

Hsa_circ_0029693 (circ-LATS2) promotes cell proliferation and regulates WNT5A via miR-4686 in breast cancer cells.

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Research

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Abstract

Background: Breast cancer (BC) is the most malignant form of tumor in women, which threatens females' health. Circular RNAs (circRNAs), a class of non-coding RNAs, can act as a disease biomarker and endogenous "sponge" molecules for microRNAs (miRNAs). circRNAs may also influence the expression of their parent gene. LATS2 is a vital suppressor gene in Hippo pathway, which is a signaling cascade composed of a group of conserved kinases. The Hippo pathway plays an important role in almost all cancer types.

Methods: Colony formation assays, MTT assays, wound healing assays, xenografts mice experiment, qRT-PCR, western blot assays, immunohistochemical staining assays, dual-luciferase reporter assays and Fluorescence in situ hybridization. Student's t-test was used to analyse the results.

Results: We discovered that circular RNA hsa_circ_0029693 (circ-LATS2), an exonic circRNA, are highly expressed in breast cancer cells. Furthermore, in BC patients' samples, higher expression of circ-LATS2 was significantly related to higher percentage of Ki-67 expression; however the expression of circ-LATS2 was higher in HER2 negative BC patients compared to HER2 positive ones. We investigated the potential function and mechanism of circ-LATS2 action in BC. The results suggested that circ-LATS2 promoted cell proliferation, growth and migration. Through western blot and immunohistochemical staining assays, we found that circ-LATS2 could influence LATS2 expression. We also discovered that there was an inverse expression relationship between miR-4686 and circ-LATS2, suggesting that circ-LATS2 might act as an endogenous "sponge" for miR-4686. Using dual-luciferase reporter assays, we confirmed that circ-LATS2 can bind miR-4686. Increased miR-4686 expression caused a reduction in the protein levels of WNT5A, which is a putative target of miR-4686. We confirm this using dual-luciferase reporter assays that revealed that miR-4686 targets WNT5A by binding its 3'-untranslated region (3'UTR).

Conclusions: Our results showed that circ-LATS2 expression in BC patients' samples were significantly related to Ki-67 expression. In addition, circ-LATS2 acted as a promoter of proliferation and growth of breast cancer cells. These indicated that circ-LATS2 is a proliferative factor, similar to Ki-67; it also acts as a co-biomarker with Ki-67 in clinical treatment.

Background

In 2018, BC is the most commonly diagnosed cancer in women contributing to 11.6% of the total cancer cases, and it is also the most common leading cause of cancer death ¹. Ever since hormone receptors and oncogene HER2 became vitally important in diagnosis and treatment, BC was classified into four subtypes, Luminal A, Luminal B, HER2-positive and triple negative breast cancer (TNBC) ². Furthermore, Ki-67, a marker of cellular proliferation in the active phases of cell cycle, is usually used as a biomarker in the clinical treatment to assess the growth function of BC ³. However, the optimal cut-off definition for Ki-67 is still controversial in clinical treatment.

CircRNAs are an atypical group of non-coding RNA molecules with closed structures, which allow them to have extraordinary stability to resist exoribonucleases. Therefore, compared to linear RNAs, circRNA are potentially a better biomarker⁴. Moreover, circRNAs are abundantly located in the cell nucleus and they might often play a role in regulating the expression of their parent gene⁵. CircRNAs also act as endogenous “decoys” for miRNAs by having common miRNA target sites. This allows circRNA to shape a regulatory network and hence, govern the movements of miRNAs⁶. MiRNAs are a class of small non-coding RNA, related to various diseases in humans including BC^{7,8}. The “seed” region of miRNA is 2–8 nucleotides from its 5'-end, which forms a complete match with the 3'-UTR of its target mRNA⁹. Targeted mRNAs by miRNA might be destroyed or their translation might be depressed¹⁰. Hence, miRNA is possibly able to destroy targeted mRNA or depress their translation.

Hippo signaling pathway is a highly conserved pathway, which performs an important role in cancers. Its main function is to regulate cell proliferation, control tissue homeostasis, and adjust organ size¹¹. Importantly, the Hippo pathway plays crucial roles in BC. LATS2, a critical Hippo pathway kinase, is significantly down-regulated in BC¹². LATS2 is an AGC kinase of the nuclear Dbf2-related family¹³. Additionally, more reduced expression of LATS2 mRNA is related to a more aggressive subtype of BC. The reduced expression of LATS2 increases cell proliferation, resulting in a worse outcome¹⁴. Thus, indicating that LATS2 has a vital role in BC.

In this research, firstly, we found that circ-LATS2 is over-expressed in BC cell lines. Although expression of circ-LATS2 showed no relationship with BC subtypes, higher expression of circ-LATS2 is significantly correlated to higher percentages of Ki-67 expression in BC patients. Therefore, we hypothesized that circ-LATS2 have the potential to be a proliferative factor as same as Ki-67. To confirm our hypothesis, circ-LATS2 biology function was tested. The results indicated that circ-LATS2 may have the potential to be a proliferation co-biomarker along with Ki-67, which help clinicians to access the growth function of BC in the clinical treatment in the future.

Methods

Methods

Bc Patient Samples

30 BC patients' cancer tissues and their paired normal adjacent tissues were obtained from the patients who have not received any anti-cancer therapy before surgery. The samples were stored in liquid nitrogen immediately after resected from patients. All patients participating in the study gave them consent forms and the procedures were approved by Institution Ethics Committees of the hospital.

Cell Culture

Human normal breast MCF-10A cells (Zhongqiaoxin Zhou Biotech, Shanghai China) were cultured in Mammary Epithelial Cell Medium (MEpiCm, ScienCell, Research Laboratories, Carlsbad, CA, USA). BC cell lines, MDA-MB-231, MDA-MB-468, HCC-1937, MCF-7 (Chinese Academy of Science, Shanghai, China) and SKBR-3 (iCell Bioscience Inc, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% Fetal Bovine Serum (FBS) (Gibco), penicillin (100 U/ml) and streptomycin (100 µg/ml) (PS, Enpromise, Hangzhou, China). Cells were incubated at 37°C supplemented with 5% CO₂, and subcultured when the cell density was around 80%.

Transfection Assay

siRNA circ-LATS2 and circ-NC, miR-4686, miR-NC, pcDNA3.1 (+) circRNA Vector-circ-LATS2 (OE-circ) and Vector-circ-NC (OE-NC) were synthesized by Intergrated Biotech Solutions (Shanghai, China) (Supplementary table 1). Cells were added into six-well-plate and cultured with DMEM medium with FBS and PS. When the cell confluency reached 30–50%, 0.8 µmol siRNA circ-LATS2, siRNA circ-NC, miR-4686, miR-NC, OE-circ-LATS2 or OE-circ-NC and 4 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were added into the cells with DMEM medium. After 4 hrs of incubation, the medium was replaced by DMEM with FBS and PS. After 48 hrs, the cells were harvested for further experiments.

Fluorescence In Situ Hybridization (fish)

Cells were cultured on coverslips, then hybridization buffer was added and incubated overnight with circ-LATS2 probe. The circ-LATS2 probe was chemically synthesized by Guangzhou RiboBio Co. Ltd (Guangzhou, China). Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The images were obtained by Thermo Fisher microscope (Thermo Fisher Scientific, Massachusetts, USA).

