

# Oncogenic miR-20b-5p contributes malignant behavior via bidirectionally regulating CCND1 and E2F1 in breast cancer stem cells

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## Research article

**Keywords:** breast cancer, miR-20b-5p, CCND1, E2F1, cancer stem cells, bidirectional regulation

**Posted Date:** April 25th, 2020

**DOI:** <https://doi.org/10.21203/rs.3.rs-18259/v1>

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**Version of Record:** A version of this preprint was published on October 2nd, 2020. See the published version at <https://doi.org/10.1186/s12885-020-07395-y>.

# Abstract

**Background** Breast cancer is the leading cause of cancer mortality in women worldwide. Therefore, it is of great significance to identify the biological mechanism of tumorigenesis and explore the development of breast cancer to achieve a better prognosis for individuals suffering from breast cancer. MicroRNAs (miRNAs) have become a hot topic in cancer research, but the underlying mechanism remains unclear.

**Methods** The miRNA profile between breast cancer stem cells (BCSCs, CD44<sup>+</sup>CD24<sup>-/low</sup>) and control MCF-7 breast cancer cells was obtained in a previous study. Based on biological analysis, miR-20b-5p was hypothesized to be a key factor due to the malignant behavior of BCSCs. Then, agomir-20b-5p and antagomir-20b-5p were transfected into MCF-7 and T47D breast cancer cells to detect cell migration, wound healing and proliferation, and lentivirus vectors silencing or overexpressing miR-20b-5p were transfected into T47D-CSCs to detect proliferation and apoptosis. The effect of miR-20b-5p on xenograft growth was investigated in vivo by transfection of a lentivirus overexpression vector in T47D cells. The target genes were predicted by the online programs picTar, miRanda and TargetScan and verified by dual luciferase assay, and changes in protein expression were detected by western blot.

**Results** miR-20b-5p had the highest degree in both the miRNA-gene network and miRNA-GO network to regulate BCSCs. Overexpression of miR-20b-5p significantly promoted the migration and wound healing ability of MCF-7 cells and T47D cells compared with the control ( $P < 0.05$ ). In addition, miR-20b-5p facilitated the proliferation of MCF-7 cells and T47D-CSCs ( $P < 0.05$ ) and inhibited the apoptosis of T47D-CSCs ( $P < 0.05$ ). Moreover, miR-20b-5p promoted xenograft growth compared with the control group ( $P < 0.05$ ). Accordingly, potential targets of both CCND1 and E2F1 were predicted by bioinformatics analysis. miR-20b-5p directly targeted both CCND1 and E2F1 in a dual luciferase assay, while antagomir-20b-5p downregulated the protein levels of CCND1 and E2F1.

**Conclusions** Oncogenic miR-20b-5p was confirmed to promote the malignant behavior of breast cancer cells and stem cells. The underlying mechanism lies in miR-20b-5p overall enhanced both CCND1 and E2F1 targets via bidirectional regulation probably involving direct downregulation and indirect upregulation.

## Background

Breast cancer is one of the most common cancers in women worldwide [1]. In recent years, although significant progress has been made in the diagnosis and treatment of malignant tumors, breast cancer remains the leading cause of cancer-related deaths in women, largely due to its high rate of relapse, metastasis, and drug resistance [2–5]. Therefore, it is of great significance to explore the mechanism of tumorigenesis in breast cancer and further identify key regulatory factors. Recent studies, including our previous findings, have demonstrated that miRNAs play an important role in regulating biological functions [6–8]. Because of their unique expression profile and special functions, miRNAs have been used as molecular markers to define the direction and processes of cell differentiation by regulating one

or several target genes. miRNAs can also control the self-renewal or proliferation of stem cells [9]. Abnormal changes in miRNAs have been discovered in breast cancer, and miRNAs have been demonstrated to be associated with the tumorigenesis and progression of breast cancer [8, 10]. Previous studies have confirmed that miRNAs play an important role in gene regulation in CD44<sup>+</sup>CD24<sup>-/low</sup> breast cancer stem cells (BCSCs). For example, we found that miR-200C was significantly downregulated in BCSCs and played a critical role in their biological features [8]. In addition, let-7a plays an important role in BCSCs' self-renewal by inhibiting the expression of H-Ras [11, 12].

In our previous research, we performed miRNA profiling between sorted CD44<sup>+</sup>CD24<sup>-/low</sup> BCSCs and the control MCF-7 breast cancer cells [8]. Finally, miR-20b-5p was chosen for further study due to having the highest degree of regulation. We found that miR-20b-5p belongs to the miR-106a-363 cluster, which together with the miR-17-92 cluster and miR-106b-25 cluster forms a large family of highly similar miRNAs called the miR-17 family [13]. In many human malignancies, members of the miR-17 family have been reported to accumulate in tumor cells and are speculated to exert oncogenic effects [14, 15], and miR-20b serves as a potential oncogene in gastric cancer [16], breast cancer [17–18] and hepatocellular carcinoma [19]. However, there has been no exploration of the regulatory role of miR-20b in BCSCs, and the underlying mechanism of miR-20b-5p remains unclear. This study aimed to investigate the role of the miR-20b-5p, a subtype of miR-20b family, in the regulation of the malignant behavior of BCSCs and to identify its target genes.

## Methods

### Induction culture of BCSCs

MCF-7 and T47D cells in this experiment were cultured in DMEM-H (HyClone, USA) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO<sub>2</sub> incubator. Cells in the logarithmic growth phase, approximately 70–80% confluent, were harvested and digested into a single cell suspension by trypsin digestion.

The complete MammoCult™ medium contained 5% MammoCult™ proliferation supplement, 4 µg/mL heparin, and 0.48 µg/mL hydrocortisone. MCF-7 cells were resuspended in complete MammoCult™ medium, and T47D cells were resuspended in complete DMEM/F-12 medium. The cell suspension (4 × 10<sup>3</sup>) was seeded into a 6-well plate and cultured at 37 °C and 5% CO<sub>2</sub>. After 7 days of culture, the spheres were collected and centrifuged at 350 g for 5 min, and the supernatant was discarded. Then, 1 mL of Accutase cell dispersion solution was added to digest the spheres, followed by adding 9 mL of sterile PBS solution and centrifugation at 350 g for 5 minutes. Finally, the supernatant was discarded, and the cells were collected.

CD44<sup>+</sup>CD24<sup>-/low</sup> BCSCs were isolated from MCF-7 and T47D cells by staining with CD44-APC, CD24-PE and ESA-FITC (BD Pharmingen, USA) antibodies via FACS as described in our previous research [8, 20].

### miRNA profile and miRNA network

Both human miRNA microarray fabrication and hybridization were performed as described previously [8]. The miRNA profiles of both BCSCs and control MCF-7 breast cancer cells were obtained from CapitalBio Corporation (Beijing, China). All microarray data were uploaded and submitted to the public repository Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68271>). The differential miRNA profiles between BCSCs and MCF-7 cells were obtained.

To identify the miRNAs that regulate BCSCs, we established a miRNA-gene network and miRNA-GO network through analyses of the significant target genes and Gene Ontology (GO) terms performed by Shanghai Qiming Corporation (Shanghai, China).

