and invasion were markedly restrained with the transfection of sh-NCK1-AS1#1/2 (Figure 2F-G). In conclusion, NCK1-AS1 knockdown inhibited BC cell growth.

NCK1-AS1 acts as a molecular sponge for miR-361-5p

LncRNAs, especially those mainly distributed in cytoplasm, have been reported to exert regulatory roles in various cancers with ceRNA network. Therefore, we carried out FISH assay to determine the cellular location of NCK1-AS1 in BT-20 and MCF-7 cells. The data manifested that NCK1-AS1 was preferentially located in the cytoplasm, suggesting it may exert regulatory function at posttranscription (Figure 3A). Hence, we conjectured that NCK1-AS1 might serve as a ceRNA to bind with specific miRNAs to exert regulatory functions. To validate this hypothesis, we searched the potential miRNAs for NCK1-AS1 and screened out 8 miRNAs via starBase website. It was revealed through RNA pull-down assay that miR-361-5p showed the most enrichment in both the BT-20 and MCF-7 cells (Figure 3B). Importantly, miR-361-5p expression was discovered to be downregulated in BC cells (Figure 3C). To verify whether there was an interplay between NCK1-AS1 and miR-361-5p, binding sequences were predicted, and luciferase reporter assay was employed (Figure 3D). The results in Figure 3E illuminated that only the luciferase activity of NCK1-AS1-WT vector was impaired by overexpression of miR-361-5p while that of NCK1-AS1-Mut vector had no response to miR-361-5p mimics. RIP assay illuminated that both NCK1-AS1 and miR-361-5p showed high enrichment in Ago2 group (Figures 3F). All these findings indicated that NCK1-AS1 served as a molecular sponge for miR-361-5p in BC cells.

SP1 serves as the downstream target of miR-361-5p

To further support ceRNA hypothesis, 23 downstream genes of miR-361-5p were screened through microT, PITA, PicTar and TargetScan, (Figure 4A). Results from RT-qPCR analysis described that miR-361-5p significantly influenced the expressions of SP1, MYCBP, PDE4B, and RHOA in MCF-7 cells. While in BT-20 cells, SP1 expression was weakened by overexpression of miR-361-5p among these four mRNAs (Figure 4B). Besides, the expression of SP1 was notably elevated in all BC cells (Figure 4C). We then measured the impacts of miR-361-5p overexpression on SP1 expression. RT-qPCR revealed the markedly decreased SP1 expression level in miR-361-5p overexpressed BC cells (Figure 4D). The predicted binding sites were exhibited in Figure 4E. Subsequent luciferase reporter assay manifested that miR-361-5p overexpression dramatically cut down the luciferase activity of SP1-WT vector, but not SP1-Mut vector in BT-20 and MCF-7 cells (Figure 4E). Additionally, NCK1-AS1, miR-361-5p and SP1 were abundantly enrichment in RNA-induced silencing complex (RISC), which was examined by RIP assay (Figure 4F). Overall, SP1 was the direct downstream target of miR-361-5p in BC cells.

NCK1-AS1/miR-361-5p/SP1 axis actuates BC cell growth

In the following experiments, we continued to explore whether NCK1-AS1 promoted BC cell growth through modulating SP1. After transfection, SP1 was overexpressed by pcDNA3.1/SP1 in BT-20 and MCF-7 cells (Figure 5A). Sphere formation assay demonstrated that the suppressive impact on spheroids number caused by NCK1-AS1 knockdown was regained by SP1 overexpression (Figure 5B). Likewise, the expressions of stemness associated proteins reduced by sh-NCK1-AS1#2 were recovered with the transfection of pcDNA3.1/SP1 (Figure 5C). By performing CCK-8 and colony formation assays, we found that SP1 overexpression reversed the inhibitory effect of NCK1-AS1 knockdown on BC cell viability and proliferation (Figure 5D-E). Moreover, transwell assays verified that NCK1-AS1 silencing-induced

suppression on cell migratory and invasive capacities was retrieved by pcDNA3.1/SP1 (Figure 5F-G). Thereby, NCK1-AS1 contributes to the malignant phenotype BC cells via regulating SP1.

Transcription factor SP1 targets NCK1-AS1 promoter region

Plenty of researches have indicated that transcription factors could bring about the aberrant expressions of lncRNAs in various carcinomas. Emerging documents indicated that SP1 plays the role of transcription factor in a serious of cancers, such as gastric cancer and cervical cancer (16-18). Therefore, we speculated whether SP1 could interact with lncRNA NCK1-AS1 promoter in BC cells. To validate this, UCSC (http://genome.ucsc.edu/) and JASPAR (http://jaspar.gener eg.net/) websites were adopted, and eight binding sites were uncovered between NCK1-AS1 promoter and SP1 (Figure 6A-B). Then, we detected the effect of SP1 knockdown on NCK1-AS1 expression and data presented that NCK1-AS1 expression was decreased in SP1-silenced cells (Figure 6C). The data from luciferase reporter assay signified that SP1 knockdown induced a decline on the luciferase activity of NCK1-AS1 promoter (Figure 6D). To identify the primary promoter region of NCK1-AS1, we constructed four non-overlapped fragments, presented as P1 (-1 to -499), P2 (-500 to -999), P3 (-1000 to -1499) and P4 (-1500 to -2000). ChIP assay unraveled that SP1 combined with P2 of NCK1-AS1 promoter (Figure 6E). Luciferase reporter assay further verified that luciferase activity of promoter-WT was increased in pcDNA3.1/SP1 transfected BC cells (Figure 6F). Therefore, results concluded that SP1 combined with NCK1-AS1 promoter.

