

Original Article

Hyaluronan synthase 2 overexpression is correlated with the tumorigenesis and metastasis of human breast cancer

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Abstract: Extracellular matrix (ECM) is closely correlated with the malignant behavior of breast cancer cells. Hyaluronan (HA) is one of the main components of ECM, and actively regulates cell adhesion, migration and proliferation by interacting with specific cell surface receptors such as CD44 and RHAMM. HA synthase 2 (HAS2) catalyzes the synthesis of HA, but its role in breast tumorigenesis remains unclear. This study assessed the roles of HAS2 in malignant behavior of human breast cancer and sought to provide mechanistic insights into the biological and pivotal roles of HAS2. We observed HAS2 was overexpressed in breast cancer cell lines and invasive duct cancer tissues, compared with the nonmalignant breast cell lines and normal breast tissues. In addition, a high level of HAS2 expression was statistically correlated with lymph node metastasis. Functional assays showed that knockdown of HAS2 expression inhibited breast tumor cell proliferation in vivo and in vitro, through the induction of apoptosis or cell cycle arrest. Further studies showed that the HA were elevated in breast cancer, and HAS2 could upregulate HA expression. In conclusion, HAS2-HA system influences the biological characteristics of human breast cancer cells, and HAS2 may be a potential prognostic marker and therapeutic target in breast cancer.

Keywords: Breast cancer, hyaluronan, hyaluronan synthase 2, metastasis, RNA interference, tumorigenesis

Introduction

Breast cancer is the most common malignancy and the major cause of cancer-related mortality of women worldwide [1]. Several studies have demonstrated a close correlation between extracellular matrix (ECM) and the malignant behavior of breast cancer cells. Hyaluronan (HA) is one of the main components of the extracellular matrix. It is a nonsulfated glycosaminoglycan made up of repeating disaccharide units, D-glucuronic acid and N-acetyl-D-glucosamine. The amount of HA is high in many tumors, where HA abundance is associated with host-tumor interactions [2], epithelial-mesenchymal transition (EMT) [3], multidrug resistance [4] and angiogenesis [5-7]. These diverse effects of HA may be due to its physical properties, e.g. rendering the environment more favorable for cell movement, or to specific signals mediated either by its own receptors, CD44

and the receptor for hyaluronan-mediated motility (RHAMM), or to modification of the responses of growth factor receptors [5-8]. In vitro studies have revealed that the most aggressive breast carcinoma cell lines both synthesize high amounts of hyaluronan and express the cell surface hyaluronan receptors, CD44 and RHAMM, unlike less aggressive cell lines [9-11].

Hyaluronan is synthesized by a multi-isoform family of transmembrane glycosyltransferases termed the HA synthases [12]. Three eukaryotic HAS isoforms have been identified: HAS1, HAS2 and HAS3. Sequence data from the HAS isoforms suggest that they contain seven membrane-associated regions and a central cytoplasmic domain possessing several consensus sequences that are substrates for phosphorylation by protein kinase C [12, 13]. Although each of the HAS isoforms is capable of hyaluronan

synthesis, they synthesize hyaluronan of different lengths. The HAS2 isoform synthesizes hyaluronan molecules with molecular weight (Mw) greater than 3.9×10^6 , HAS3 synthesizes polydisperse hyaluronan (Mw of $0.12\text{--}1 \times 10^6$) and HAS1 synthesizes much smaller chains (Mw of 0.12×10^6) [14]. Moreover, the HAS isoforms exhibit different catalytic activities: HAS3 is catalytically more active than HAS2, which in turn is more active than HAS1 [14].

Within the tumor environment, the production of high molecular weight HA is thought to provide a hydrated matrix which forces gaps in the ECM, enabling tumor cells to migrate and metastasize to other tissues [15]. Several studies have demonstrated that the manipulation of HAS genes alters the tumorigenicity of malignant tumors [16–21], including breast cancer [22, 23]. Manipulation of the HAS genes has enabled the over-expression or inhibition of the different HA synthase isoforms, which has provided a preliminary insight into the role of HA synthesis in cancer [15]. The over-expression or inhibition of HAS2 has generated more profound results; several studies have demonstrated that, in a variety of cancer cells, HAS2 is responsible for the generation of an HA pericellular coat, anchorage-independent growth and tumor formation [24–26]. The contrary has also been observed, where very high levels of HAS2 expression inhibited tumor growth [26].

To determine whether HAS2 is correlated with the malignant behavior of human breast cancer, in this study we investigated HAS2 mRNA and protein expression in the breast cancer cells BT549, Hs578T, MCF-7, MDA-MB-231, MDA-MB-468, SK-BR-3 and T47D, nonmalignant breast epithelial cells MCF-10A and HBL-100, 55 invasive duct cancer tissues and 55 relative normal breast tissues. We also suppressed HAS2 expression by the use of specific HAS2-shRNA in Hs578T cells, and thus evaluated the relationship between HAS2 and the malignant behavior of breast cancer in vitro and in vivo.

Materials and methods

Tissue specimens, cell lines and cell culture

A total of 55 invasive ductal carcinoma breast tissue samples and 55 relative normal breast tissue samples were obtained from patients who underwent surgical treatment at the First

Affiliated Hospital of Chongqing Medical University from January to July in 2012, after obtaining informed consent. None of the patients received therapy before surgery. All samples were evaluated and subject to histological diagnosis by pathologists. Clinical information, including age, tumor grade, tumor size, and follow-up data after initial diagnosis, and treatment, was obtained for the majority of tumor cases. Grading of tumors was achieved by staining with hematoxylin and eosin (H&E). All patients provided written consent for participation in the study. The study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (Approval notice: 2010/2012 (23)).

Seven breast tumor cell lines (BT549, Hs578T, MCF-7, MDA-MB-231, MDA-MB-468, SK-BR-3, T47D) were used. Human normal mammary epithelial cell lines MCF-10A and HBL-100 were used as controls. All cell lines were acquired from the cell bank of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. These cells were maintained in RPMI 1640 (Gibco-BRL, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories, Linz, Austria), 100 U/ml penicillin and 100 mg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂.