## Target prediction by bioinformatics

Chromosome localization, sequence analysis and target prediction of the miRNAs were carried out by the online programs picTar (<http://pictar.mdc-berlin.de/>), miRanda (<http://microrna.sanger.ac.uk>), and TargetScan (<http://www.targetscan.org>). The mRNAs associated with cell proliferation and the cell cycle predicted by at least three algorithms were selected as putative targets. Then, the binding free energy ( $\Delta G$ ) of the hybridization between miRNAs and their 3' UTR complementary sites was analyzed with the aid of Mfold software. The mRNAs that could bind with lower free energy at both 5'-70 bp and 3'-70 bp than their average random free energy were deemed to be accessible to specific miRNAs [21].

## RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. After the concentration and purity of total RNA were determined, reverse transcription was performed using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China). For qPCR analysis, cDNA was amplified with a SYBR Premix Ex Taq (TaKaRa) kit by using an AB 7500 Real-time PCR system. The relative gene expression was calculated by the  $2^{-\Delta\Delta Ct}$  method. For miR-20b-5p qRT-PCR, the primers for miR-20b-5p (Forward: 5'-CAAAGTGCTCATAGTGCAG GTAG-3', Reverse 5'-GCAAAGTGCTCATAGTGCAGG-3') and U6 (Forward 5'-CTCGCTTCGGCAGCACA-3', Reverse 5'-AACGCTTCACGAATTTGCGT-3') were used. For qRT-PCR of potential target genes, primers are listed in Table 1.

Table 1  
Primers of potential target genes

Gene	Forward primer	Reverse primer
CCND1	5'-CCCGCACGATTTTCATTGAAC-3'	5'-GGCGGATTGGAAATGAACTTC-3'
E2F1	5'-ACCTCTTCGACTGTGACTTTG-3'	5'-GAGCATCTCTGGAAACCCTG-3'
MAPK1	5'-AGCGTATCAGCATGCCAC-3'	5'-GACCTCGAAGACGTTTCTCC-3'
STAT3	5'-GCTTCCCTGATTGTGACTG-3'	5'-CTGACAGATGTTGGAGATCACC-3'
R2b23b	5'-CTGACAGAGCAGTGGAGTATC-3'	5'-TGTTGCTGTCGTAGTTGCTG-3'
RAB5BR	5'-GCCAGTCCTAGCATCGTTATTG-3'	5'-TCACGTTCATAGCTGTCTTGG-3'
RABEP1	5'-GCCACAGTCTCTGAGAACACCAAG-3'	5'-GGAAGTGGTGCTCATAGTCACGAA-3'
TAOK3	5'-CAAGAGACACGGAATGGACC-3'	5'-TCACGGACATGCTTGGAAATG-3'
PPARRDR	5'-GCTTCCACTACGGTGTTTCATG-3'	5'-CTTCTCGTACTCCAGCTTCATG-3'
XIAP	5'-AAACACCATCACTAACTAGAAGAATTG-3'	5'-CAAGTGATTTATAGTTGCTCCCAG-3'

## Transwell migration assay

Transient transfection of agomir-20b-5p and antagomir-20b-5p in MCF-7 and T47D breast cancer cells was performed as follows. After digestion, breast cancer cells were mixed with 1 mL DMEM-H containing 10% FBS and subjected to resuspension followed by cell counting. Then, the 24-well plates were inoculated at  $4 \times 10^4$  cells per well, containing 500  $\mu$ L of medium, and placed in a 37 °C, 5% CO<sub>2</sub> incubator overnight. The medium was removed the next day, the cells were washed with PBS three times, and then 500  $\mu$ L of Opti-MEM medium was added to each well. Then, Lipofectamine 2000 (Invitrogen, USA) was mixed with 1  $\mu$ L of agomir of miR-20b-5p (agomir-20b-5p), antagomir of miR-20b-5p (antagomir-20b-5p) (Dharmacon, USA), or miR-control at a final concentration of 30 nM, and the cells were incubated for 24–48 h to achieve successful transfection.

The transwell chamber was placed in a 24-well plate, 800  $\mu$ L of DMEM-H containing 15% FBS was added to the lower chamber, and 200  $\mu$ L transfected MCF-7 or T47D breast cancer cells ( $4 \times 10^4$  cells resuspended in 3% DMEM-H) was added to the upper chamber and cultured in a 37 °C, 5% CO<sub>2</sub> incubator for 24 h. After removal of the medium, the cells were fixed with 1 mL of 95% ethanol solution for 10 min, washed 3 times with 1 mL of PBS for 5 min each, and then stained with 1% crystal violet dye solution for 10 min. The crystal violet dye in the upper chamber was washed off under a small stream of water and gently wiped with a cotton swab. Finally, the cells were observed under an inverted microscope followed by imaging and counting. The test was repeated three times.

## Scratch assay

Transient transfection of agomir-20b-5p and antagomir-20b-5p in MCF-7 and T47D breast cancer cells was performed as described above. First, 3–4 straight lines were drawn vertically on the back of the 6-well plate. Then, the cells were seeded according to the grouping and cultured in DMEM-H containing 10% FBS. When the transfected MCF-7 and T47D breast cancer cells reached 60–70% confluence, mitomycin was added for 2 h (final concentration was 10 ng/mL). After washing 3 times with PBS, the cells were scratched gently with a 100  $\mu$ L pipette tip according to the marked lines, and then the detached cells were removed by washing with PBS. Under an inverted microscope, the imaging position was recorded at the 24 h and 48 h time points. The test was repeated three times.

## **Proliferation assay in MCF-7 cells and T47D-CSCs**

Transient transfection of agomir-20b-5p and antagomir-20b-5p in MCF-7 breast cancer cells was performed as described above. MCF-7 cells were incubated with 30  $\mu$ M EdU, and cell proliferation was evaluated by flow cytometry using an EdU assay kit (GeneCopoeia, USA) according to the manufacturer's instructions. The test was repeated five times.

Stable transfection of a lentivirus overexpression system was used in the T47D-CSC proliferation assays. The lentiviral vector (GV369) overexpressing miR-20b-5p was purchased from GeneChem (China). The miR-20b-5p overexpression vector was labeled T47D-CSCs/LV-miR-20b-5p, and the control vector was labeled T47D-CSCs/LV-NC. The induced T47D-CSCs were suspended in EpiCult-B serum-free medium, and Lipofectamine 2000 (Invitrogen, USA) was added together with T47D-CSCs/LV-miR-20b-5p or T47D-CSCs/LV-NC. Then, the viruses were harvested. Cultured cells were infected with 5  $\mu$ g/mL polybrene and lentivirus (MOI = 50) for 24 h, and then the cells were incubated with fresh medium for another 48 h to establish stable cell lines. The proliferation assay was conducted as described above. The test was repeated five times.