Discussion

An increasing magnitude of documents have illustrated that lncRNAs exert vital functions in multiple malignancies by regulating extensive biological progressions, such as cell stemness, apoptosis, and tumor angiogenesis (19). In recent years, NCK1-AS1 was stressed for its dysregulated expression and carcinogenic function in several cancers like nasopharyngeal carcinoma (14), ovarian cancer (20) and cervical cancer (21); nonetheless, little is known about the biological function and molecular regulatory mechanism of NCK1-AS1 in BC. In our study, the expression of NCK1-AS1 was conspicuously upregulated in BC tissues and cells. In addition, NCK1-AS1 silencing impeded the malignant phenotype of BC cells via restraining cell stemness, viability, proliferation, migration, and invasion as well as accelerating cell apoptosis.

As another subgroup of noncoding RNAs, microRNAs (miRNAs) are verified to be important regulators with 21-25 nucleotides and in close correlation with the cellular processes of malignant tumors (22). Experimental research manifested that miRNAs could function as tumor suppressor or promoter to regulate various cancer progressions. It is worth noting that dysregulated miRNAs such as miR-383-5p (23), miR-4472 (24), and miR-532-5p (25), have been discovered in a variety of cancers, including BC. Mechanistically, lncRNA could play as a ceRNA to absorb downstream miRNAs and regulate biological processes of cancers. For instance, lncRNA SBF2-AS1 mediated FOXM1 upregulation promotes cell proliferation in cervical cancer by sequestering miR-361-5p (26). LncRNA MEG3 obstructed osteosarcoma cell growth by modifying miR-361-5p and FoxM1 (27). Nevertheless, the molecular mechanism of miR-

361-5p in BC are elusive. Our present investigation manifested that miR-361-5p was downregulated in BC cells and it combined with NCK1-AS1.

Emerging researches have reported that SP1 exerts the cancer-promoting function in the biological processes of cancer progressions. Additionally, SP1 was also received considerable concern for its another character as transcription factor carcinomas including BC. SP1-induced upregulation of lncRNA TINCR conduces to BC tumorigenesis (28). Via activating transcription factor SP1, the oncoprotein HBXIP elevates PDGFB expression to facilitate BC cell proliferation (29). Our investigation unveiled that SP1 was targeted by miR-361-5p. Moreover, restoration experiments illuminated that overexpression of SP1 countervailed the inhibitory effects of NCK1-AS1 deficiency on the malignant phenotype of BC cells. In addition, SP1 was validated to bind with NCK1-AS1 promoter, suggesting a positive feedback loop between NCK1-AS1 and SP1.

To summarize, our findings verified that NCK1-AS1 acted as an oncogene to regulate BC cell growth via sponging miR-361-5p to upregulate SP1. Further, SP1 was discovered to combine with NCK1-AS1 promoter, indicating a NCK1-AS1/miR-361-5p/SP1 feedback loop in BC. This discovery offered a theoretical basis for the exploration of treatment strategies for BC.

Conclusion

Overall, these findings showed for the first time that NCK1-AS1 served as a carcinogenic gene in BC cells via combining with miR-361-5p to upregulate SP1.

Declarations

Ethics approval and consent to participate

This research was approved by the Ethics Committee of The First Hospital of Jilin University (Jilin, China). Verbal informed consent was obtained from the patients for their anonymized information to be published in this article.

Consent for publication

Not applicable.

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We appreciate all participants in this work.

Competing interests

The authors declare that no conflict of interests exists.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Authors' contributions