RNA extraction and quantitative real-time PCR

Total RNA from cells and tissues was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was performed using random hexamers, and reverse transcription-PCR was performed using Go-Taq (Promega, Madison, WI, USA), with GAPDH as a control, and the following primers: HAS2-F: 5'-TCGC-AACACGTAACGCAAT-3' and HAS2-R: 5'-ACTTCTCTTTTCCACCCCATTT-3'. The PCR program consisted of initial denaturation at 95°C for 2 min, followed by 32 cycles (for HAS2) or 23 cycles (for GAPDH) of the reaction (94°C for 30 s, 55°C for 30 s and 72°C for 30 s), with a final extension at 72°C for 10 min. Real-time PCR (rtPCR) was performed using Maxima SYBR Green/ROX qPCR Master Mix (MBI Fermentas, St. Leon-Rot, Germany). The thermal-cycling reaction was performed in the 7500 Real-Time PCR System (Applied Biosystems). The nucleotide sequences of the primers used for PCR amplification were as follows: (1) HAS2-F: 5'-TCGCAACACGTAACGCAAT-3' and HAS2-R:

5'-ACTTCTCTTTTCCACCCCATTT-3' (GenBank accession No. NM_005328), (2) GAPDH-F: 5'-TCCTGTGGCATCCACGAACT-3' and GAPDH-R: 5'-GAAGCATTG CCGTGGACGAT-3' (GenBank accession No. NM_001101). Melting-curve analysis and agarose gel electrophoresis of PCR products were performed.

RNA interference

The sequence of human HAS2 mRNA was obtained from Genbank (accession No. NM_005328). The siRNA targeting HAS2 were designed using a program available online (<http://jura.wi.mit.edu/bioc/siRNAext/>). The selected sequences were screened further against the human genome using BLAST searches to avoid nonspecific silencing. The top-ranked gene sequence was chosen for synthesis of short hairpin RNA (shRNA) in this study. The shRNA was synthesized and cloned into the pGenesil-1 vector (Genesil Corp, Shanghai, China) according to the manufacturer's instructions. The HAS2-siRNA sequence was 5'-GCGATTATCACTGGATTCT-3' (target nucleotide sites: 1676-1694 bp) and the negative-siRNA (N-siRNA) sequence was 5'-GTTCT-CCGAACGTGTACAGT-3'. All constructs were verified by DNA sequencing. The plasmids were transfected into breast cancer cells with lipofectamine™ 2000 transfection reagent (Invitrogen). Stably transfected cells were identified by selection for 3 weeks with 0.2 mg/ml G418. The same volumes of culture medium were used for the transfection control. Thus, three groups of cell were produced (control, N-shRNA, HAS2-shRNA).

Immunohistochemical staining

Immunohistochemical staining was performed on paraffin-embedded specimens. Slides were routinely deparaffinized and hydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min, and the deparaffinized sections in 10 mM citrate buffer were microwaved for 30 min for epitope retrieval. Subsequently, the sections were incubated with an antibody against HAS2 (goat polyclonal, 1:50 dilution, Santa Cruz Biotechnology, USA) for 18 h at 4°C in 2% bovine serum albumin in phosphate-buffered saline (PBS). A secondary antibody was added and incubated for 1 h at 37°C. The sections were counterstained with hematoxylin for 3-5 min. PBS, instead of primary antibody, was used as a negative control.

Evaluation of HAS2 expression by IPP (version 6.0, Media Cybernetics, Silver Spring, MD) was performed as described previously [27]. Briefly, five digital images at 1360 × 1024 pixel resolution and 400 × magnification were captured by a LEICA DM500 ICC50 microscope (Leica microsystems, Germany).

Western blot

The cells and fresh tissue were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Thermo Scientific, Cramlington, UK) containing a protease inhibitor cocktail (Sigma Aldrich). A total of 50 µg of protein lysate for each sample was separated using sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE). The lysates were transferred to PVDF membranes for antibody incubation. After blocking with 5% nonfat milk and 0.1% Tween 20 in TBS, the membranes were incubated with HAS2 antibody (Santa Cruz Biotechnology, USA). The immunoblots were visualized using an enhanced chemiluminescence detection system. Beta-actin was used as a control.

Cell proliferation assay

A Cell Count Kit-8 (CCK-8, Beyotime, China) was employed for quantitative evaluation of cell viability. Briefly, 2×10^3 cells/well were seeded in 96-well flat-bottomed plates, and then grown at 37°C for 24, 48, 72, and 96 h. Subsequently, the original medium in each well was replaced by 200 µl 10% FBS/RPMI 1640 medium contain 20 µl CCK-8. The cells were incubated at 37°C for 2 h, and the absorbance was determined at wavelengths of 450 nm using a microplate reader. RPMI 1640 containing 10% CCK-8 was used as a control.

Wound-healing assay

HAS2-shRNA and N-shRNA transfected cells (Hs578T) were selected using G418, and cultured in six-well plates until confluent. After scratching the monolayer, cells were photographed at 0, 12, and 24 hours under a 10 × objective (Leica DMI4000B, Milton Keynes, Bucks, UK).