Qrt-pcr

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). Nuclear and cytoplasm RNA was extracted from MCF-10A and MDA-MB-231 cells using Paris™ kit (Thermo Fisher Scientific, Massachusetts, USA). cDNA was generated by reverse transcription using the PrimeScript RT-PCR kit in accordance with the manufacturer's instructions (Takara, Tokyo, Japan). Real-time PCR was performed on a 7900HT Fast RT-PCR instrument (Applied Biosystems, Singapore). The primer of circ-LATS2 and miR-4686 were synthesized by Intergrated Biotech Solutions (Shanghai, China). The qRT-PCR results were analysed with the $2^{-\Delta\Delta CT}$ method.

Colony Formation Assay

The treated cells were then harvested and plated into a six-well-plate at 500 cells/well. The plates were incubated for 7 to 10 days. When the colonies were visible, the medium was removed, and the plates were

washed with phosphate buffered saline and allowed to dry. After that, the dried colonies were fixed in 95% ethanol, then dried and stained with 0.1% crystal violet solution. Finally, the colonies were washed, dried and immediately photographed.

Mtt Assay

The treated cells were seeded into 96-well-plate at 500 cells/well. Cell viability was estimated using an MTT assay kit (Sangon, Shanghai, China). After 4 hrs incubation in MTT assay reagent, the medium was replaced with 150 μ l dimethylsulfoxide (DMSO, Sangon, Shanghai, China). The absorbance at 490 nm was measured using a microplate spectrophotometer (BioTek, Vermont USA).

Wound-healing Assay

Cells were transfected in 6-well-plate with a range of constructs as indicated. When cells reached about 90% confluent, a scratch was produced in the cell monolayer by drawing a 200- μ l-pipette tip over the surface of each well, holding the tip perpendicular to the plate. The monolayers were washed and cultured with DMEM medium without FBS and PS. Wound-healing was observed under a light microscope and pictures were taken at 0, 24 and 48 hrs at the same position to observe cell movement.

Western Blotting

Whole cell protein extracts were prepared by lysis of cells using cold RIPA buffer (100 μ l/well, Beyotime, Shanghai, China). The concentration was measured using a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Shanghai, China). Equal protein amounts of each of the samples were denatured with 6 \times sodium dodecyl sulfate (SDS) loading buffer (Beyotime, Shanghai, China) at 100°C for 10 min. Protein samples were separated by electrophoresis on a 10% polyacrylamide SDS gel (Beyotime, Shanghai, China) and transferred onto 0.45- μ m nitrocellulose membranes (Beyotime, Shanghai, China). Following 60 min of blocking with 5% fat-free milk in double distillation H₂O, membranes were incubated with the primary antibodies in antibody diluent (Beyotime, Shanghai, China) overnight at 4 °C. Blots were washed with PBST (PBS with Tween), and incubated for 1 hr with the anti-rabbit or anti-mouse secondary antibody, as appropriate (1:2000, Santa Cruz Biotechnology, USA). Immunoreactive protein bands were detected with an Odyssey Scanning system (Li-Cor, USA). The density of the bands was measured by ImageStudio.

The following primary antibodies and dilutions were used:

β -actin (1:2000, Santa Cruz Biotechnology, USA), LATS2 (1:1000, Bioworld, Shanghai, China), PCNA (1:2000, Danvers, MA, USA) and WNT5A (1:1000, Proteintech, Chicago, USA).

Dual-luciferase Reporter Assay

PmirGLO-hsa_circ_0029693 mutant and wild type reporter plasmids, pmirGLO-WNT5A 3'-UTR mutant and wild type reporter plasmids were purchased from Integrated Biotech Solutions (Shanghai, China). 293T cells were seeded in 48-well plates, and co-transfected with pmirGLO-hsa_circ_0029693 mutant or wild type reporter plasmids, and miR-4686 or NC and vector using Lipofectamine 2000 reagent. These reagents were added into Opti-MEM according to the manufacturer's instruction. 293T cells were co-transfected with pmirGLO-WNT5A 3'-UTR wild type or mutant reporter plasmids, and miR-4686 or NC using Lipofectamine 2000 reagent. After 24 hours, firefly and Renilla luciferase activities were measured using a Dual Luciferase Reporter Assay (Promega, Madison, WI, USA). Firefly luciferase activity was normalized to the Renilla control, and the ratio of firefly/Renilla was recorded.

Animal Experiment

The five-week-old female athymic nude mice were used to do tumorigenicity in vivo. MDA-MB-231 cells were infected by lentiviruses. Mice were divided into two groups randomly. Over expressing circ-LATS2 or circ-NC MDA-MB-231 cells were implanted into the right side of second mammary fat pad of the mice (3×10^6 cells/mouse). After 5 weeks of observation, mice were sacrificed. The mice tumors were harvested, weighed and photographed.

Immunohistochemical Staining (ihc)

The mice tumors were formalin-fixed and paraffin-embedded (FFPE). 5 μ m FFPE sections were cut. Antibody LATS2 was used to do IHC following the protocol. The images were captured using Thermo Fisher microscope (Thermo Fisher Scientific, Massachusetts, USA). The ratio of brown areas was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

Databases Analysis

RegRNA 2.0 (<http://regrna2.mbc.nctu.edu.tw>) was used to identify regulatory circRNA motif and its binding site for miRNA ¹⁵. CircBase (<http://www.circbase.org>) was used to search for the sequence of circRNA ¹⁶. USCS Human Genome Browser (<https://genome.ucsc.edu/index.html>) was used to identify the position and the component of circRNA ¹⁷. Data from GEO, EGA, TCGA and Pubmed were combined in KMplotter (<https://kmplot.com/analysis/>), and Kaplan-Meier survival analysis was used to determine the association between expression levels of potential prognostic biomarkers and clinical outcome in a range of cancers including BC ¹⁸. KMplotter was used to evaluate the association between LATS2 expression and BC survival. TargetScan 7.2 (http://www.targetscan.org/vert_72/) was used to predict potential target genes of miRNAs ¹⁹. The Global Cancer Observatory (GCO) is a web-based program presenting global

cancer statistics²⁰. GCO was used to estimate age-standardized cancer incidence rates in 2018 worldwide.

Statistical analysis

Data were obtained from three independent experiments which are presented as the means \pm standard deviation. Differences were considered significant for *P*-values less than 0.05. GraphPad Prism version 8.0 was used for statistical analyses.

Results

Positive association between LATS2 and survival in BC

The association between LATS2 expression and survival in the TCGA breast cancer cohort was queried using KMplotter. This revealed that high expression of LATS2 from this cohort was associated with better survival than in which LATS2 expression was lower (Fig. S1). These results affirmed that LATS2 acts as a tumour suppressor in BC. To further understand the relationship between circRNA and its symbol gene, we searched for circ-LATS2 sequence on CircBase. It is reported that hsa_circ_0029693, position on chromosome 13:21619823-21620369, is a circRNA of LATS2.