Hongyao Jia and Di Wu conceived and designed the experiments. Hongyao Jia, Di Wu, and Zhiru Zhang carried out the experiments. Hongyao Jia and Sijie Li analyzed the data. Hongyao Jia, Di Wu and Sijie Li drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA: a cancer journal for clinicians. 2016;66(1):7-30.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394-424.
3. Xu Y, Lin X, Xu J, Jing H, Qin Y, et al. SULT1E1 inhibits cell proliferation and invasion by activating PPARgamma in breast cancer. J Cancer. 2018;9(6):1078-87.
4. A MB, R VS, J ME, A AO. Breast cancer biomarkers: risk assessment, diagnosis, prognosis, prediction of treatment efficacy and toxicity, and recurrence. Current pharmaceutical design. 2014;20(30):4879-98.
5. Nagano T, Fraser P. No-nonsense functions for long noncoding RNAs. Cell. 2011;145(2):178-81.
6. Gui X, Li H, Li T, Pu H, Lu D. Long Noncoding RNA CUDR Regulates HULC and β-Catenin to Govern Human Liver Stem Cell Malignant Differentiation. Molecular therapy : the journal of the American Society of Gene Therapy. 2015;23(12):1843-53.
7. Zhu X, Luo C, Bu F, Lin K, Zhu Z. Long non-coding RNA RP11-59H7.3 promotes cell proliferation and invasion metastasis in colorectal cancer by miR-139-5p/NOTCH1 axis. Aging. 2020;12.
8. Song EL, Xing L, Wang L, Song WT, Li DB, et al. LncRNA ADAMTS9-AS2 inhibits cell proliferation and decreases chemoresistance in clear cell renal cell carcinoma via the miR-27a-3p/FOXO1 axis. Aging. 2019;11(15):5705-25.
9. Zhou Y, Shi H, Du Y, Zhao G, Wang X, et al. lncRNA DLEU2 modulates cell proliferation and invasion of non-small cell lung cancer by regulating miR-30c-5p/SOX9 axis. Aging. 2019;11(18):7386-401.
10. Misawa A, Takayama K, Urano T, Inoue S. Androgen-induced Long Noncoding RNA (lncRNA) SOCS2-AS1 Promotes Cell Growth and Inhibits Apoptosis in Prostate Cancer Cells. The Journal of biological

chemistry. 2016;291(34):17861-80.

11. Zhou Q, Guo J, Huang W, Yu X, Xu C, et al. Linc-ROR promotes the progression of breast cancer and decreases the sensitivity to rapamycin through miR-194-3p targeting MECP2. Molecular oncology. 2020.

12. Xia W, Liu Y, Cheng T, Xu T, Dong M, et al. Down-regulated lncRNA SBF2-AS1 inhibits tumorigenesis and progression of breast cancer by sponging microRNA-143 and repressing RRS1. Journal of experimental & clinical cancer research : CR. 2020;39(1):18.

13. Qiao K, Ning S, Wan L, Wu H, Wang Q, et al. LINC00673 is activated by YY1 and promotes the proliferation of breast cancer cells via the miR-515-5p/MARK4/Hippo signaling pathway. Journal of experimental & clinical cancer research : CR. 2019;38(1):418.

14. Hu H, Li H, Feng X. Downregulation of lncRNA NCK1-AS1 Inhibits Cancer Cell Migration and Invasion in Nasopharyngeal Carcinoma by Upregulating miR-135a. Cancer Manag Res. 2019;11:10531-7.

15. Li H, Jia Y, Cheng J, Liu G, Song F. LncRNA NCK1-AS1 promotes proliferation and induces cell cycle progression by crosstalk NCK1-AS1/miR-6857/CDK1 pathway. Cell Death Dis. 2018;9(2):198.

16. Tian Y, Ma R, Sun Y, Liu H, Zhang H, et al. SP1-activated long noncoding RNA lncRNA GCMA functions as a competing endogenous RNA to promote tumor metastasis by sponging miR-124 and miR-34a in gastric cancer. Oncogene. 2020.

17. Wang ZQ, Cai Q, Hu L, He CY, Li JF, et al. Long noncoding RNA UCA1 induced by SP1 promotes cell proliferation via recruiting EZH2 and activating AKT pathway in gastric cancer. Cell death & disease. 2017;8(6):e2839.

18. Lin CL, Ying TH, Yang SF, Wang SW, Cheng SP, et al. Transcriptional Suppression of miR-7 by MTA2 Induces Sp1-Mediated KLK10 Expression and Metastasis of Cervical Cancer. Molecular therapy Nucleic acids. 2020;20:699-710.

19. Wu H, Liu B, Chen Z, Li G, Zhang Z. MSC-induced lncRNA HCP5 drove fatty acid oxidation through miR-3619-5p/AMPK/PGC1alpha/CEBPB axis to promote stemness and chemo-resistance of gastric cancer. Cell Death Dis. 2020;11(4):233.

20. Chang H, Li B, Zhang X, Meng X. NCK1-AS1 promotes NCK1 expression to facilitate tumorigenesis and chemo-resistance in ovarian cancer. Biochem Biophys Res Commun. 2020;522(2):292-9.

21. Huang L, Gan X, He L, Wang L, Yu J. Silencing of long non-coding RNA NCK1-AS1 inhibits cell proliferation and migration via inhibition of microRNA-134 in cervical cancer. Exp Ther Med. 2019;18(3):2314-22.

22. Osada H, Takahashi T. MicroRNAs in biological processes and carcinogenesis. Carcinogenesis. 2007;28(1):2-12.

23. Zhang J, Kong X, Shi Q, Zhao B. MicroRNA-383-5p acts as a potential prognostic biomarker and an inhibitor of tumor cell proliferation, migration, and invasion in breast cancer. Cancer biomarkers : section A of Disease markers. 2020;27(4):423-32.

24. Li Y, Wang YW, Chen X, Ma RR, Guo XY, et al. MicroRNA-4472 Promotes Tumor Proliferation and Aggressiveness in Breast Cancer by Targeting RGMA and Inducing EMT. Clinical breast cancer.