Flow cytometry analysis of cell cycle and apoptosis

Flow cytometry analyses of cell cycle and apoptosis was performed as described previously

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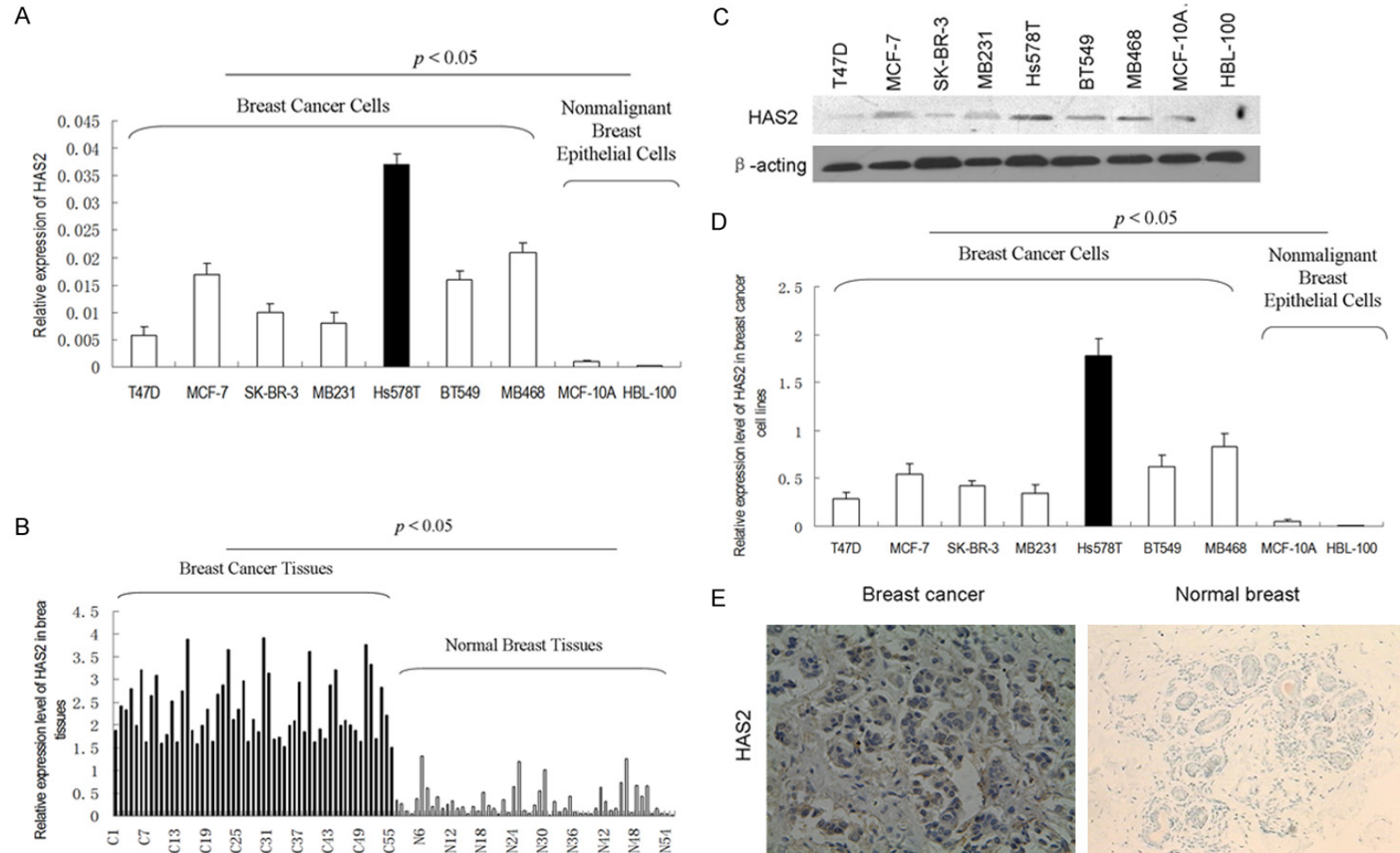


Figure 1. Overexpression of HAS2 in breast cancer cells and tissues. Expression of HAS2 mRNA and protein were examined by quantitative real-time PCR (A, B), western blotting (C, D) and immunohistochemistry (E); Magnification, 400 \times). HAS2 was overexpressed in breast cancer cell lines and invasive duct cancer tissues, compared with nonmalignant breast cells and normal tissues (* $P < 0.05$).

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Table 1. Relationship of HAS2 expression with clinicopathologic factors of breast cancer

| Clinicopathological features | Number (N=55) | HAS2 expression status | | P value [#] |
|-----------------------------------|------------------|------------------------|-----|----------------------|
| | | (-) | (+) | |
| Age | | | | |
| ≤ 40 | 7 | 2 | 5 | > 0.05 |
| > 40 | 48 | 18 | 30 | |
| Stage | | | | |
| I | 7 | 2 | 5 | > 0.05 |
| II | 29 | 13 | 16 | |
| III | 19 | 11 | 8 | |
| Lymph node metastasis | | | | |
| Positive | 29 | 9 | 20 | < 0.05 |
| Negative | 26 | 16 | 10 | |
| Oestrogen receptor (ER) status | | | | |
| Positive | 21 | 10 | 11 | > 0.05 |
| Negative | 34 | 13 | 21 | |
| Progesterone receptor (PR) status | | | | |
| Positive | 23 | 10 | 13 | > 0.05 |
| Negative | 32 | 15 | 17 | |
| HER-2 | | | | |
| Positive | 35 | 17 | 18 | > 0.05 |
| Negative | 20 | 7 | 13 | |
| P53 | | | | |
| Positive | 36 | 15 | 21 | > 0.05 |
| Negative | 19 | 8 | 11 | |

[#]P values are from χ^2 or Fisher's exact test and were statistically significant when < 0.05.

[28, 29]. For cell cycle analysis, the cells were fixed in ice-cold 70% ethanol and stained with propidium iodide (PI). The cell-cycle profiles were assayed using an Elite ESP flow cytometer at 488 nm, and the data were analyzed using CELL Quest software (BD Biosciences, San Jose, CA). For analysis of apoptosis, annexin V-FITC/PI staining was also performed using flow cytometry according to the manufacturer's guidelines. Briefly, cells were incubated with PI and annexin V-fluorescein isothiocyanate in the dark at room temperature. Flow cytometric analysis was performed immediately.

Cell invasion assay

Cell invasion was investigated using a transwell (Costar) with a pore size of 0.8 μ m; 1×10^5 cells were seeded in serum-free medium in the upper chamber (a matrigel-coated chamber for invasion assay), with medium containing 10%

FBS in the lower chamber. After incubation for 8 h at 37°C, the cells in the upper chamber were removed carefully with a cotton swab, and the cells that had traversed to the reverse face of the membrane were fixed in methanol, stained with Giemsa, and counted.