The expression of circ-LATS2 in BC cells and BC patient samples

qRT-PCR revealed that circ-LATS2 was expressed at high level in HCC-1937, MDA-MB-231, MDA-MB-468, MCF-7 and SKBR-3 BC cells compared to the MCF-10A normal breast cell line. (Fig 1A). We randomly chose 30 BC patients, and divided them into three cohorts based on American Joint Committee on Cancer eighth edition, which are basal-like, Her2-like and luminal-like cohort. (Supplementary table 2). BC tissues as well as paired normal adjacent tissues were taken from these 30 patients. circ-LATS2 was mostly high expression in basal-like cohort (6 in 9, 67%), while it was low expression in luminal-like cohort (7 in 9, 78%) and Her2-like cohort (10 in 12, 83%) (Fig 1B-E, Fig S2 A-E). Moreover, we found that there was a relationship between circ-LATS2 expression and HER2 amplified. The expression of circ-LATS2 was higher in HER2 negative BC patients compared with HER2 positive ones. But there was not a significant difference between ER and PR groups. We also found that the expression of circ-LATS2 was higher in younger age group (age<60) (Fig S3). Based on each patient's circ-LATS2 expression, 30 patients were separated into two cohorts, low and high circ-LATS2 expression cohort. It is demonstrated that higher expression of circ-LATS2 was significantly related to higher percentage of Ki-67 expression (Fig 1F). These results demonstrated that though circ-LATS2 expression has no strong correlation with BC subtypes, it has significant correlation with Ki-67, suggesting that circ-LATS2 could be used as a co-biomarker with Ki-67 in clinical diagnosis and treatment.

The characteristic of circ-LATS2 in breast cells

UCSC was used to search the component of circ-LATS2, we found that circ-LATS2 was composed by one exon only (Fig 2A). Due to the closed loop structures, there is no 3' poly-A tail in circRNAs²¹. To confirm it, random hexamer or oligo (dT)₁₈ primers were used in MDA-MB-231 and SKBR-3 cell lines. When oligo (dT)₁₈ primers were used, the relative expression of circ-LATS2 was significantly downregulated compared with random hexamer primers. (Fig 2B). This result proved that circ-LATS2 has no 3' poly-A tail. In addition, we did nuclear and cytoplasmic isolation RNA extraction for MCF-10A and MDA-MB-231 cell lines. The results showed that 94% and 80% of circ-LATS2 was in cell nuclear in MCF-10A and in MDA-MB-231, respectively (Fig 2C). FISH experiment also confirmed the predominant nuclear distribution of circ-LATS2 (Fig 2D).

Silenced circ-LATS2 inhibited proliferation, viability and migration of BC cells

circ-LATS2 was silenced in MDA-MB-231 and HCC-1937 cells and cell proliferation and viability were estimated in colony formation and MTT assays (Fig 3A-B). circ-LATS2 siRNAs reduced colony number and lowered MTT activity compared with NC. This revealed that inhibiting circ-LATS2 suppressed cell proliferation and viability of BC cells. Western blotting analysis demonstrated that, in MDA-MB-231 cells, proliferation marker PCNA expression was inhibited by circ-LATS2 siRNAs, while it was promoted by over expressing circ-LATS2 (Fig SF4B). The influence of circ-LATS2 on migration was tested by silencing in MDA-MB-231 cells and performing a wound healing assay (Fig. 4). Cells low expression circ-LATS2 migrated markedly slower than the control group. After 24 and 48 hrs, limited migration was seen in the circ-LATS2 low-expression group, while the wound area in the control group was 77% filled. These results proved that silenced circ-LATS2 could inhibit cell proliferation, viability and migration in BC cells.

Over-expressed circ-LATS2 in nude mice accelerated BC tumor growth

MDA-MB-231 cells were stably transfected with circ-LATS2 or control. The expression of circ-LATS2 was affirmed by qPCR (Fig SF4A). The mice tumors were photographed, measured and weighted. The mice tumors of circ-LATS2 overexpression group is larger and heavier than control group (Fig 5A). The results proved that circ-LATS2 can accelerate BC tumor growth.

circ-LATS2 influence the expression of LATS2

IHC and western blot analysis were used to explore the relationship with circ-LATS2 and its parent gene, LATS2, in BC cells. The mice tumors were used to detect changes of LATS2 expression by IHC staining.

The results suggested that LATS2 expression in the circ-LATS2 over-expression group was higher than control group (Fig 5B). Also, through western blot, after silencing circ-LATS2 expression, the results showed that the expression of LATS2 was down-regulated, vice versa (Fig 6). These results suggested that circ-LATS2 influenced LATS2 protein expression.

circ-LATS2 could bind miR-4686 in BC cells

RegRNA 2.0 analysis predicted that miR-4686 was a potential miRNA-target of circ-LATS2 as shown in Fig 7A. miR-4686 is low expressed in MDA-MB-468, MDA-MB-231, MCF-7 and HCC-1937 BC cells compared with MCF-10A cells. (Fig 7B). Our observations suggested an inverse relationship between the expression levels of circ-LATS2 and miR-4686. A dual-luciferase reporter assay, using constructs containing wild-type and mutant sequences spanning the predicted binding sites in circ-LATS2, revealed a 36% reduction in luciferase activity from the wild-type circ-LATS2 reporter when co-transfected with miR-4686, and no change with the mutant sequence-miR-4686 combination compared to the control. (Fig 7C). This suggested that circ-LATS2 might act as a RNA “sponge” targeting miR-4686. Furthermore, we test miR-4686 expression in 10 BC patients (chose randomly from 30 BC patients), the results demonstrated that miR-4686 was significantly low expression in BC patient tissues (Fig 7D).

miR-4686 directly inhibited WNT5A expression, and inhibited proliferation in BC cells

To explore the potential consequences of down-regulating miR-4686 in BC cells, a search was performed for putative miR-4686 targets. The database TargetScan 7.2 predicted that WNT5A was a potential target of miR-4686, and this was confirmed using a dual-luciferase reporter assay. Constructs containing wild-type and mutant sequences spanning the predicted binding sites of miR-4686 were combined in the assay with WNT5A (Fig 8A). This revealed a 31% decrease in luciferase activity when the wild type miR-4686 construct was combined with WNT5A while no significant change in activity was observed with the mutant sequence. Western blotting analysis (Fig 8B), revealed that increased expression of miR-4686 decreased WNT5A protein levels relative to NC, in MDA-MB-231 cells. In summary, miR-4686 can target WNT5A to suppress WNT5A expression in BC. miR-4686 were over-expressed in MDA-MB-231 cells. We found that miR-4686 reduced colony numbers and lowered MTT activity (Fig 8C-D). This revealed that miR-4686 inhibited cell proliferation and viability of MDA-MB-231.

Discussion

The world incidence rates of BC are around 46 per 100,000 person in both sexes and all ages. That is 1.5 times doubled than the second one, prostate cancer²² (Fig SF5). Ki-67 is a proliferative index in BC for acting as predictive and prognostic parameter. However, Ki-67 is not recommend in routine practice because it lacks standardization and reproducible assessment method²³.

CircRNAs are special having neither 5' caps nor 3' poly-A tails. Also, a covalently closed ring structure is possessed by circRNAs. Therefore, they are insensitive to exonuclease R and stable expression²⁴. Compared with linear RNA, the continuous loops make them more suitable as biomarkers²⁵. In our research, we confirmed that circ-LATS2 has no 3' poly-A tail and we also found that circ-LATS2 is highly expressed in BC cell lines and 67% TNBC tissues. Whereas, circ-LATS2 is expressed in low level in 81% luminal and HER2 positive BC tissues. Briefly, the expression of circ-LATS2 has no notable relationship with BC subtypes. However, circ-LATS2 shows significantly difference between HER2 negative and positive groups. Moreover, higher expression of circ-LATS2 has strong relationship with Ki-67 expression percentage. These results suggest that circ-LATS2 is a potential co-biomarker with Ki-67 as a proliferative factor in clinical treatment. To validate the suggestions, we tested proliferation function of circ-LATS2. We confirmed that silenced circ-LATS2 expression decreased cell proliferation and viability in BC cells. We also confirmed that over-expressed circ-LATS2 promoted tumor growth *in vivo*. These results suggested that circ-LATS2 have strong proliferation function and could be used as a potential proliferative factor.