2020;20(2):e113-e26.

25. Huang L, Tang X, Shi X, Su L. miR-532-5p promotes breast cancer proliferation and migration by targeting RERG. Exp Ther Med. 2020;19(1):400-8.

26. Gao F, Feng J, Yao H, Li Y, Xi J, et al. LncRNA SBF2-AS1 promotes the progression of cervical cancer by regulating miR-361-5p/FOXM1 axis. Artif Cells Nanomed Biotechnol. 2019;47(1):776-82.

27. Shen B, Zhou N, Hu T, Zhao W, Wu D, et al. LncRNA MEG3 negatively modified osteosarcoma development through regulation of miR-361-5p and FoxM1. Journal of cellular physiology. 2019;234(8):13464-80.

28. Liu Y, Du Y, Hu X, Zhao L, Xia W. Up-regulation of ceRNA TINCR by SP1 contributes to tumorigenesis in breast cancer. BMC Cancer. 2018;18(1):367.

29. Zhang Y, Zhao Y, Li L, Shen Y, Cai X, et al. The oncoprotein HBXIP upregulates PDGFB via activating transcription factor Sp1 to promote the proliferation of breast cancer cells. Biochem Biophys Res Commun. 2013;434(2):305-10.
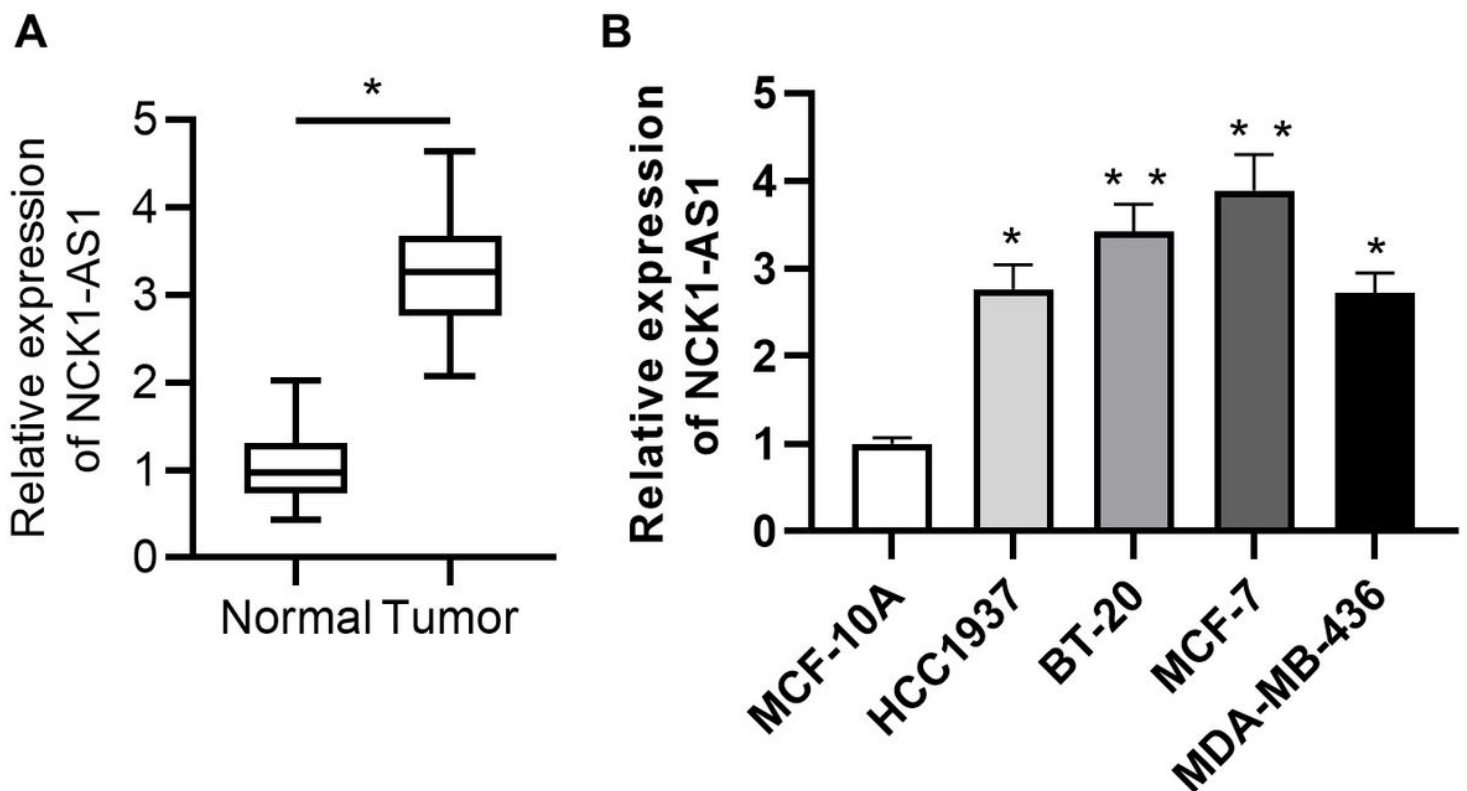
Figures