In vivo assay

All experimental procedures were carried out in accordance with the National Institute of Health Guidelines for the care and use of laboratory animals. Hs578T cells, at 1×10^7 cells per 100 μ l of serum-free medium, were injected into the right flanks of 4-week-old female athymic mice. The mice were divided randomly into three groups (control, N-shRNA and HAS2-shRNA) with six mice in each group. Tumor size was measured externally every 3 days using a caliper, and tumor volume was estimated using the equation: length (mm) \times width² (mm) \times 0.52. The mice were sacrificed 4 weeks after the transplant, and their tumors were weighed after dissection. Samples from each area were snap-frozen at -80°C for protein preparation, and

the corresponding tissue samples were fixed in 4% formalin to obtain paraffin-embedded sections.

ELISA-like assay for HA

Cells were grown in 24-well plates, washed twice with serum-free culture medium to remove HA accumulated during cell growth and incubated for 48 h. At the end of incubation time, aliquots of serum-free culture medium were collected and tested for the quantity of HA by ELISA kit (Echelon Bio).

Statistical analysis

Statistical analyses were performed using SPSS statistical software (SPSS, Chicago, Illinois). The results were expressed as mean \pm standard deviation (SD). Student's t test followed by least significant difference t test (LSD-

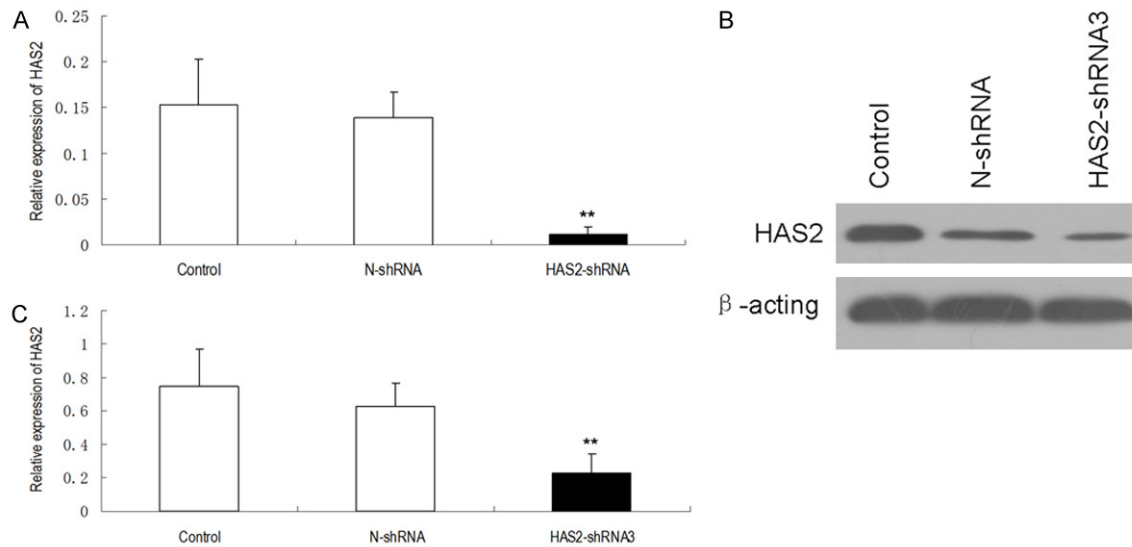


Figure 2. Knockdown of HAS2 expression by HAS2-shRNA. Real-time PCR showed the HAS2 mRNA was effectively knockdown by HAS2-shRNA in Hs578T breast cancer cells (A; $^{**}P < 0.01$). The HAS2 protein levels were examined by western blot analysis (B). The Hs578T cells showed a significant decrease in HAS2 expression levels, compared with control and the N-shRNA group (C; $^{**}P < 0.01$).

t) was used for group comparison, $P < 0.05$ was considered statistically significant.

Results

Expression of HAS2 in breast cancer cells and tissues

We first evaluated the levels of expression of HAS2 transcripts and protein in breast cell lines and tissues by quantitative real-time PCR, western blot analysis and immunohistochemistry staining. Our results confirmed that HAS2 was overexpressed in breast cancer cell lines and invasive duct cancer tissues, compared with nonmalignant breast cells and normal tissues. Next, quantitative real-time PCR suggested that the level of expression of HAS2 mRNA in Hs578T breast cancer cells was significantly higher than that in other breast cancer cells (Figure 1A-E).

We further analyzed the correlation between HAS2 expression and clinicopathological features including age, tumor stage, lymph node metastasis, estrogen receptor (ER) status, progesterone receptor (PR) status, HER-2 and P53. It was found that the expression of HAS2 was statistically correlated with lymph node metastasis; while there was no significant correlation between HAS2 expression and other factors (Table 1). These results suggest that the HAS2

may be involved in the progression and metastasis of breast cancer.

Knockdown of HAS2 expression by HAS2-shRNA

The shRNA-expressing (HAS2-shRNA, N-shRNA) plasmids were constructed using the pGene-sil-1 vector to target different regions of human HAS2 mRNA. These plasmids were transfected into Hs578T cells. After stably transfected cells were individually selected, the HAS2 and the housekeeping gene beta-actin mRNA and protein levels were measured using quantitative real-time PCR and western blotting. Compared with the control group, the HAS2 transcripts and protein levels were sharply reduced in HAS2-shRNA ($P < 0.01$, Figure 2A-C). The N-shRNA did not substantially affect the endogenous HAS2 expression ($P > 0.05$), and there was no difference in the beta-actin expression among groups. These results demonstrated that HAS2 was downregulated specifically and effectively by HAS2-shRNA showed higher efficiency to knockdown HAS2 expression.