Stable transfection of a lentivirus-miR-sponge construct was used in T47D-CSC proliferation assays. The miR-20b-5p shRNA sponge lentivirus for inhibition of miR-20b-5p was purchased from Hanbio Biotechnology (China). The tandem antisense sequence of miR-20b-5p (CTACCTGCACTATGAGCACTTTG) was synthesized and cloned into the shRNA lentiviral vector pHBLV-U6-mCherry-Puro (Hanbio Biotechnology). Then, the miR-20b-5p sponge construct and the empty vector were packaged and labeled T47D-CSCs/miR-20b-5p-sp and T47D-CSCs/vector-sp, respectively. The miR-20b-5p sponge shRNA lentiviral vector (Hanbio Biotechnology) or control lentiviral vector was incubated at a final concentration of 30 nM for 24–48 h. The proliferation assay in induced T47D-CSCs was conducted as described above. The test was repeated five times.

## **Apoptosis assay in T47D-CSCs**

The stable transfection of lentivirus overexpression and lentivirus-miR-sponge constructs was used to assay apoptosis in T47D-CSCs as described above. The induced T47D-CSCs were stained with anti-annexin V/7-AAD antibodies in binding buffer for 15 min and then analyzed for apoptosis by flow cytometry. The test was repeated five times.

# Animal experiments

The stable transfection of T47D cells overexpressing lentivirus was conducted as described above, and the cells were used in animal experiments. The vector overexpressing miR-20b-5p was labeled as T47D/LV-miR-20b-5p, and the control vector was labeled as T47D/LV-NC. All animal experiments were approved by the Institutional Animal Care and Use Committee of Xinqiao Hospital. A total of 15 nude mice (4–6 weeks old) from the SPF Laboratory Animal Center of Xinqiao Hospital were randomly divided into the following three groups with 5 mice in each group: T47D control, T47D/LV-NC and T47D/LV-miR-20b-5p. A total of  $1 \times 10^7$  cells/100  $\mu$ L of PBS were subcutaneously injected to establish a xenograft. The tumor size was measured every 3 days and calculated according to the formula  $V = (\text{length} \times \text{width}^2)/2$ . After four weeks, the mice were sacrificed by cut off the heads after injected Chloral hydrate, and the tumor tissues were collected for miR-20b-5p detection by qRT-PCR. All protocols involving mice were conducted in accordance with the animal care guidelines of Xinqiao Hospital, Army Medical University.

## Dual luciferase assay

293T cells were cultured with DMEM (supplemented with 10% FBS) to a cell density of 70%-80%. Then, the transfection reagent was diluted in serum-free DMEM, and the plasmid was diluted in serum-free DMEM. The cells were incubated for 20 min at room temperature and then supplemented with serum-free DMEM. The culture medium in the dish was discarded, and the transfection mix was added and cultured at 37 °C for 5 h. The culture medium was removed, changed to complete culture medium (0.5 mL), and the cells were incubated at 37 °C for 48 h. Then, 200  $\mu$ L of cell lysate was added into each well, and the cells were incubated for 10 min at room temperature. The lysate was centrifuged at 10,000 g for 5 min, and the supernatant was collected. The firefly luciferase assay reagent and the Renilla luciferase assay buffer were dissolved to room temperature. Then, 100  $\mu$ L lysis buffer was added to the 96-well plate, and 100  $\mu$ L firefly luciferase detection solution was added and mixed completely. With Renilla luciferase as the internal reference, the RLU value determined for firefly luciferase was divided by the Renilla luciferase value. The vectors (GV306) for CCND1 and E2F1 were purchased from GeneChem (China). The insert sequences were as follows: wild type CCND1 (CCND1-WT) and mutated CCND1 (CCND1-MT), wild type E2F1 (E2F1-WT) and mutated E2F1 (E2F1-MT) (Supplementary Fig. 1). A random sequence and miR-20b-5p mimics were used for negative control (NC) and miRNA overexpression, respectively. The experiment was divided into 4 groups: NC + CCND1-WT, MIMICS + CCND1-WT, NC + CCND1-MT, and MIMICS + CCND1-MT. The same experiment was performed with E2F1 reagents.

## Western blot analysis of target genes

Transfected cells were washed twice with PBS, lysed in RIPA buffer (Beyotime, China) containing a protease inhibitor cocktail (Roche, Switzerland) for 30 min and then centrifuged at 13000 xg for 10 min. After boiling in loading buffer for 10 min, the supernatants were loaded onto SDS-PAGE gels and then transferred onto PVDF membranes (Millipore, USA). After blocking, the membranes were incubated separately with specific primary antibodies overnight at 4 °C, followed by incubation with goat anti-rabbit IgG or goat anti-mouse IgG antibodies (ZSGB-Bio, China) for 1 h at room temperature. Finally,

immunoreactive proteins were visualized using a chemiluminescence detection system (FluorChem HD2, USA).

## Results

### The miRNA profile screened by miRNA networks

In the miRNA-gene network (Fig. 1A), the degrees of connectivity of miR-106a-5p, miR-20b-5p, miR-106b-3p, let-7b-5p, miR-7e-5p, miR-29b-3p, and miR-98-5p ranked at the top, with an average degree of 42.86. Among them, the degrees of miR-106a-5p and miR-20b-5p reached 48 (Fig. 1B). In the miRNA-GO network (Fig. 1C), the degrees of miR-106a-5p, miR-20b-5p, miR-106b-3p, let-7b-5p, miR-7e-5p, miR-29b-3p and miR-98-5p were ranked at the top, with an average degree of 34.14. Among them, the degrees of miR-106a-5p and miR-20b-5p reached 36 (Fig. 1D). Based on the two networks above, both miR-106a-5p and miR-20b-5p had the highest regulatory degrees. They are both located on the X chromosome (Xq26.2, GRCh37) and are less than 1 kb apart. Moreover, miR-106a-5p and miR-20b-5p together with other molecules, such as miR-92a-2 and miR-363, constitute a cluster of miRNAs [22]. Our previous study demonstrated that miR-106a-5p acted as a tumor suppressor gene and significantly inhibited the invasion and migration of breast cancer cells [23]. In the current study, we focused on miR-20b-5p.

### Bioinformatics analysis of target genes

We explored the target genes of miR-20b-5p related to cell proliferation and cell cycle and found 10 potential targets of miR-20b-5p, namely, CyclinD1 (CCND1), E2F1, MAPK1, STAT3, R2b23b, RAB5BR, RABEP1, TAOK3, PPARDR and XIAP. After transfection of agomir-20b-5p in MCF-7 cells, the qRT-PCR results showed that miR-20b-5p increased the expression of some of the potential target genes and inhibited the others (Fig. 2A). The dramatically increased expression of CCND1 and E2F1 was intriguing and unexpected, since miRNAs usually negatively regulate their target genes. Therefore, the contradictory preliminary results of qRT-PCR compelled us to choose CCND1 and E2F1 for subsequent validation and functional studies. The binding free energies between CCND1 and miR-20b-5p at both the 5' UTR and 3' UTR were - 14.50 kcal/mol and - 12.10 kcal/mol, respectively, lower than the average random free energy of CCND1 (0 kcal/mol). Likewise, the binding free energies between E2F1 and miR-20b-5p at both the 5' UTR and 3' UTR were - 18.10 kcal/mol and - 19.00 kcal/mol, respectively, lower than the average random free energy of E2F1 (- 3.60 kcal/mol) (Fig. 2B) (Supplementary Fig. 2).