Figure 1

LncRNA NCK1-AS1 is highly expressed in BC tissues and cells. (A) NCK1-AS1 expressions in BC tissues and the corresponding normal tissues were examined using RT-qPCR analysis. (B) The expression of

NCK1-AS1 in BC cells (HCC1937, BT-20, MCF-7, and MDB-MB-436) and human normal breast cells (MCF-10A) was tested by the means of RT-qPCR. *P < 0.05, **P < 0.01.
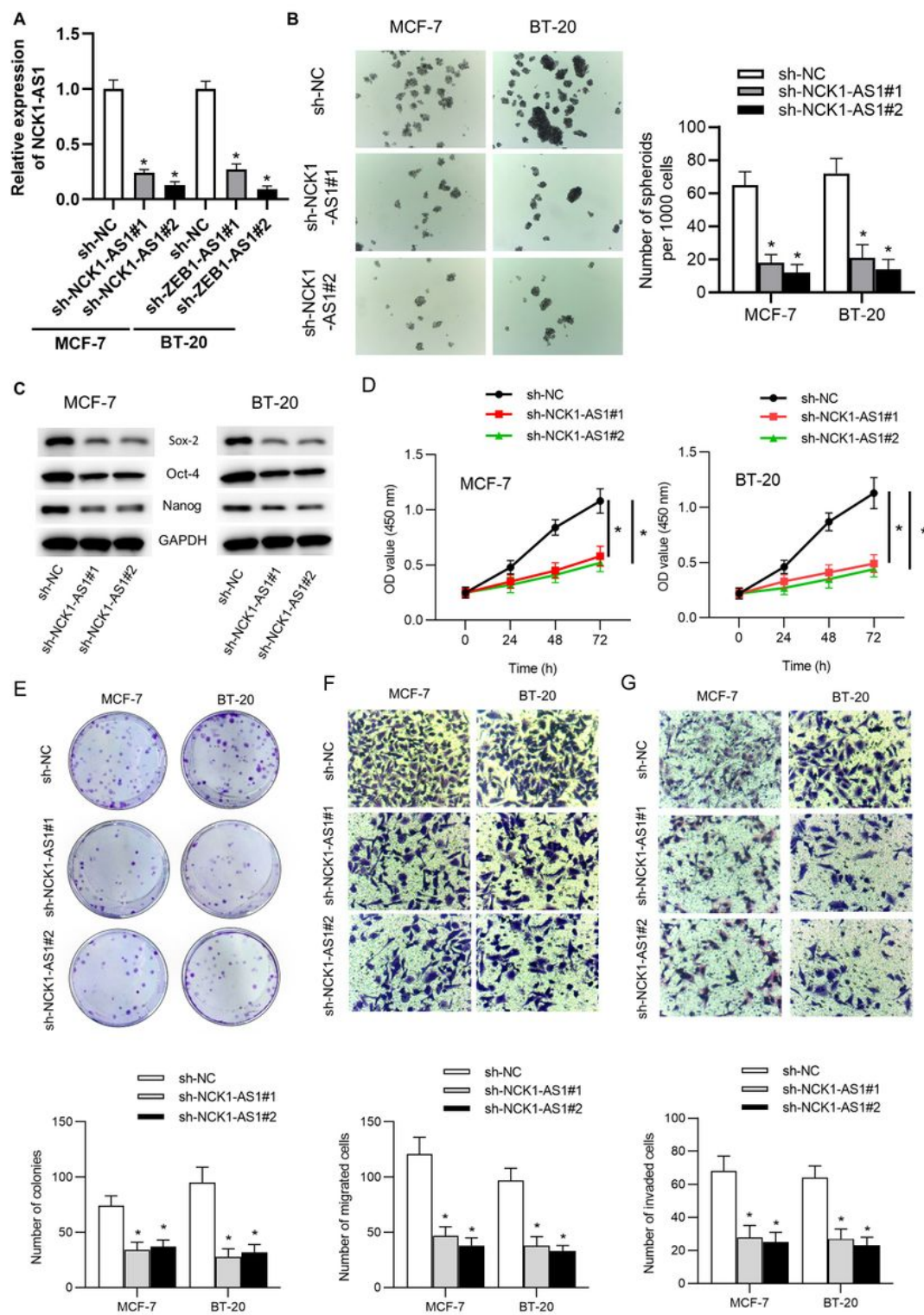

Figure 2

NCK1-AS1 contributes to BC cell stemness, proliferation, and metastasis. (A) RT-qPCR was conducted to examine the interference of NCK1-AS1 knockdown in BT-20 and MCF-7 cells. (B) The effect of NCK1-AS1 silencing on the number of spheroids in BT-20 and MCF-7 cells was examined by sphere formation assay.

(C) Western blot analysis was adopted to check the expressions of proteins (Sox-2, Oct-4 and Nanog) related to stemness in BC cells after transfection of sh-NCK1-AS1#1/2. (D) CCK-8 assay was utilized to confirm the viability and proliferation of sh-NCK1-AS1#1/2 transfected BC cells. (E) The proliferation of BC cells was examined in sh-NCK1-AS1#1/2 transfected BC cells by colony formation assay. (F-G) Cell migratory and invasive capacities were determined through transwell assays after sh-NCK1-AS1#1/2 transfection. *P < 0.05.