Another function of circRNAs is that it has the ability to affect their parent genes expression. The intron-derived circRNAs regulate their parent genes expression by RNA Pol II machinery²⁶, additionally, the exon-intron circRNAs promote the transcription of their parent gene by interacting with U1 snRNP and RNA Pol II machinery in the nucleus²⁷. Although circ-LATS2 is an exonic circRNA, the results showed that circ-LATS2 could promote LATS2 expression. However, the mechanism of this needs to be explored further.

Furthermore, circRNAs act as a miRNA "sponge" to modulate gene transcription²⁸. The circRNA-miRNA construction could eventually regulate cell proliferation, differentiation, invasion and metastasis through cell cycle regulation, signal transduction, epigenetic modulation or transcriptional regulation²⁹. For instance, circ-ITCH-miR-17/miR-224-p21 could target PTEN in bladder cancer to inhibit cancer progression³⁰. Hsa_circRNA_101996-miR-8075 could activate TPX2 in cervical cancer to promote cancer proliferation and invasion³¹. In our study, we predicted that miR-4686 was a potential target for circ-LATS2. This was supported by the finding that there is an inverse relationship between the expression circ-LATS2 and miR-4686 in breast cell lines. We confirmed that circ-LATS2 can bind miR-4686 by targeting its 3'UTR.

MiRNAs are short non-coding RNAs which are reputed to cause mRNA degradation by directly targeting its 3'UTR³². Our study suggested that miR-4686 acted as a tumor suppressor in BC cell lines. In dual-luciferase assay, we confirmed that miR-4686 could target WNT5A which sharply decreased luciferase activity in the wild-type group.

Conclusions

Circ-LATS2 is an exonic circRNA mainly located in the cell nuclear. It expressed highly in BC cell lines. Although the expression of circ-LATS2 in BC patients tissues showed no relationship with BC subtypes, there was a strong relation between circ-LATS2 expression with Ki-67 expression percentage. circ-LATS2

acted as a tumor promoter in BC cells. Silencing circ-LATS2 inhibited BC cell proliferation and migration. Furthermore, through tumorigenicity *in vivo*, over-expression circ-LATS2 in nude mice accelerated BC growth. Through IHC and western blot, we found that circ-LATS2 can influence LATS2 expression. circ-LATS2 can also act as a miRNA sponge to bind miR-4686. Moreover, miR-4686 can directly regulated the expression of WNT5A. Based on our results, it is suggested that circ-LATS2 could be a proliferative factor in clinical treatment and might be a co-biomarker with Ki-67. Our group will continue to test circ-LATS2 expression in more patient samples. To further support the notion that circ-LATS2 could act as a co-biomarker, we will keep on collecting the patients' follow-up data to explicit useful information. With a better understanding of circRNAs, there could be a potential more effective biomarkers in cancer treatment.

List Of Abbreviations

Breast cancer (BC), Circular RNAs (circRNAs), microRNAs (miRNAs), hsa_circ_0029693 (circ-LATS2), 3'-untranslated region (3'UTR), triple negative breast cancer (TNBC), pcDNA3.1 (+) circRNA Vector-circ-LATS2 (OE-circ), pcDNA3.1 (+) circRNA Vector-circ-NC (OE-NC).

Declarations

Ethics approval and consent to participate: Shanghai Tenth People's Hospital ethics committee approved the study. Human tissues ethics reference number: SHSY-IEC-KY-4.0/18-105/01. Mice experiment ethics reference number: SHDSYY-2018-0600.

Consent for publication: Not applicable.

Availability of data and materials: Results shown here are include analysis based upon data generated by the TCGA Research Network: <https://www.cancer.gov/tcga>.

Competing interest: There is no conflict of interest in this research.

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Authors' contributions: Jiashu Hu designed, performed the experiment and wrote this manuscript; Kaiyao Hua co-performed the experiment; Changle Ji did western blot assays; Xuehui Wang did qRT-PCR, Hongming Song did part of cell line work; Tianqi Wu did part of cell line work; Dan Xie analysed data; Twingle Daniel and Mariah Tahan did language editing; Xueyu Zheng collected patients' clinical information; Lin Fang guided the experiment.

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Authors' contributions: JH designed, performed the experiment and wrote this manuscript; KH co-performed the experiment; CJ performed western blot assays and analysed the data; XW did performed RT-qPCR and analysed the data of them; HS did part of cell line work; TW did part of cell line work; DX acquired data by performing cell transfection, colony formation assays and counted colony numbers, then analysed and interpreted this data.; TD and MT assisted in interpreting the data and helped language editing of this manuscript; XZ collected patients' samples and clinical information; LF helped to perform the experiment and contributed to the design of experiments.. All authors read and approved the final manuscript.

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Supplemental Information

Figure S1: Expression of LATS2 in TCGA database. (A-D) Association between LATS2 expression and overall survival in BC cases in the TCGA database. (red line – high expression, black line – low expression, logrank test) (There are three Affymetrix ID for LATS2 in TCGA database, 223379_s_at, 223380_s_at, 227013_at).

Figure S2: Expression of circ-LATS2 in BC patients' samples. (A-E) Expression of circ-LATS2 in thirty BC patients' samples. (Student's t-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure S3: Expression of circ-LATS2 in different types of BC. Expression of circ-LATS2 in HER2 negative (-) or positive (+), ER (-) or (+), PR (-) or (+), age < 60 or age ≥ 60 . (Sidak's multiple comparisons test, * $p < 0.05$, ** $p < 0.01$).

Figure S4: circ-LATS2 influenced PNCA expression in BC cells. (A) Relative circ-LATS2 expression after transfection of pcDNA3.1 (+) circRNA Vector-circ-LATS2 (OE-circ) and pcDNA3.1 (+) circRNA Vector-circ-

NC (OE-NC) in MDA-MB-231 cells (Student's t-test, **** $p < 0.0001$). (B) Effect of circ-LATS2 siRNA1 (si-circ1), circ-LATS2 siRNA2 (si-circ2) and over-expressed circ-LATS2 (OE-circ) level of PNCA proteins in MDA-MB-231 cells (paired Student's t-test, * $p < 0.05$).

Figure S5: BC incidence rate in 2018 worldwide. The lateral axis of bar chart is age-standardised cancer incidence rates (per 100,000). The vertical axis is different types of cancer.

Supplementary table 1: The sequences of siRNA and miRNA.

Supplementary table 2: The clinical data of 30 breast cancer patients.