Knockdown of HAS2 expression inhibited proliferation of breast cancer cells

The effects of HAS2 knockdown on cell growth at 24, 48 and 72 h were assessed by CCK-8 assay. In HAS2-shRNA3 transfected Hs578T

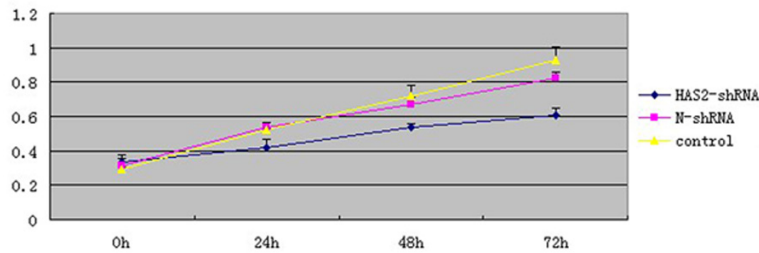


Figure 3. Knockdown of HAS2 inhibited the proliferation of breast cancer cells. The effects of HAS2 knockdown on cell growth at 24, 48 and 72 h were assessed by CCK-8 assay. In HAS2-shRNA3 transfected Hs578T cells, cell growth was significantly decreased at 48 h and 72 h, compared with control and the N-shRNA group (* $P < 0.05$).

cells, cell growth was significantly decreased at 48 h and 72 h, compared with control and the N-shRNA group ($P < 0.05$, **Figure 3**).

Knockdown of HAS2 expression inhibited the migration and invasion potential of breast cancer cells

One of the most distinct features of breast cancer cell is the invasive growth pattern, which prevents total surgical resection. Therefore, we evaluated the effect of HAS2 knockdown on the properties of cell migration and invasion.

The role of HAS2-knocked down tumor cells in migration was analyzed by the wound healing assay. We found that the relative migration distance of cells was significantly shorter in HAS2-shRNA3 transfected Hs578T cells when compared with the control and N-shRNA groups ($P < 0.01$, **Figure 4A, 4B**).

Matrigel-coated transwell assay was performed to assess the effect of HAS2 knockdown on the cell invasion tendency. The percentages of HAS2-shRNA3 transfected Hs578T cells that invaded through the matrigel were significantly lower than those of the control and N-shRNA groups ($P < 0.01$, **Figure 4C, 4D**). This indicated that Hs578T breast cancer cells exhibited significantly reduced invasive tendencies after HAS2 knockdown.

Knockdown of HAS2 expression induced G0/G1 cell cycle arrest and apoptosis of breast tumor cells

We further investigated the effects of HAS2 knockdown on the cell cycle and apoptosis of breast cancer cells. Representative results of cell-cycle distribution in control, N-shRNA and

HAS2-shRNA3 groups are shown in **Figure 5A**. Flow cytometry analysis revealed a statistically significant increase in the number of HAS2-shRNA3 transfected Hs578T cells in the G0/G1 phase, accompanied by a decrease in S cells, compared with control and the N-shRNA group ($P < 0.01$, **Figure 5B**).

Next, we evaluated the apoptotic effect of HAS2 knockdown in breast cancer cells using annexin V-FITC/PI staining assays. The percentage of annexin V-PI-positive cells was increased in HAS2-shRNA3 transfected Hs578T cells and compared with controls ($P < 0.01$, **Figure 5C, 5D**). These results indicated that the inhibitory effect of cell proliferation produced by knockdown of HAS2 expression was most likely mediated by G0/G1 cell cycle arrest and apoptosis.

Knockdown of HAS2 expression inhibited the tumorigenesis of breast cancer cells in vivo

To evaluate further the tumor-suppressive functions of HAS2 knockdown in vivo, tumorigenicity of Hs578T cells transfected with HAS2-shRNA3 was evaluated in nude mice. The growth of subcutaneous tumors in three groups of nude mice is shown in **Figure 6A**. Three weeks after injection, tumors were excised from tested mice for further analysis. The average volume of tumors induced by HAS2-shRNA3 transfected Hs578T cells was significantly decreased, compared with control tumors ($P < 0.01$, **Figure 6B**). The average tumor weight in the HAS2-shRNA3 group was less than that in the N-shRNA and control groups ($P < 0.05$, **Figure 6C**). Immunohistochemistry analyses confirmed that the expression of HAS2 protein was significantly inhibited in the HAS2-shRNA3 group (**Figure 6D**). These results indicate that HAS2 is correlated with the tumor proliferation in vivo.

HAS2 knockdown downregulated HA expression in the breast cancer cells

ELISA-like assay was used to measure HA secretion by breast cancer cells. As shown in **Figure 7**, compared with MCF-10A and HBL-100