Figure 3

NCK1-AS1 serves as a molecular sponge for miR-361-5p. (A) The location of NCK1-AS1 in BT-20 and MCF-7 cells was evaluated using FISH assay. (B) The underlying miRNAs for NCK1-AS1 were obtained through searching starBase online website. RNA pull-down assay was used to assess the binding situation between miRNAs and NCK1-AS1. (C) MiR-361-5p expression profile in normal breast cells and BC cells. (D) Predicted binding sequences between NCK1-AS1 and miR-361-5p. (E-F) Luciferase reporter assay and RIP assay were carried out to detect the combination between NCK1-AS1 and miR-361-5p. *P < 0.05, **P < 0.01.
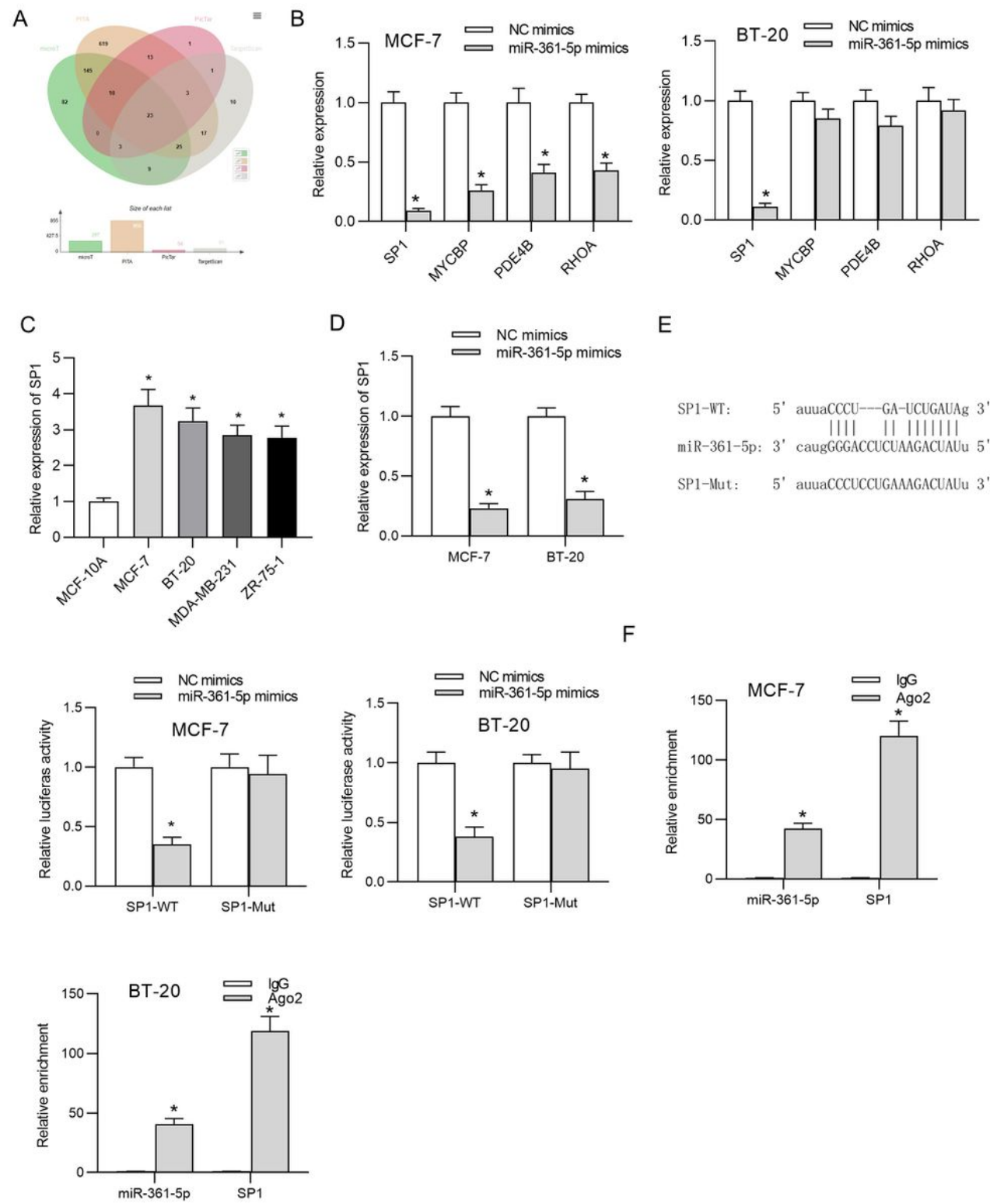
Figure 4