### The effects on migration and wound healing ability

miR-20b-5p expression was increased 75-fold and 77-fold by agomir-20b-5p transfection in MCF-7 cells and T47D cells, respectively and was downregulated 65-fold and 60-fold by transfection of antagomir-20b-5p in MCF-7 cells and T47D cells, respectively (Fig. 3A). Transwell migration experiments ( $n = 3$ ) showed that agomir-20b-5p significantly promoted the migration ability of MCF-7 cells ( $85 \pm 3$  vs.  $185 \pm 2$ ) ( $P < 0.01$ ), and antagomir-20b-5p significantly inhibited the migration ability of MCF-7 cells ( $85 \pm 3$  vs. 62

$\pm 2$ ) ( $P < 0.01$ ) (Fig. 3B). Similarly, agomir-20b-5p significantly promoted the migration ability of T47D cells ( $20 \pm 1$  vs.  $33 \pm 2$ ) ( $P < 0.01$ ,  $n = 3$ ), and antagomir-20b-5p markedly inhibited the migration ability of T47D cells ( $20 \pm 1$  vs.  $14 \pm 1$ ) ( $P < 0.05$ ,  $n = 3$ ) (Fig. 3C).

For the scratch test, the ImageJ tool was used to measure the scratched surface, and the upper, middle and lower positions for the scratch were measured at 0 h and 24 h, respectively, and recorded as  $L_0$  and  $L_{24}$ , based on which  $L = L_0 - L_{24}$  was calculated. The results showed that agomir-20b-5p significantly increased the wound healing ability of MCF-7 cells ( $161.6257 \pm 11.23752$  vs.  $339.0160 \pm 18.26367$ ) ( $P < 0.01$ ,  $n = 3$ ), and antagomir-20b-5p inhibited the wound healing ability of MCF-7 cells ( $161.6257 \pm 11.23752$  vs.  $120.0483 \pm 16.72730$ ), but the difference was not statistically significant ( $P > 0.05$ ,  $n = 3$ ) (Fig. 3D). Similarly, agomir-20b-5p also significantly promoted the wound healing ability of T47D cells ( $108.0030 \pm 9.64574$  vs.  $435.9533 \pm 37.53868$ ) ( $P < 0.05$ ,  $n = 3$ ), while antagomir-20b-5p inhibited the wound healing ability of T47D cells but without a statistically significant difference ( $108.0030 \pm 9.64574$  vs.  $87.0583 \pm 9.04403$ ) ( $P > 0.05$ ,  $n = 3$ ) (Fig. 3E).

## The effects of miR-20b-5p on proliferation and apoptosis in BCSCs

In MCF-7 cells, EdU proliferation experiments indicated that agomir-20b-5p promoted cell proliferation, while antagomir-20b-5p inhibited cell proliferation, with a proliferation rate of  $16.6\% \pm 0.68\%$  in the control group,  $24.3\% \pm 0.59\%$  in the agomir-20b-5p group ( $P < 0.05$ ,  $n = 3$ ) and  $14.7\% \pm 0.88\%$  in the antagomir-20b-5p group (Fig. 3F).

The proliferation rate of the  $CD44^+CD24^{-/low}$  subpopulation was 1.04% and 96.4% in T47D cells and induced T47D-CSCs, respectively (Fig. 4A). qRT-PCR analysis showed that miR-20b-5p expression was 11.20 times higher in T47D-CSCs/miR-20b-5p compared with T47D-CSCs/LV-NC, while there was no significant difference between T47D-CSCs/miR-20b-5p-sp and T47D-CSCs/vector-sp (Fig. 4B). The EdU proliferation experiment in T47D-CSCs showed that the proliferation rates of the T47D-CSCs/LV-NC group and T47D-CSCs/LV-miR-20b-5p group (the proportion of cells in the S phase of the cell cycle) were  $36.47\% \pm 1.87\%$  and  $41.63\% \pm 0.64\%$ , respectively ( $P = 0.011$ ,  $n = 3$ ). The proliferation rates of the T47D-CSCs/vector-sp group and T47D-CSCs/miR-20b-5p-sp group were  $41.80\% \pm 1.14\%$  and  $37.87\% \pm 0.95\%$ , respectively ( $P = 0.010$ ,  $n = 3$ ), indicating the increase in proliferation by miR-20b-5p in T47D-CSCs (Fig. 4C).

The apoptosis rates of the T47D-CSCs/LV-NC group and T47D-CSCs/LV-miR-20b-5p group were  $8.56\% \pm 0.48\%$  and  $4.37\% \pm 0.45\%$ , respectively ( $P = 0.004$ ,  $n = 3$ ), while the apoptosis rates of the T47D-CSCs/vector-sp group and T47D-CSCs/miR-20b-5p-sp group were  $7.43\% \pm 0.75\%$  and  $12.90\% \pm 0.54\%$ , respectively ( $P = 0.001$ ,  $n = 3$ ), indicating apoptosis inhibition by miR-20b-5p in T47D-CSCs (Fig. 4D).

## Xenograft growth impacted by miR-20b-5p

After lentiviral transfection, miR-20b-5p expression was 5.618 times higher (Fig. 5A). The xenograft tumor volume in the miR-20b-5p overexpression group (T47D/LV-miR-20b-5p group) was significantly larger than that in the blank group and negative control group (T47D/LV-NC group) (Fig. 5B), and the growth curve also showed that the tumors in the T47D/LV-miR-20b-5p group grew significantly faster than those in the blank group and T47D/LV-NC group ( $P < 0.05$ ,  $n = 3$ ) (Fig. 5C). The expression of miR-20b-5p in xenografts in the T47D/LV-miR-20b-5p group was 107.68 times higher than that in the T47D/LV-NC group ( $P < 0.001$ ,  $n = 3$ ) (Fig. 5D).

## Verification of target genes

To verify the target genes of miR-20b-5p, we conducted three experiments.

First, we compared the correlation between miR-20b-5p expression and CCND1 and E2F1 mRNA levels in BCSCs and MCF-7 cells or T47D cells. The CD44<sup>+</sup>CD24<sup>-/low</sup> subpopulation was 2.58% and 64.3% of MCF-7 and induced MCF-7-CSCs, respectively (Fig. 4A). The expression of miR-20b-5p was 3.98 times and 2.21 times higher in BCSCs than in the control MCF-7 and T47D cells, respectively. Accordingly, the mRNA expression levels of E2F1 and CCND1 were higher in BCSCs than in MCF-7 and T47D cells, although the difference was not statistically significant (Fig. 6A).

Second, the potential targeting of CCND1 and E2F1-WT was further verified by dual luciferase assay. Luciferase activity was significantly decreased in cells cotransfected with miR-20b-5p mimics plus CCND1-WT compared with cells cotransfected with NC plus CCND1-WT ( $0.966 \pm 0.027$  vs.  $1.156 \pm 0.026$ ,  $P < 0.0001$ ,  $n = 5$ ). There was no significant difference between NC plus CCND1-MT and mimics plus CCND1-MT ( $1.164 \pm 0.015$  vs.  $1.177 \pm 0.051$ ,  $P > 0.05$ ,  $n = 5$ ) (Fig. 6B). Likewise, the luciferase activity was significantly decreased in cells cotransfected with miR-20b-5p mimics plus E2F1-WT compared with cells cotransfected with NC plus E2F1-WT ( $0.636 \pm 0.020$  vs.  $0.874 \pm 0.015$ ,  $P < 0.0001$ ,  $n = 5$ ). There was no significant difference between NC plus E2F1-MT and mimics plus E2F1-MT ( $1.015 \pm 0.026$  vs.  $1.073 \pm 0.018$ ,  $P > 0.05$ ,  $n = 5$ ) (Fig. 6B). These results indicated that miR-20b-5p negatively regulated CCND1 and E2F1.