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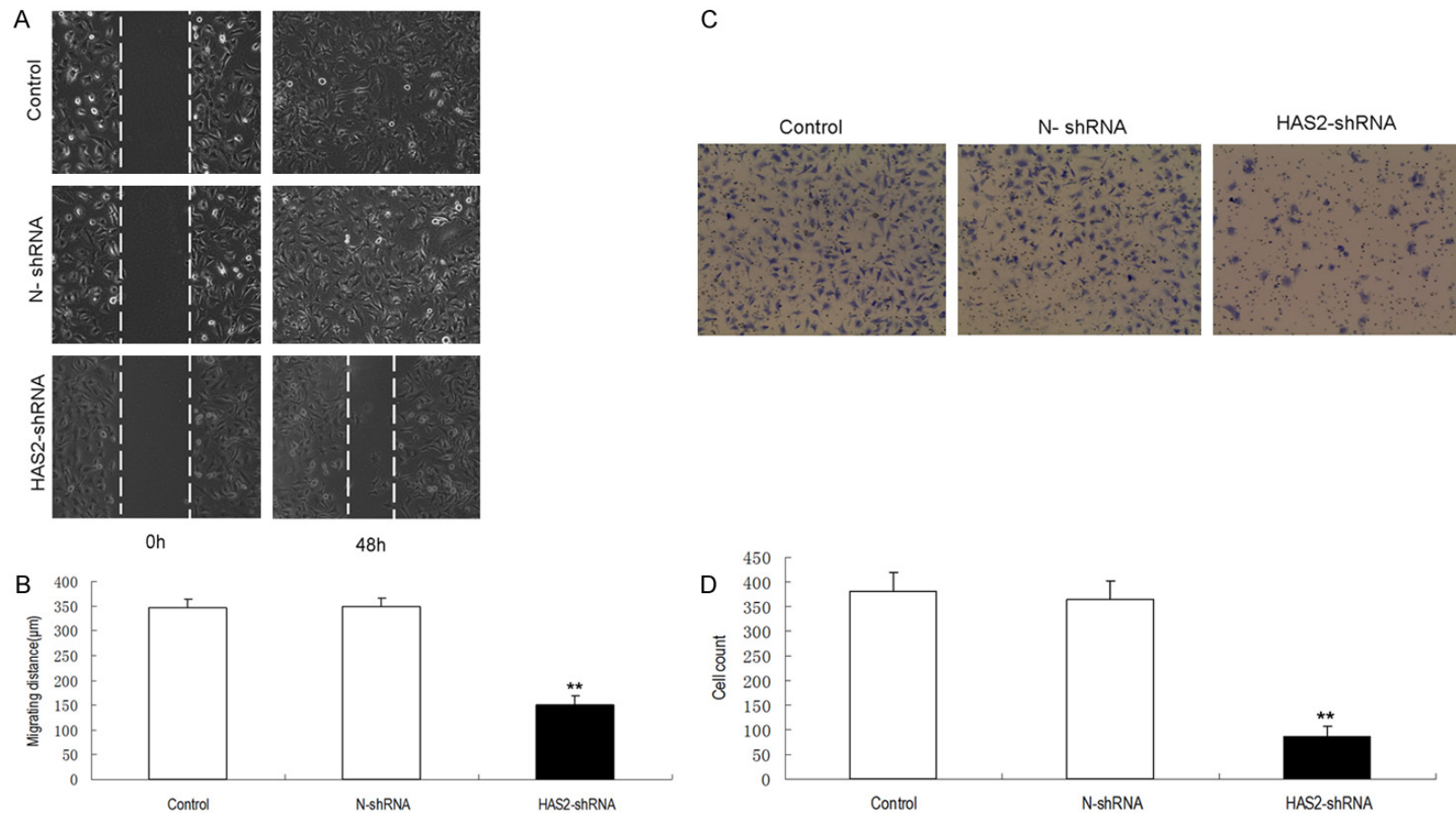


Figure 4. Knockdown of HAS2 inhibited the migration, invasion potential of breast cancer cells. A: Wound healing assay. Photographs show cells that have migrated into the wounded area and the histogram showed the relative migration distance of the cells. Compared with the control and N-shRNA groups, the relative migration distance of cells was significantly shorter in HAS2-shRNA3 transfected Hs578T cells. B: Quantitative analysis of wound healing (** $P < 0.01$). C: Matrigel-coated transwell assay. Photographs show cells that have invaded through the matrigel and the histogram shows the percentage of invasive cells. The percentages of HAS2-shRNA3 transfected Hs578T cells that invaded through the matrigel were significantly lower than those of the control and N-shRNA groups. D: Quantitative analysis of matrigel-coated transwell (** $P < 0.01$, Magnification, 40 \times).

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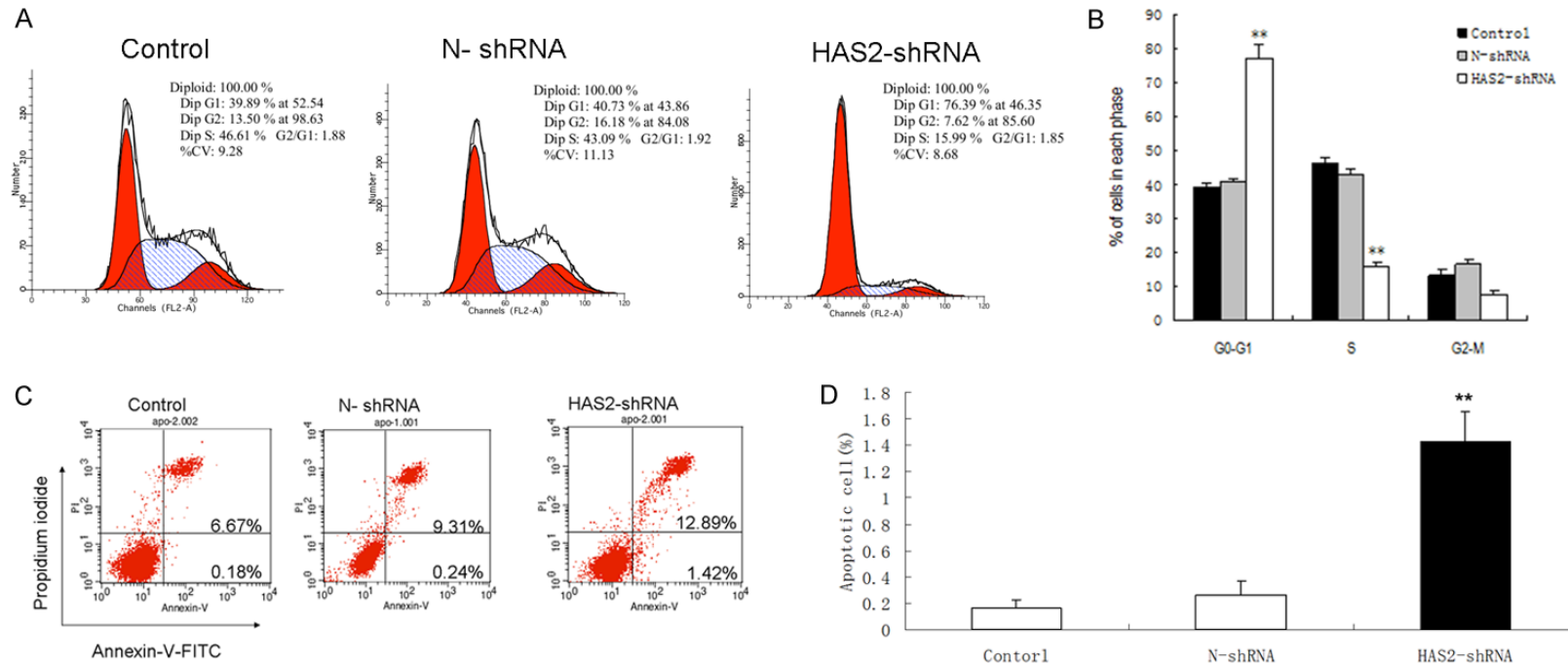