SP1 is the downstream target gene of miR-361-5p. (A) Twenty-three potential downstream targets of miR-361-5p were screened out through microT, PITA, PicTar and TargetScan and exhibited by Venn diagram. (B) RT-qPCR was conducted to examine the expressions of four potential mRNAs (SP1, MYCBP, PDE4B, RHOA) in BT-20 and MCF-7 cells downregulated miR-361-5p mimics. (C) The four mRNAs expressions in normal breast cells or BC cells was tested by RT-qPCR. (D) RT-qPCR was conducted to examine SP1 expression in cells after transfecting miR-361-5p mimics. (E) Predicted binding sites between miR-361-5p and SP1 was presented. The mutual effect between miR-361-5p and SP1 was determined by luciferase reporter assay. (F) RIP assay further detected the interplay between miR-361-5p and SP1. $*P < 0.05$.
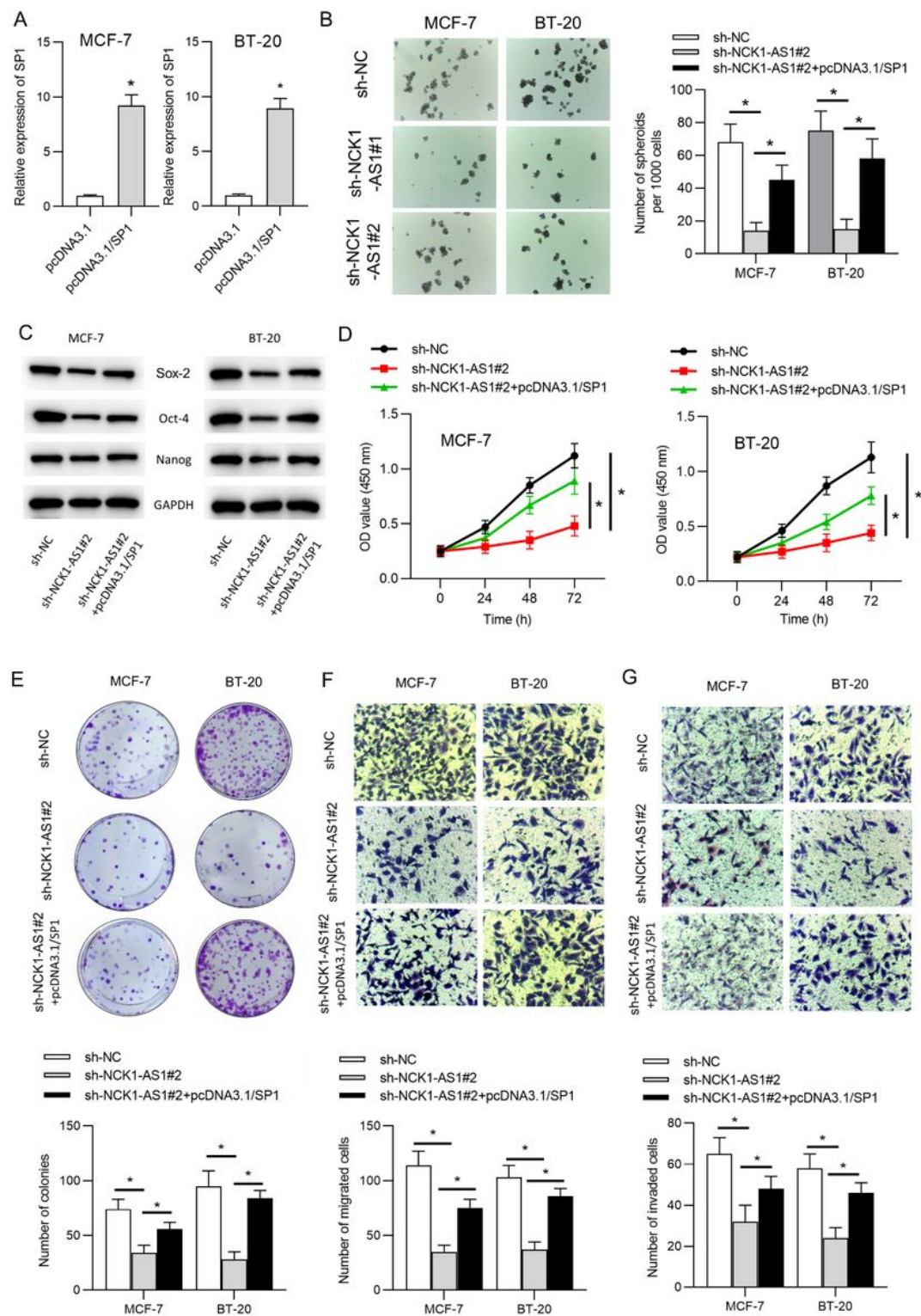
Figure 5