Third, the protein expression levels of CCND1 and E2F1 were detected via western blot assay after agomir-20b-5p or antagomir-20b-5p transfection in MCF-7 cells. When the relative expression level of the control group was defined as 1, the relative expression levels of E2F1 and CCND1 were both 1.04 in the agomir-20b-5p group. The relative expression levels of E2F1 and CCND1 in the antagomir-20b-5p group were decreased to 0.46 and 0.45, respectively (Fig. 6C).

## Discussion

To determine the normal function of miR-20b-5p, we searched for miR-20b expression in normal tissues using the public website miRNAmap (<http://mirnamap.mbc.nctu.edu.tw/>). The highest expression level

was in the thymus, and the expression level was also high in the prostate, kidney, lung and other normal tissues (Supplementary Fig. 3).

Furthermore, we researched the reports on the miR-20b family in different tumors. The expression level of miR-20b was reported to be increased in gastric cancer [16], breast cancer [17–18] and hepatocellular carcinoma [19]. Wang B et al. [24] found that miR-20b promoted proliferation, migration, invasion and tumorigenicity in esophageal cancer cells. It was also reported that aberrant expression of miR-20b contributed to tumorigenesis and progression of breast cancer [25]. In the above literature, miR-20b is shown to play an oncogenic role. However, miR-20b includes both miR-20b-3p and miR-20b-5p, which might have different biological functions. Only one report showed that overexpressed miR-20b-5p in cancer tissue and patient serum had a regulatory effect on the proliferation and migration of breast cancer cells [26]. In addition, there has been no exploration of the regulatory role of miR-20b in BCSCs. In the current study, we focused on the function of miR-20b-5p and found that miR-20b-5p acted as an oncogene in the regulation of the malignant behavior of breast cancer and BCSCs.

Cancer stem cells (CSCs) have the ability of self-renewal and multidirectional differentiation [27]. Studies have shown that the molecular regulation of BCSCs is different from that of breast cancer cells [28]. BCSCs are associated with epithelial-mesenchymal transformation (EMT), miRNAs, the tumor microenvironment and other factors [29]. Our experimental results indicated that the upregulation of miR-20b-5p in MCF-7 and T47D cells significantly promoted the migration ability of breast cancer cells. Furthermore, overexpression and knockdown systems of miR-20b-5p in BCSCs were established. It was confirmed that miR-20b-5p promoted the proliferation of BCSCs and inhibited the apoptosis of BCSCs. As an integrated outcome of miR-20b-5p on breast cancer cells and stem cells, we observed that the xenografts of breast cancer cells were significantly enlarged by miR-20b-5p overexpression, which indicated that miR-20b-5p acted as an oncogene *in vivo*.

To further clarify the underlying mechanism of miR-20b-5p in the regulation of breast cancer cells and stem cells, we chose CCND1 and E2F1 as potential targets based on bioinformatics analysis and preliminary screening by qRT-PCR. CCND1, as an oncogene, is overexpressed in tumors and plays an important regulatory role in normal breast development, damage repair, maintenance of breast epithelial stem cell proliferation and self-renewal [30–32]. E2F1, a member of the E2F transcription factor family, regulates gene expression related to cell proliferation, differentiation and apoptosis and controls the cell cycle via a two-way regulatory mechanism. As a tumor suppressor gene or oncogene, E2F1 is closely related to tumor progression and drug resistance [33–36]. The literature shows that CCND1 and E2F1 are regulated by the miR-17-92 cluster, which is a miRNA cluster located on chromosome 13 that is composed of miR-17, miR-20a and miR-92a-1 [8, 9]. This suggests that E2F1 is targeted by certain miRNAs and is involved in the regulation of tumor cells [37]. To confirm that miR-20b-5p regulated CCND1 and E2F1 expression, we performed three experiments, namely, a comparison between BCSCs and the control breast cancer cells, a dual luciferase assay and western blot analysis after overexpression or knockdown of miR-20b-5p. The dual-luciferase reporting system showed that CCND1 and E2F1 were real

targets of miR-20b-5p. Moreover, our western blot analysis showed that antagomir-20b-5p significantly decreased the protein levels of both CCND1 and E2F1.

This seems a contradictory but interesting phenomenon. Our results revealed an indirect upregulation of CCND1 and E2F1 by miR-20b-5p. There likely are unknown targets (X and Y) negatively regulated by miR-20b-5p. The unknown targets (X and Y) could inhibit CCND1 and E2F1 protein expression. Overall, miR-20b-5p regulated both CCND1 and E2F1 via bidirectional regulation, namely, direct downregulation and indirect upregulation. As a tug-of-war mechanism, the indirect promoting effect of miR-20b-5p on CCND1 and E2F1 may eclipse its direct downregulation of CCND1 and E2F1. However, it is still necessary to reveal the underlying mechanism, which will be explored in further studies. The hypothesis involving the effect of miR-20b-5p on CCND1 and E2F1 is shown in a regulatory network (Supplementary Fig. 4).

As above, we conclude that miR-20b-5p has a cancer-promoting function in malignant behavior in both breast cancer cells and stem cells. We speculate that the underlying mechanism of miR-20b-5p contributing to the malignant behavior of breast cancer is that miR-20b-5p overall upregulates both CCND1 and E2F1 via bidirectional regulation.

## Conclusion

As above, we conclude that miR-20b-5p has a cancer-promoting function in malignant behavior in both breast cancer cells and stem cells. We speculate that the underlying mechanism lies in miR-20b-5p overall enhanced both CCND1 and E2F1 targets via bidirectional regulation probably involving direct downregulation and indirect upregulation.

## Abbreviations

miRNAs: MicroRNA; BCSCs: breast cancer stem cells; FBS: fetal bovine serum; GO: Gene Ontology; CCND1: Cyclin D1; E2F1: E2F transcription factor 1; Edu: 5-ethynyl-2'-deoxyuridine; T47D-CSCs: T47D cancer stem cells; sp: sponge; LV: lentivirus; NC: negative control.

## Declarations

### Ethic approval and consent to participate

The study was approved by the animal ethics committee of the Xinqiao Hospital of Army Medical University.

### Consent for publication

Applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this article.

### **Competing interests**

The authors declare that they have no competing interests.

### **Funding**

The work was supported by National Science Foundation of China (No: 30772108 and 81272910); and Chongqing Natural Science Foundation (No: cstc2012jjB10003). These are earmarked funds for cancer research. The funding bodies have no role in study design, data collection, data analysis, data interpretation, or manuscript preparation.