Figures

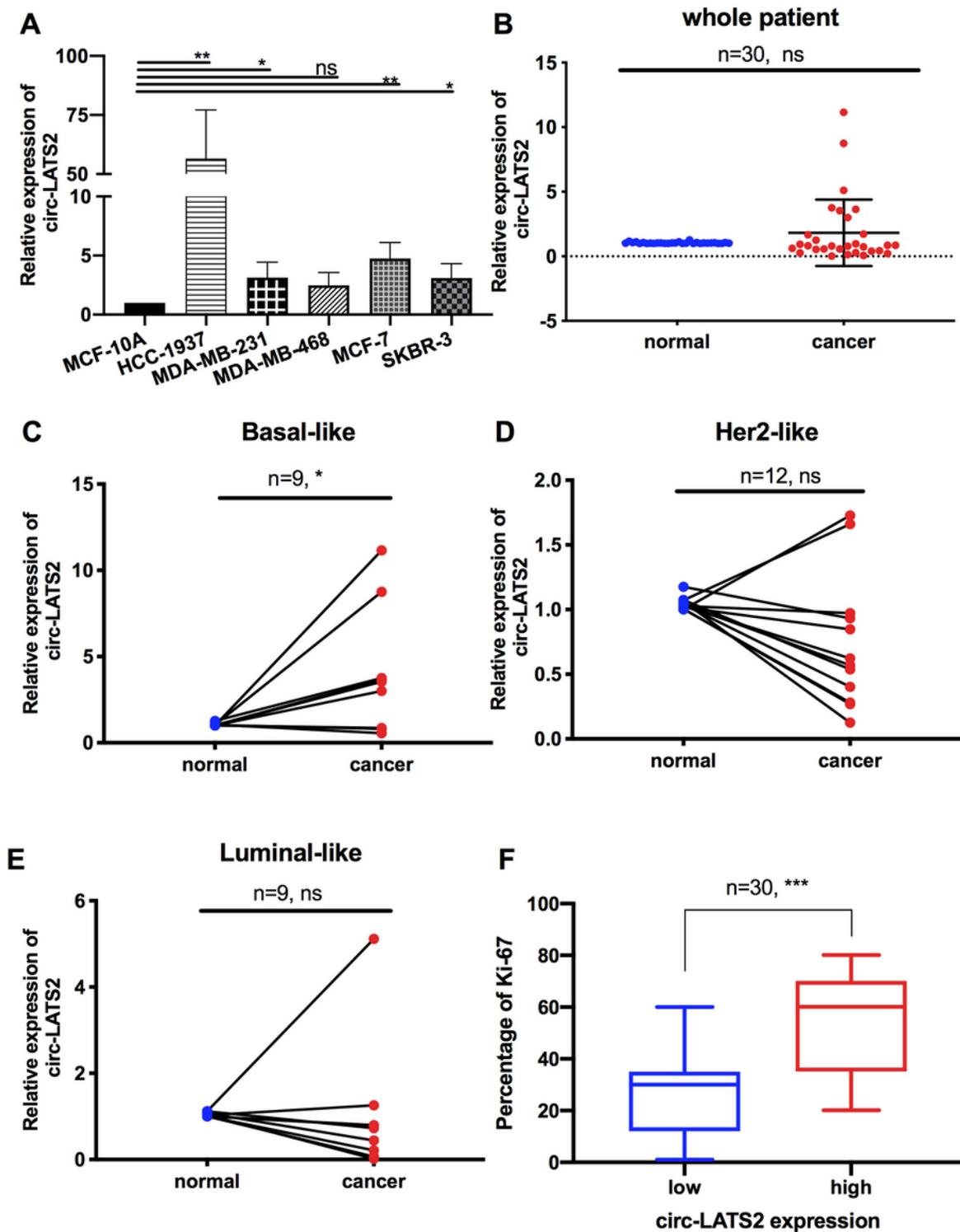


Figure 1

Expression of circ-LATS2 in BC lines and BC patients' samples. (A) Expression of circ-LATS2 in HCC-1937, MDA-MB-231, MDA-MB-468, MCF-7 and SKBR-3 breast cancer, and MCF-10A transformed normal breast cells. (B-E) Expression of circ-LATS2 in thirty BC patients samples, basal-like cohort, Her2-like cohort and luminal-like cohort, respectively. (F) Percentage of patients' Ki-67 expression in low and high circ-LATS2 expression cohort. (Student's t-test, ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

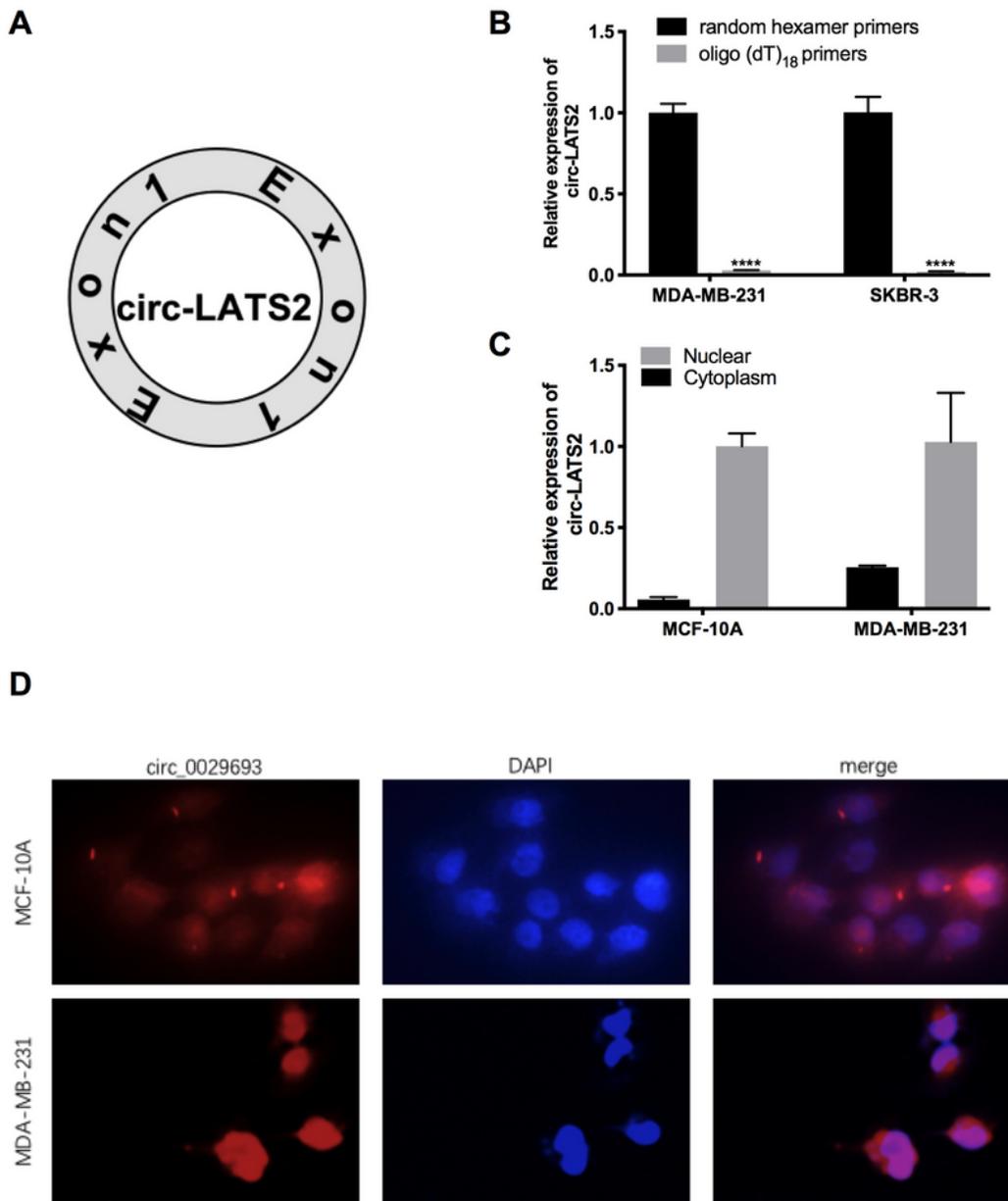


Figure 2

The characteristic of circ-LATS2 in BC. (A) circ-LATS2 composed by one exon. (B) Expression of circ-LATS2 in MDA-MB-231 and SKBR-3 cells with random hexamer or oligo (dT)₁₈ primers. (Student's t-test, **** $p < 0.0001$) (C) Expression of circRNA in cell nuclear and cytoplasm in MCF-10 and MDA-MB-231 cells. (D) RNA FISH for circ-LATS2. Nuclei was stained with DAPI.

