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ARTICLE

Cellular and Molecular Biology

SBSN drives bladder cancer metastasis via EGFR/SRC/STAT3 signalling

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BACKGROUND: Patients with metastatic bladder cancer have very poor prognosis and predictive biomarkers are urgently needed for early clinical detection and intervention. In this study, we evaluate the effect and mechanism of Suprabasin (SBSN) on bladder cancer metastasis.

METHODS: A tissue array was used to detect SBSN expression by immunohistochemistry. A tumour-bearing mouse model was used for metastasis evaluation in vivo. Transwell and wound-healing assays were used for in vitro evaluation of migration and invasion. Comprehensive molecular screening was achieved by western blotting, immunofluorescence, luciferase reporter assay, and ELISA.

RESULTS: SBSN was found markedly overexpressed in bladder cancer, and indicated poor prognosis of patients. SBSN promoted invasion and metastasis of bladder cancer cells both in vivo and in vitro. The secreted SBSN exhibited identical biological function and regulation in bladder cancer metastasis, and the interaction of secreted SBSN and EGFR could play an essential role in activating the signalling in which SBSN enhanced the phosphorylation of EGFR and SRC kinase, followed with phosphorylation and nuclear location of STAT3.

CONCLUSIONS: Our findings highlight that SBSN, and secreted SBSN, promote bladder cancer metastasis through activation of EGFR/SRC/STAT3 pathway and identify SBSN as a potential diagnostic and therapeutic target for bladder cancer.

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INTRODUCTION

Bladder cancer is one of the most common malignant tumours in the urinary system, and accounts for 90% to 95% of urothelial carcinoma, with ~500,000 new cases and 200,000 deaths worldwide annually [1, 2]. The survival of patients with non-muscle-invasive bladder cancer is favourable, as the 5-year progression-free survival reaches 93% in the low-risk group and 54% even in high-risk group [3]. However, invasion and metastasis promote the disease to a worse outcome: the overall 5-year survival rate of patients with nonmetastatic muscle-invasive disease drops to 36–48%, while in cases of distant metastasis, the 5-year relative survival is only 5% and the median survival time is less than one year [3, 4]. Nevertheless, metastatic disease only accounts for 4% of newly diagnosed bladder cancer [1]. It is imperative to study the mechanism of bladder cancer invasion and metastasis and find new targets for early clinical detection and intervention.

Epidermal growth factor receptor (EGFR), interacting with its ligand EGF, activates multiple intracellular signalling pathways [5]. EGFR has been found to reduce tumour suppressors and activate oncogenes to promote cancer progression and metastasis [6, 7]. Studies have shown that EGFR is highly expressed in more than half of bladder cancer cases and is closely related to high tumour grade, clinical stage, high recurrence rate and poor clinical prognosis [8–10]. EGFR is also considered a prognostic indicator of disease progression for bladder cancer patients with local recurrence or metastatic muscle-invasive bladder cancer, and the EGFR signalling pathway is found to be abnormally activated and directly promotes metastasis of bladder cancer [11, 12]. Many ongoing clinical trials are evaluating inhibitors against the EGFR family of proteins to treat cancers [7]. However, EGFR inhibitors designed and developed for bladder cancer have not achieved satisfactory results, due to low response or drug resistance [13].

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The effectiveness and mechanism of activation of EGFR signalling activation need to be further explored to improve the efficiency of bladder cancer therapy.

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family, has been confirmed to be closely associated with the occurrence and development of solid tumours and haematological tumours [14]. Phosphorylated STAT3 can regulate EGFR signal transduction from the cell surface to the nucleus and improve adhesion and invasion of bladder cancer cells to local tissues [15]. In addition, STAT3 activation has been identified in a variety of EGFR-Tyrosine kinase inhibitor (TKI) resistance models. Sen et al. [16] showed that STAT3 activation contributed to EGFR inhibitor resistance and targeting STAT3 enhanced the anti-tumour effects of EGFR blocking strategies or reversed cell resistance to EGFR inhibitor.

Suprabasin (SBSN), a gene expressed in mouse and human differentiating keratinocytes, is a marker of epidermal differentiation and a potential precursor of the keratinised envelope [17]. SBSN promotes the malignant progression of salivary gland adenoid cystic carcinoma [18], and may be involved in the carcinogenesis of glioblastoma and non-small cell lung cancer [19, 20]. Our previous study found that SBSN was highly expressed in oesophageal squamous cell carcinoma, and was involved in tumour cell proliferation and tumour formation of oesophageal cancer by activating the Wnt/β-catenin signalling pathway [21]. Studies have shown that SBSN is abnormally expressed in bladder cancer [22, 23]. However, the detailed function and regulation of SBSN in bladder cancer is not yet clear.

The present study aimed to evaluate the effects and regulation of SBSN on bladder cancer progression. We found that SBSN is abnormally highly expressed in bladder cancer and is associated with the poor prognosis of patients. We observed that SBSN promotes malignant invasion and metastasis of bladder cancer cells both in vivo and in vitro. We further confirmed that secreted SBSN achieves the identical biological function and regulation in bladder cancer metastasis as its overexpression, and the interaction of secreted SBSN and EGFR might play an essential role of signal transduction to activate the EGFR/SRC/STAT3 pathway. Collectively, these findings highlight that SBSN drives bladder cancer metastasis and could be a potential diagnostic and therapeutic target for bladder cancer.