### **Authors' contributions**

LX, FL and JQ contributed equally to this work. LX, FL, JQ carried out the experiments. LX, FL, ZF, ZX analyzed the data. ZC, JS conceived and designed the experiments. FL wrote the manuscript. All authors read and approved the final manuscript.

### **Acknowledgements**

Not applicable

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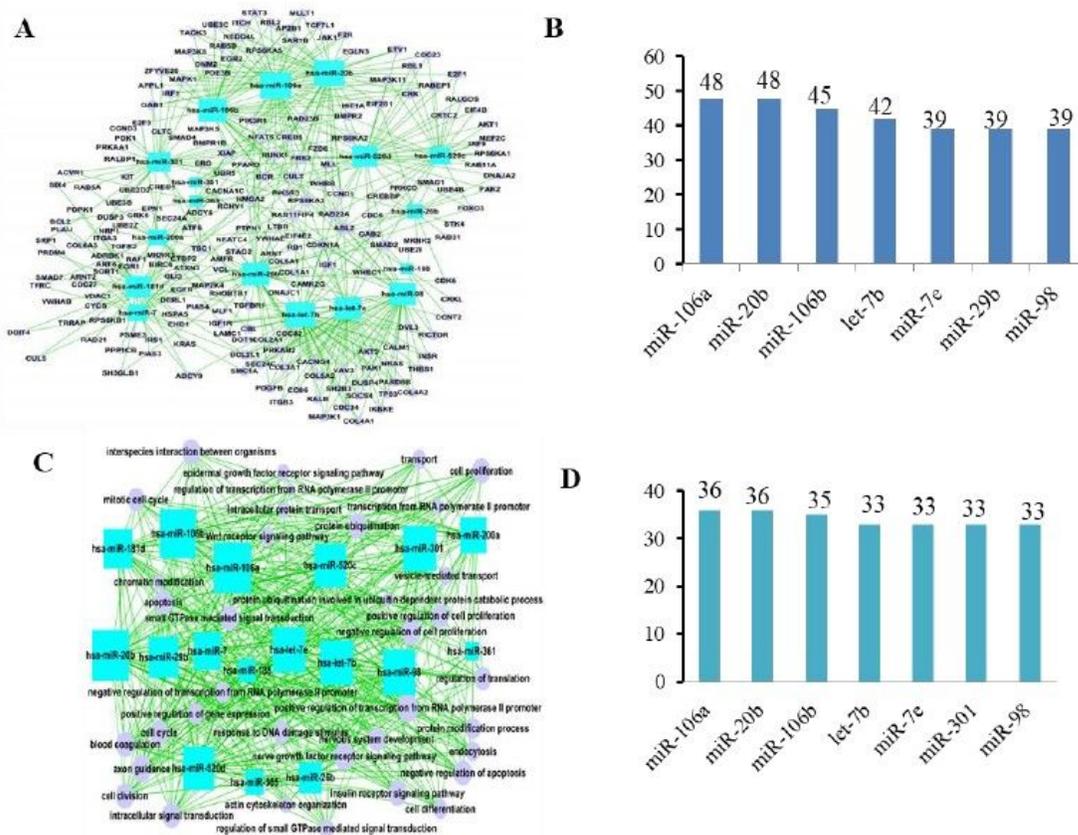
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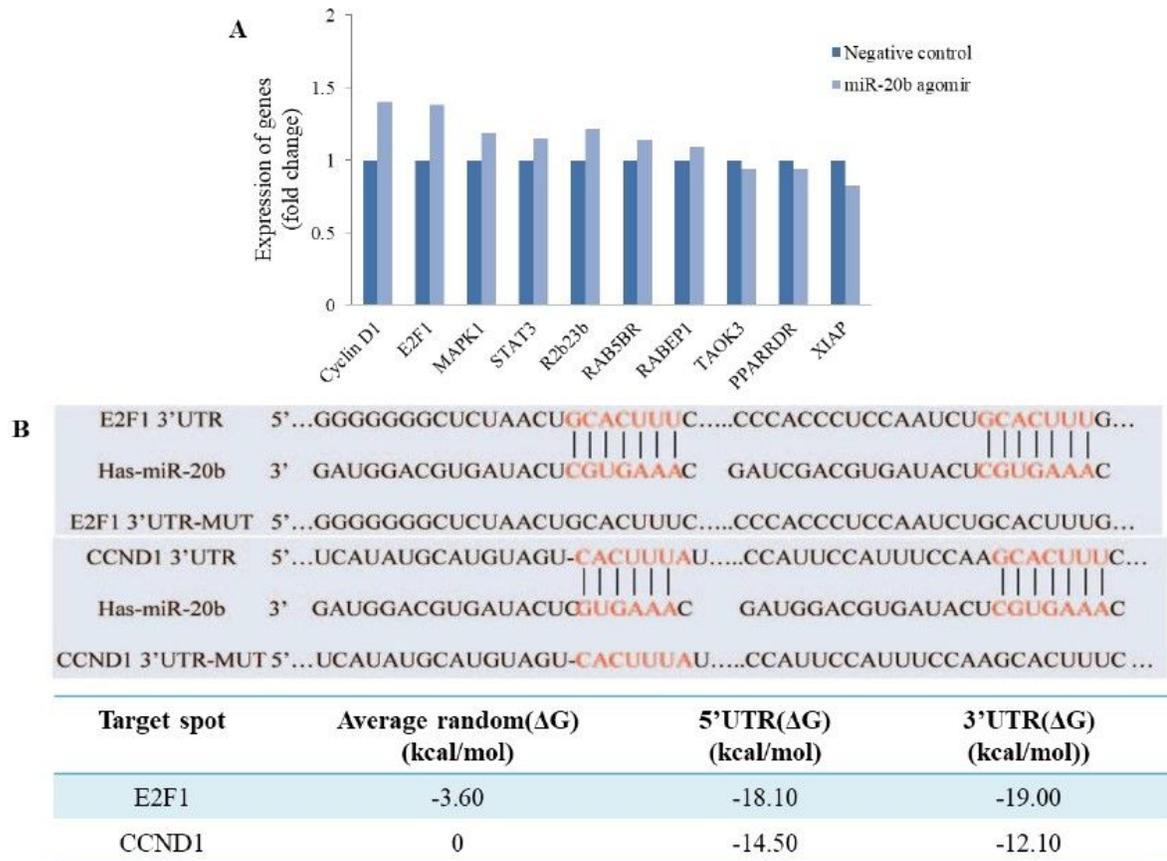
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## Figures