Figure 5. Knockdown of HAS2 expression induced cycle arrest and apoptosis of breast tumor cells. A: Representative cell cycle analysis. Flow cytometry analysis revealed a statistically significant increase in the number of HAS2-shRNA3 transfected Hs578T cells in the G0/G1 phase, accompanied by a decrease in S cells, compared with control and the N-shRNA group. B: Summarized flow cytometry data (** $P < 0.01$). C: Induction of apoptosis detected by flow cytometric analysis with annexin V-FITC and PI-staining. The percentage of annexin V-PI-positive cells was increased in HAS2-shRNA3 transfected Hs578T cells and compared with controls. D: Quantitative analysis of apoptosis (** $P < 0.01$).

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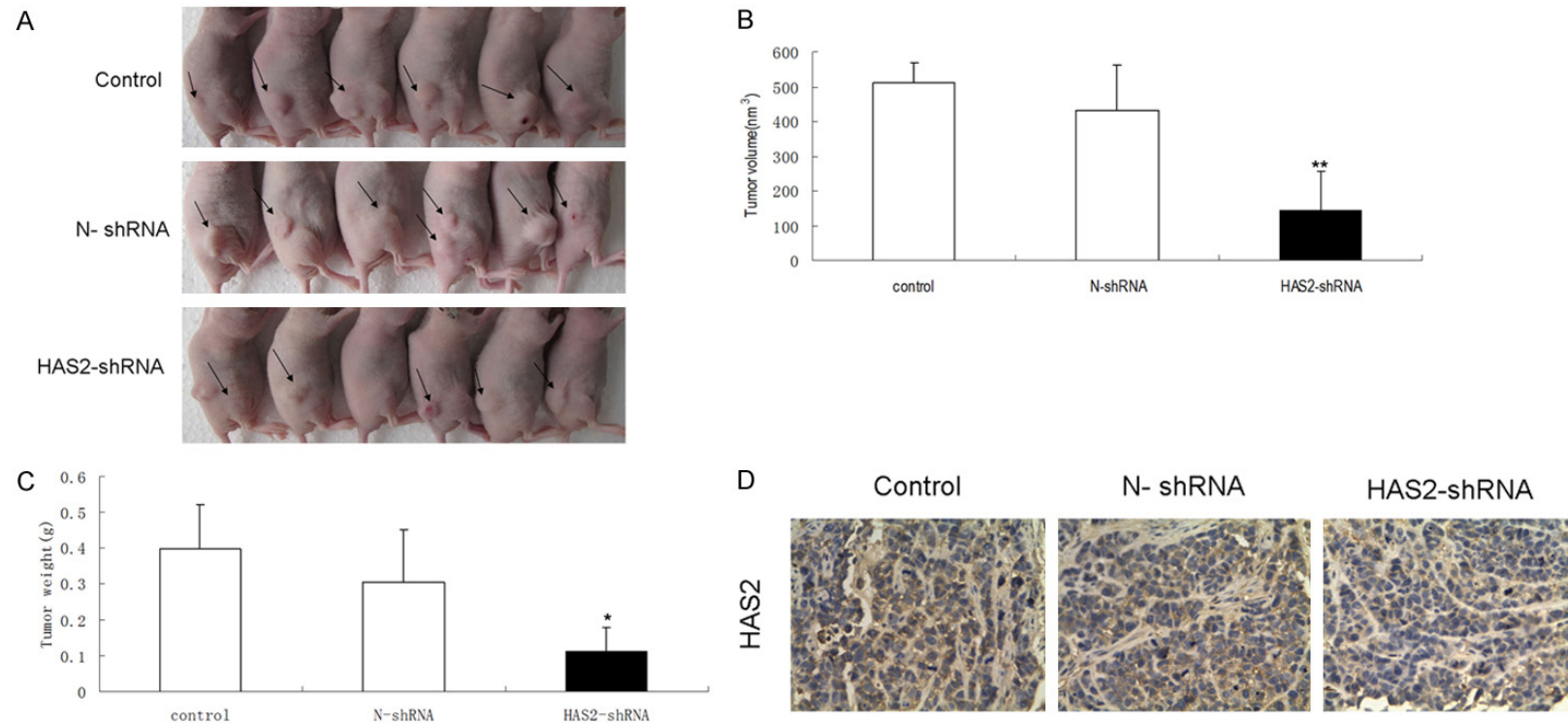


Figure 6. Knockdown of HAS2 expression inhibited the tumorigenicity of breast cancer in vivo. A: Tumors derived from three groups of nude mice. The average volume of tumors induced by HAS2-shRNA3 transfected Hs578T cells was significantly decreased, compared with control tumors (B; $**P < 0.01$). The average tumor weight in the HAS2-shRNA3 group was less than that in the N-shRNA and control groups (C; $*P < 0.05$). D: Representative photographs of immunohistochemistry (IHC) analysis of HAS2 expression in tumors of nude mice (Magnification, 400 \times).