NCK1-AS1/miR-361-5p/SP1 axis actuates BC cell growth. (A) The overexpression efficiency of SP1 in BT-20 and MCF-7 cells was tested by RT-qPCR. (B) The number of spheroids derived from BT-20 and MCF-7 cells was evaluated after transfecting sh-NC, sh-NCK1-AS1#2 or sh-NCK1-AS1#2+pcDNA3.1/SP1 through sphere formation assay. (C) Western blot analysis was implemented to check the effect of transfecting the indicated plasmids on proteins expressions associated with stemness. (D-E) The viability and

proliferation of BC cells were estimated after transfecting sh-NC, sh-NCK1-AS1#2 or sh-NCK1-AS1#2+pcDNA3.1/SP1 through CCK-8 and colony formation assays. (F-G) Cell migration and invasion in BC cells transfected with sh-NC, sh-NCK1-AS1#2 or sh-NCK1-AS1#2+pcDNA3.1/SP1 were evaluated by transwell assays. *P < 0.05.
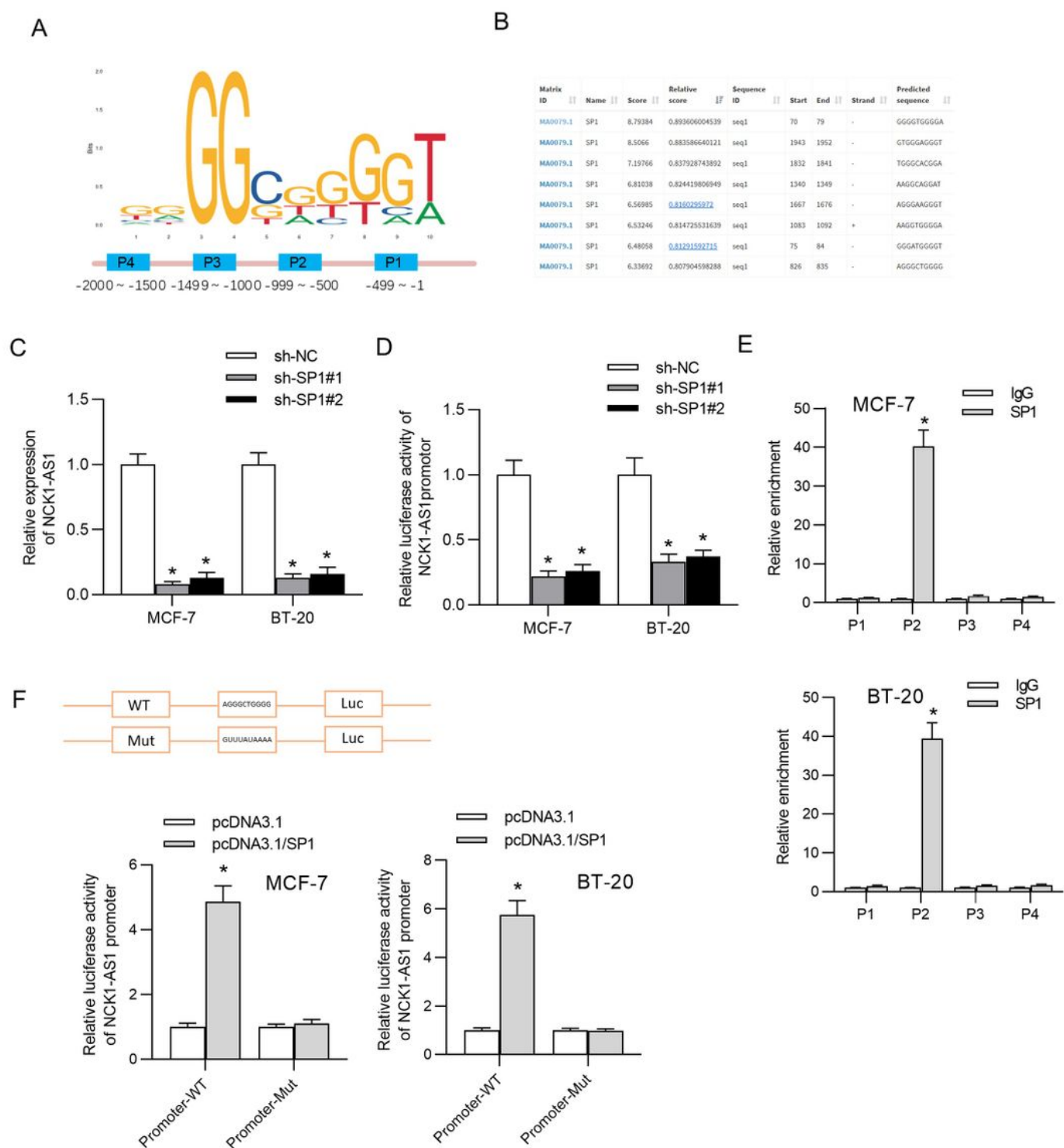

Figure 6

Transcription factor SP1 targets NCK1-AS1 promoter region. (A-B) SP1 DNA motif and binding sites in NCK1-AS1 promoter. (C) The influence of transfecting sh-SP1#1/2 on NCK1-AS1 expression level was calculated with RT-qPCR. (D) Luciferase reporter assay was applied to detect the luciferase activity of NCK1-AS1 promoter in cells transfected with sh-SP1#1/2. (E) ChIP assay were used to verify the interplay between SP1 and P2 in NCK1-AS1 promoter region. (F) Luciferase reporter assay was applied to further check whether SP1 bound to NCK1-AS1 promoter. *P < 0.05.