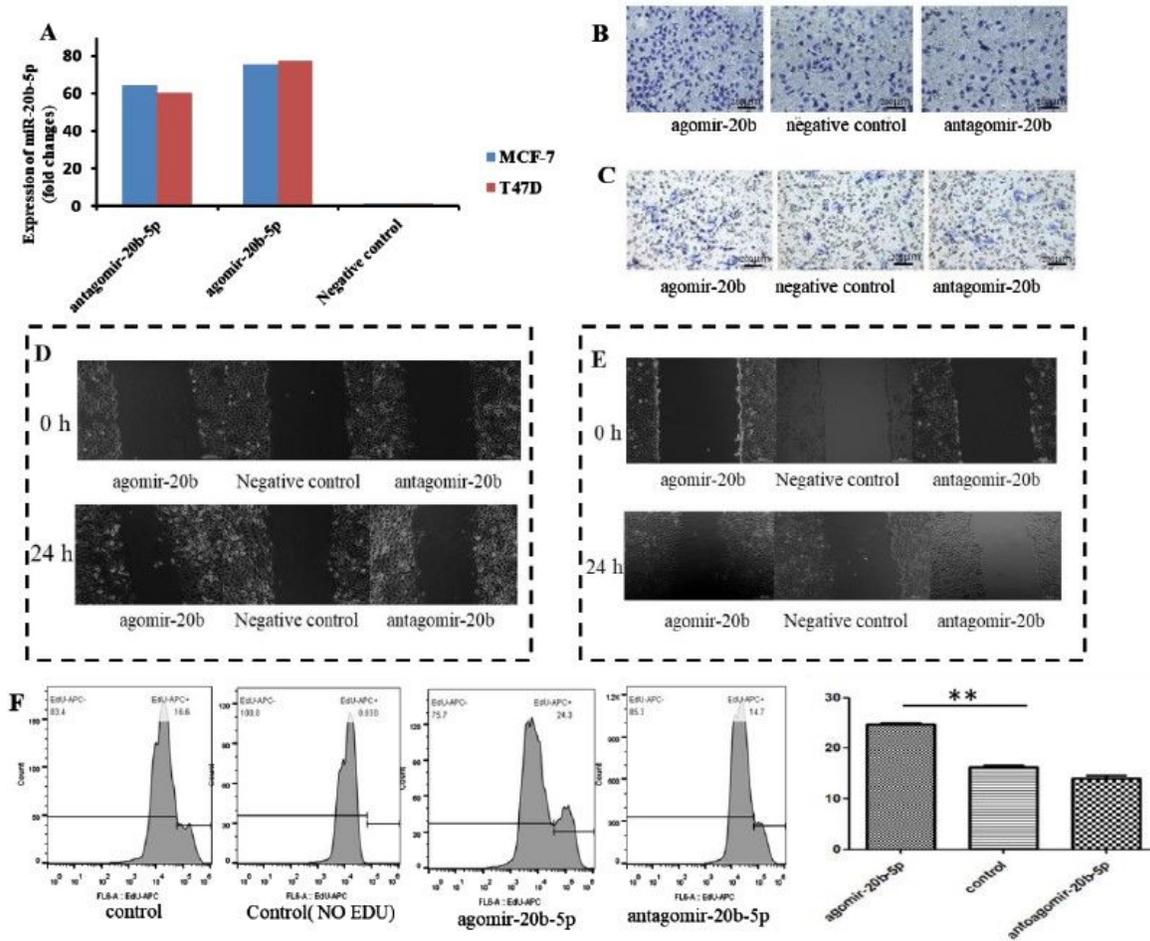
**Figure 1**

miRNA screening via miRNA networks. A In the miRNA-gene network, the rectangle in the figure represents upregulated miRNAs, the circle represents genes, and the line represents the regulatory relationship between miRNAs and genes; B The regulatory degrees of miRNAs in the miRNA-gene network; C In the miRNA-GO network, the rectangle in the figure represents miRNAs, the circle represents GO terms, and the straight line represents the regulatory relationship between miRNAs and GO terms. The more miRNAs that regulate a GO term, the larger its area; D The regulatory degrees of miRNAs in the miRNAs-GO network.



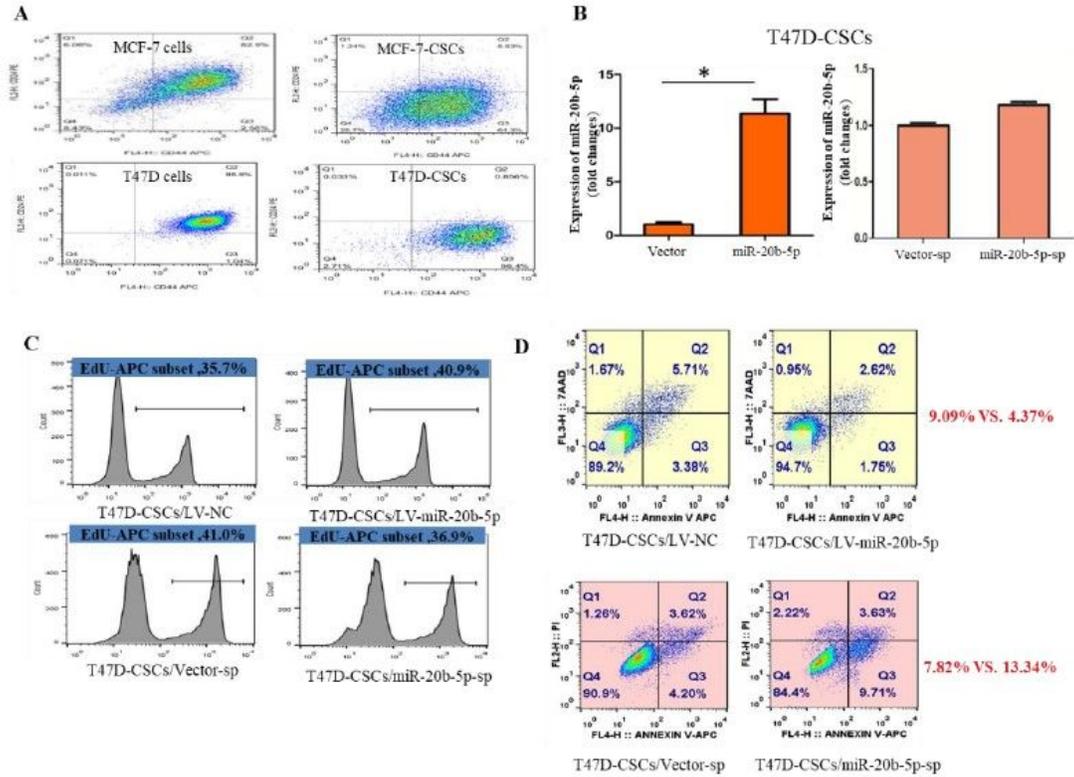
**Figure 2**

Bioinformatics analysis of target genes. A: qRT-PCR screening of potential target genes; B: binding site of miR-20b-5p to the E2F1-3' UTR and CCND1-3' UTR and free energy analysis.