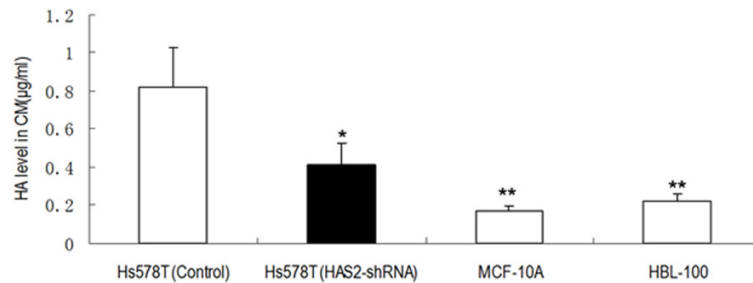


Figure 7. HA overexpression in the breast cancer cells, the HAS2 could up-regulate the level of HA. ELISA-like assay measured HA levels present in the culture medium (CM) of the cells. Compared with the MCF-10A and HBL-100, the HA levels were higher in Hs578T cells (** $P < 0.01$), which were further downregulated after the knockdown of HAS2 (* $P < 0.05$).

cells, the HA level was significantly higher in Hs578T cells ($P < 0.01$). Furthermore, the HA level was downregulated significantly in HAS2-shRNA3 group ($P < 0.05$). These results demonstrated that HAS2 could upregulate HA expression. HAS-HA system is involved in the regulation of tumor growth and invasion, influences the biology characteristics of human breast cancer cells.

Discussion

Increased synthesis of hyaluronan (HA) is often associated with malignant progression in certain types of human tumor, including colon cancer, lung cancer, breast cancer, mesotheliomas and gliomas, and the levels of HA in the sera of some cancer patients are significantly higher than those of normal individuals [30-32]. Previous studies have suggested that the HAS isoforms are involved in different stages of malignant tumor progression [33]. An abnormal accumulation of HA matrix induced by the overexpression of HAS genes diminished contact inhibition of non-transformed cells [24]. It has been proven that overexpression of HAS2 enhances both the anchorage-independent growth and tumorigenicity of a human fibrosarcoma cell line [34]. Rika Kosaki *et al.* showed that increased production of HA directed by human HAS2 promotes anchorage-independent growth and tumorigenicity of human HT1080 cells [34]. HAS2 may be a potential prognostic marker and therapeutic target in human cancer.

We first evaluated the levels of expression of HAS2 transcripts and protein in breast cancer cell lines BT549, Hs578T, MCF-7, MDA-MB-231, MDA-MB-468, SK-BR-3 and T47D, nonmalignant

breast cell lines MCF-10A and HBL-100, 55 invasive ductal carcinoma breast tissues and 55 relative normal breast tissues. Our results confirmed that HAS2 was overexpressed in breast cancer cell lines and invasive duct cancer tissues compared with normal breast tissues. These results demonstrated that HAS2 is widely expressed in breast cancer.

In the current study, four shRNA that targeted HAS2

and one negative control shRNA were designed to block HAS2 expression in Hs578T breast cancer cells. CCK-8 assay demonstrated that the proliferation and growth of the cells were inhibited after the transfection of HAS2-shRNA into Hs578T cells. In addition, blockage of HAS2 expression induced cell cycle arrest and apoptosis of breast tumor cells. Based on these findings, we predicted that HAS2 affected breast cancer cell proliferation, probably by regulating the cell cycle and apoptosis. The wound healing and matrigel-coated transwell assays showed that knockdown of HAS2 expression resulted in reduced capacity for cell migration and invasion in vitro. Our results indicated that HAS2 is correlated with the potential of breast cancer cells for migration and invasion. We also analyzed the correlation between HAS2 and clinicopathological features and found that the HAS2 was statistically correlated with lymph node metastasis, which further demonstrates that HAS2 may be involved in the progression and metastasis of breast cancer. Further to evaluate the functions of HAS2 in vivo, the tumorigenicity of Hs578T cells transfected with HAS2-shRNA3 was evaluated in nude mice, and the results were consistent with our in vitro results.

At present, whether HA synthase is a tumor promoter or a repressor remains controversial. The results presented in this study show that blocking HAS2 expression reduced tumor growth and invasion, supporting its role as a tumor promoter. Intriguingly, Naoki Itano *et al.* reported that the activity and transcriptional levels of hyaluronan synthase (HAS) were significantly increased after oncogenic malignant transformation of a rat 3Y1 fibroblast cell line. Of the

three HAS isoforms (HAS1, HAS2, and HAS3), only HAS2 gene expression was increased in the v-Ha-ras transformed 3Y1 cells, which showed lower malignancy [33]. Moreover, the growth stimulation was only seen within a narrow range of HAS2 expression, and high levels of HAS2 expression even inhibited tumor growth [33]. Thus, it is possible that proper regulation of the expression of HAS2 is required for optimal malignant transformation and tumor growth. It is also noteworthy that other proteins related to HA receptors (CD44 and RHAMM) and HAases are also involved in tumor growth and metastasis. Interaction between RHAMM and HA fragments is known to induce the mitogen-activated protein kinase pathway, and overexpression of RHAMM is a useful prognostic indicator for breast cancer [35]. Knockdown of HYAL1 expression in breast cancer cells resulted in decreased cell growth, adhesion, invasion and angiogenesis potential. Meanwhile, HYAL1 knockdown markedly inhibited breast cancer cell xenograft tumor growth and microvessel density [36]. It is possible that the effect of HAS2 (and possibly other HAS isoforms) on tumor growth and invasion is independent, a complex system is involved in the malignant behavior of human breast cancer.

To date, the pattern of expression and function of the HAS2 gene in human tumors have not been elucidated. Summarizing the observations made by ourselves and others, we favor the hypothesis that HAS2 may play a critical role in the longevity of a wide spectrum of breast cancer cells. Our study also showed that the HA was downregulated by blocking the HAS2 expression, which could inhibit the proliferation of breast cancer cells, raising a possibility for targeted therapy. To identify the mechanism by which the HAS2-HA dynamic system influences the biological characteristics of human breast cancer cells more investigations will be performed in the future.

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Disclosure of conflict of interest

None.

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