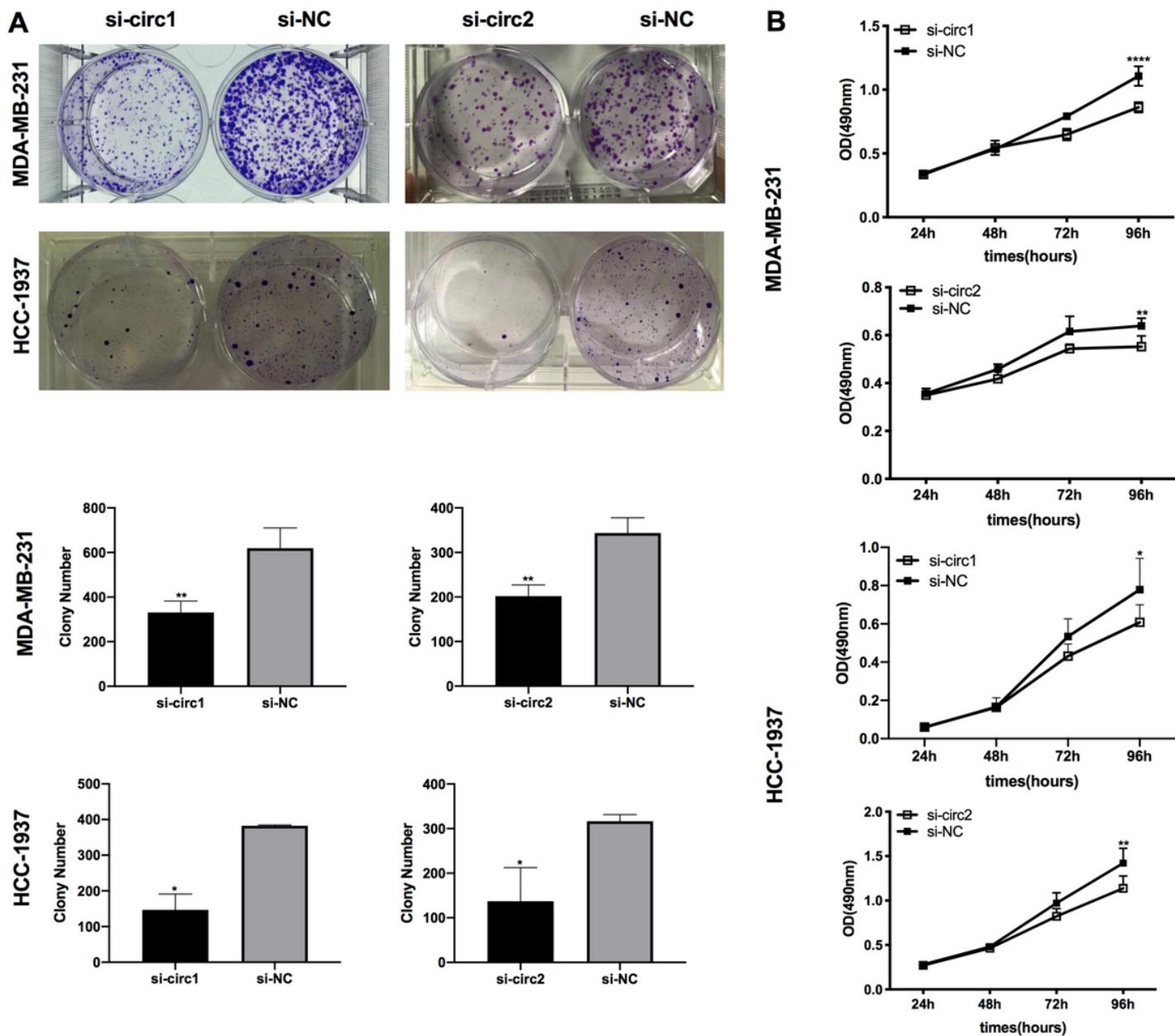


Figure 3

circ-LATS2 siRNA suppressed proliferation and viability of BC cells. (A) Effect of circ-LATS2 siRNA1 (si-circ1) and circ-LATS2 siRNA2 (si-circ2) on proliferation in MDA-MB-231 and HCC-1937 cells by colony formation assay (Student's t-test, * $p < 0.05$, ** $p < 0.01$). (B) Effect of si-circ1 and si-circ2 on viability in MDA-MB-231 and HCC-1937 cells by MTT assay (Student's t-test, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

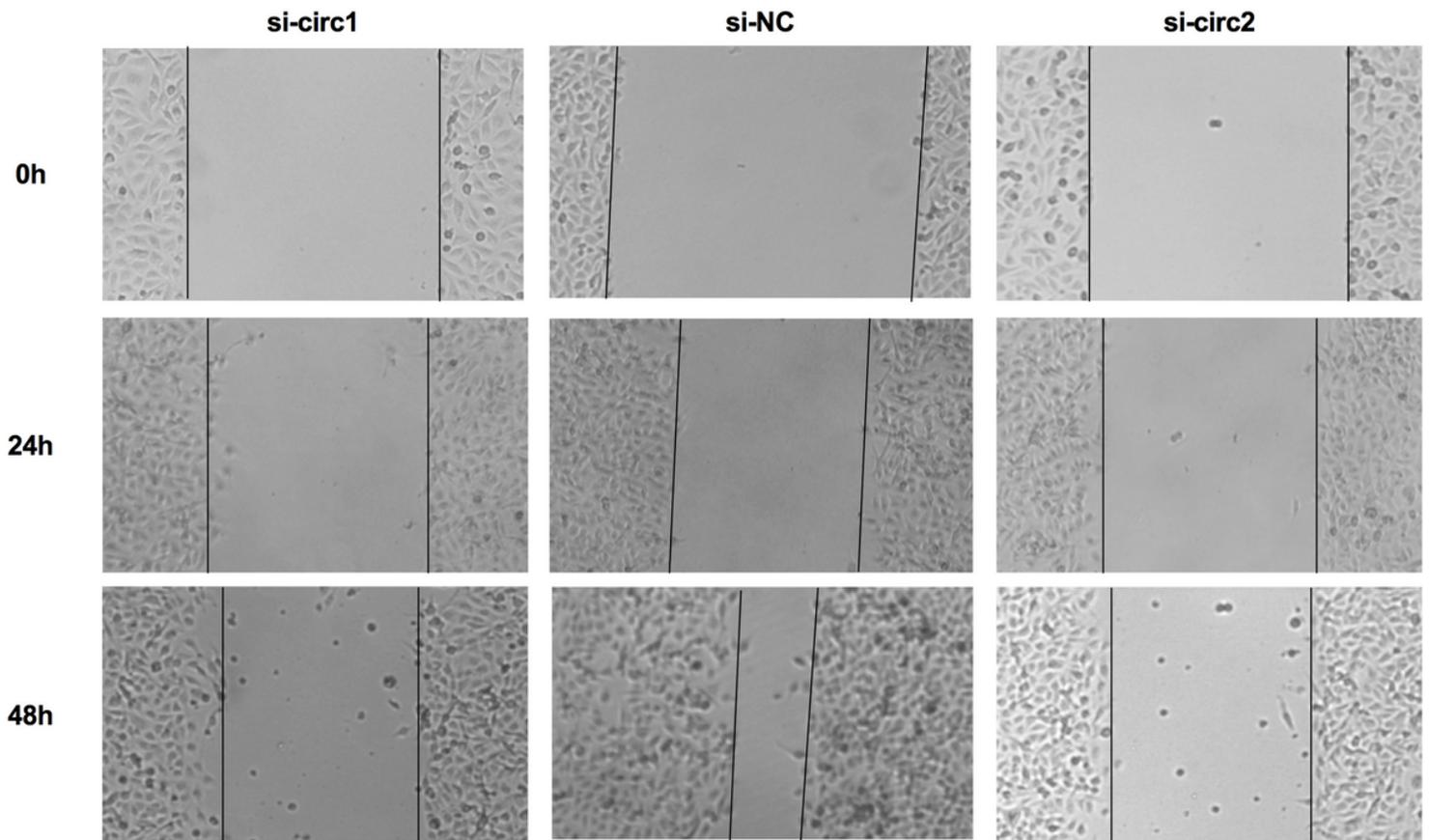


Figure 4

circ-LATS2 siRNA suppressed migration of BC cells. Effect of circ-LATS2 siRNA1 (si-circ1) and circ-LATS2 siRNA2 (si-circ2) on wound healing in MDA-MB-231 cells.