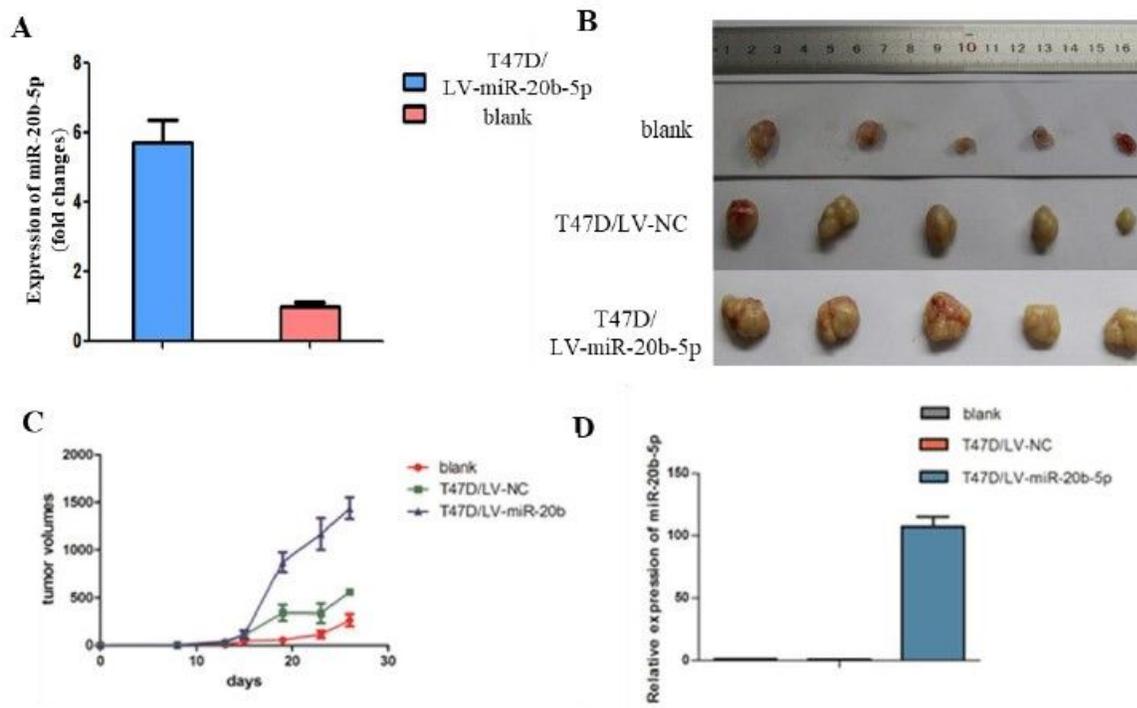
**Figure 3**

The effects of miR-20b-5p on migration. A: Expression of miRNA after transient transfection; B: Transwell migration experiment in MCF-7 cells; C: Transwell migration experiment in T47D cells; D: scratch test in MCF-7 cells; E: scratch test in T47D cells; F: effects of miR-20b-5p on the proliferation of MCF-7 cells.



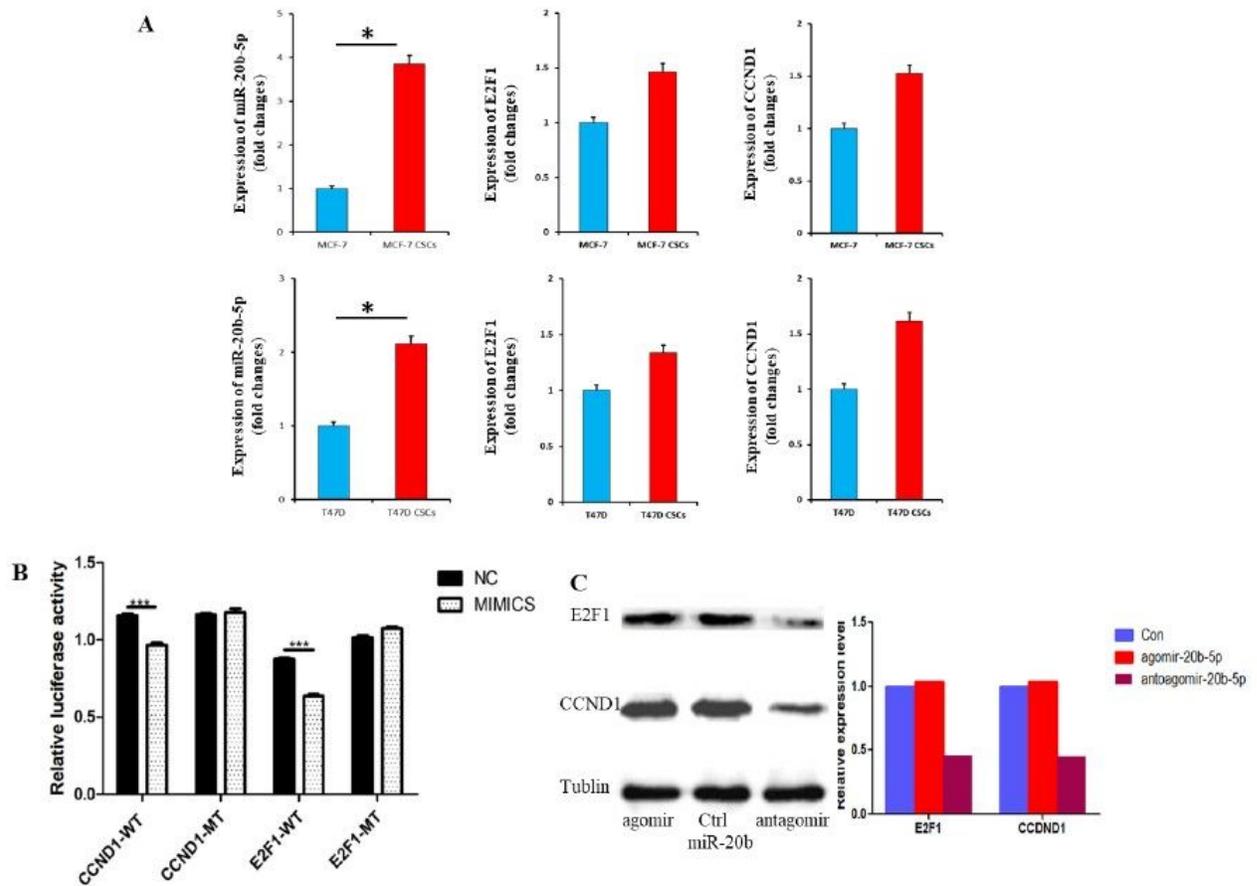
**Figure 4**

The effects of miR-20b-5p on proliferation and apoptosis in BCSCs. A: The ratio of CD44+CD24-/low subpopulation in breast cancer cells and BCSCs induced from MCF-7 and T47D cells; B: the expression of miR-20b-5p between lentivirus vector and miR-20b-5p and lentivirus-sp and miR-20b-5p-sp cells; C: effect of miR-20b-5p on the proliferation of T47D-CSCs; D: effect of miR-20b-5p on apoptosis of T47D-CSCs (Q2+Q3).



**Figure 5**

Xenograft growth impacted by miR-20b-5p. A: Expression of miR-20b-5p after lentivirus transfection; B: effect of miR-20b-5p on the tumorigenic ability of T47D cells; C: tumor growth curve; D: qRT-PCR verified the expression of miR-20b-5p in xenografts.



**Figure 6**

Verification of target genes. A: Expression of miR-20b-5p, E2F1 and CCND1 between MCF-7 and MCF-7-CSCs, T47D and T47D-CSCs; B: Dual luciferase assay to assess the regulatory effect of miR-20b-5p on CCND1 and E2F1; C: CCND1 and E2F1 are regulated by miR-20b-5p overexpressed in MCF-7 cells, as demonstrated by western blot. ( full-length blots/gels are presented in Supplementary Figure 5).

## Supplementary Files

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