Figure 5

Over-expressed circ-LATS2 promoted BC growth in nude mice models. (A) Xenografts tumors from over-expressed circ-LATS2 (OE-circ) and negative control (OE-NC) group were harvested and photographed (n=3). Tumor volume and weight were measured. (Student's t-test, * $p < 0.05$) (B) IHC staining of LATS2 for mice tumors from OE-circ and OE-NC group. (n=5, Student's t-test, *** $p < 0.001$)

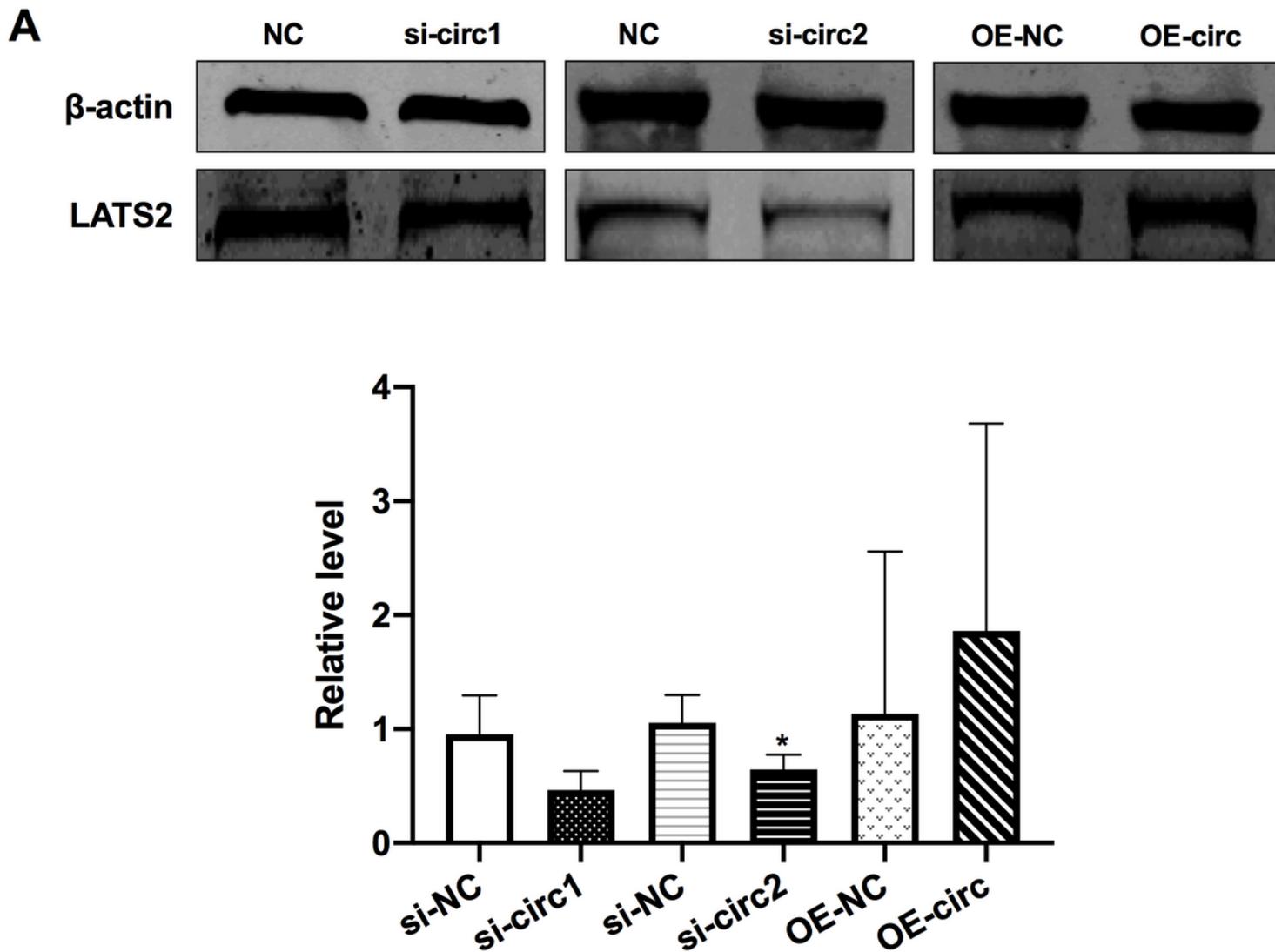


Figure 6

circ-LATS2 influenced expression of LATS2 in BC cells. Impact of circ-LATS2 siRNA1 (si-circ1), circ-LATS2 siRNA2 (si-circ2) and over-expressed circ-LATS2 (OE-circ) level on the expression of LATS2 proteins in MDA-MB-231 (paired Student's t-test, * $p < 0.05$).

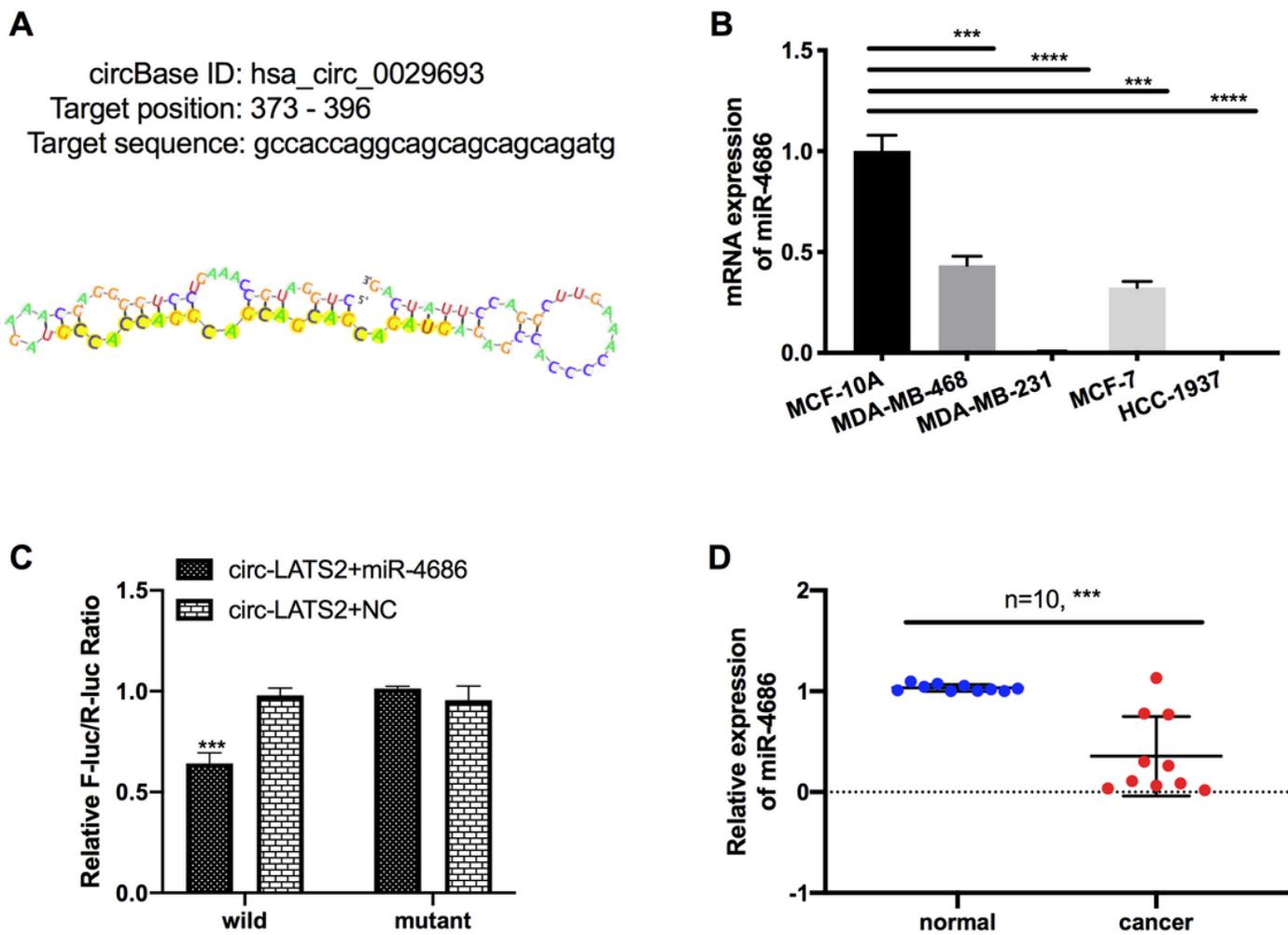


Figure 7

Expression of miR-4686 in BC cells: miR-4686 is a miRNA-target of circ-LATS2. (A) Predicted binding site of circ-LATS2 for miR-4686. (B) Expression of miR-4686 in MDA-MB-468, MDA-MB-231, MCF-7 and HCC-1937 breast cancer, and MCF-10A transformed normal breast cells. (Student's t-test, ***p < 0.001, **** p < 0.0001). (C) Dual-luciferase reporter assay evaluating interaction between wild-type circ-LATS2 + miR-4686 reporter compared with mutant circ-LATS2 + miR-4686 reporter. (D) Expression of miR-4686 in ten BC patients' samples . (Student's t-test, ***p < 0.001).

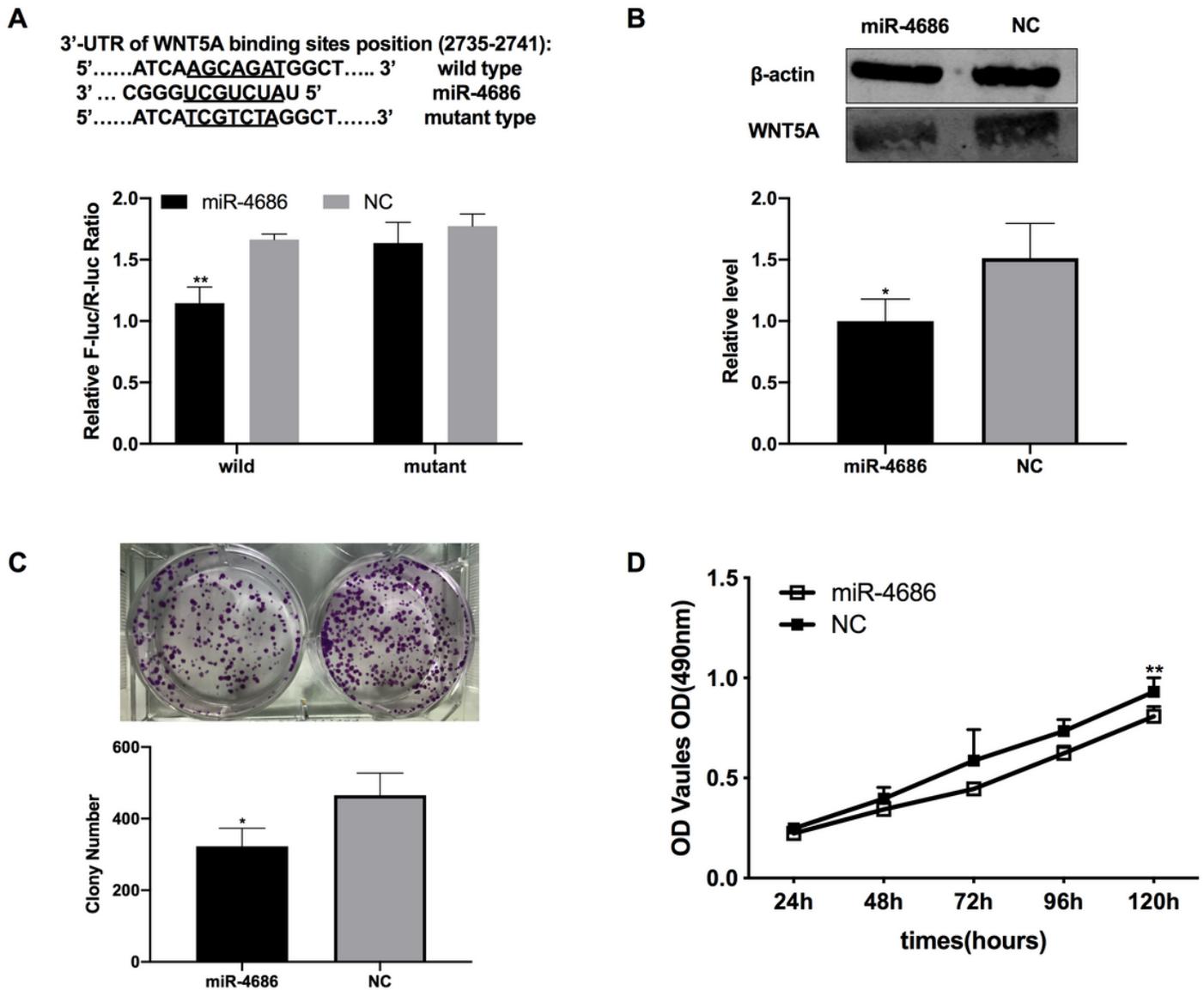


Figure 8

WNT5A is a direct target gene of miR-4686 and miR-4686 suppressed proliferation of BC cells. (A) miR-4686 binding sites homology for the 3'-UTR of wild type and mutant WNT5A. Dual-luciferase reporter assay for miR-4686 interaction with wild type and mutant WNT5A (Student's t-test, ** $p < 0.01$). (B) Impact of miR-4686 level on the expression of WNT5A in MDA-MB-231 (paired Student's t-test, * $p < 0.05$). (C) Effect of miR-4686 on colony formation in MDA-MB-231 cells (Student's t-test, * $p < 0.05$). (D) Effect of miR-4686 on proliferation in MDA-MB-231 cells by MTT assay (Student's t-test, ** $p < 0.01$).

Supplementary Files

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