MATERIALS AND METHODS

Cells and cell culture

The bladder cancer cell lines RT4, ScaBER, TCCSUP, 5637, SW780, J82, T24, and UM-UC-3 were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) (#C11995500BT, Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS) (#0270–106, Gibco). The immortalised human bladder epithelial cell SV-HUC-1 was purchased from Guangzhou Xiandu Biotechnology company. (Guangzhou, China). The culture medium was Ham's F-12K (#21127–022, Gibco) supplemented with 10% FBS. The murine bladder cancer cell line MB49 was purchased from Guangzhou Xiandu Biotechnology Company and cultured in DMEM supplemented with 10% FBS. All cells were authenticated, tested for mycoplasma contamination, and cultured in a humidified incubator at 37 °C and 5% CO_2. The EGFR inhibitor Erlotinib (1 μg/mL) and STAT3 inhibitor STATTIC (5 μM) were purchased from Selleck (Houston, TX, USA) and were dissolved in DMSO.

Tissue specimens

A tissue array (No. HBlaU066Su01) (Outdo Biotech, Shanghai, China) was used to detect SBSN expression. Clinical and pathological classification and clinical staging were determined according to the standards of the American Joint Committee on Cancer. The investigation was conducted in accordance with the Declaration of Helsinki and the ethical standards of national and international standards. For the use of clinical material used, the institutional research ethics committee were obtained. The clinical information of patients' samples is shown in Supplementary Tables 1, 2.

Immunohistochemistry

The paraffin-embedded specimen was cut into 4-μm sections, deparaffinised with xylene, rehydrated, and then immersed in an antigen retrieval buffer containing EDTA, and subjected to antigen retrieval by microwave. A 1% bovine serum albumin solution was used to block nonspecific binding. The sections were then incubated with anti-SBSN (ab232771) (Abcam, Cambridge, MA, USA), anti-EGFR (#4267), anti-phospho-EGFR (Tyr1068) (#3777), anti-STAT3 (#9139) (Cell Signaling, Danvers, MA, USA) and MMP9 (#10375–2-AP, Proteintech, Rosemont, IL, USA) antibodies at 4 °C overnight. Tissue sections were treated with biotinylated anti-rabbit/mouse secondary antibody (#PV8000, ZSGB-BIO, Beijing, China), and then further incubated with streptavidin-horseradish peroxidase complex (#PV8000, ZSGB-BIO) and immersed in 3,3′-diaminobenzidine (#ZLI9017, ZSGB-BIO), counterstaining with 10% Mayer's hematoxylin (#G1080, Solarbio, Beijing, China), followed by dehydration, and mounting. The stained tumour sections were examined and scored independently by two observers to determine the immunohistochemical signals. The staining index (SI) and the mean optical density (MOD) value determined by Image J software (National Institutes of Health, Bethesda, USA) were used for quantitative analysis of IHC results. The SI was calculated as the product of the staining intensity score and the proportion of positive tumour cells. According to the proportion of positively stained tumour cells, the sections were scored as follows: 1, <25% positive tumour cells; 2, 25–50% positive tumour cells; 3, 50–75% positive tumour cells; 4, >75% positive tumour cells. The intensity of staining was graded according to the following criteria: 1, weak staining (light yellow); 2, moderate staining (yellow brown); 3, strong staining (brown); and 4, very strong staining (dark brown). The expression of the indicated protein in IHC-stained tumour sections was assessed based on the SI scores as 1, 2, 3, 4, 6, 8, 9, 12, and 16. Cutoff values were chosen to stratify the high and low SI score, where $SI \geq 8$ was considered as high expression.

Western blotting assay

The cells were pre-treated with RIPA lysis buffer (#9806, Cell Signaling), and the protein was quantified using the BCA protein detection kit (#23227, Thermo, Waltham, MA, USA), and denatured at 100 °C for 5 min. Equal quantities of protein were electrophoresed by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (#IPVH00010, Millipore, Billerica, MA, USA). The blots were blocked with 5% milk for 1 h. The following primary antibodies were used: SBSN (ab232771) (Abcam), GAPDH (#5174), β-actin (#3700), p-EGFR (Tyr1068) (#3777), EGFR (#4267), p-SRC (Tyr416) (#2101), p-SRC (Tyr527) (#2105), SRC (#2109), p-STAT3 (Tyr705) (#9145), STAT3 (#9139) and p-STAT3(Tyr705) (#9131) (Cell Signaling), following incubation with specific HRP-conjugated antibody (#7074, #7076, Cell Signaling). FDbio-Feto ECL Western Blotting Detection Reagent (#FD8030, Fdbio science, Hangzhou, China) was used to detect the chemiluminescence signal.

In vivo tumour model, hematoxylin and eosin, and IHC staining

All animal experiments complied with the ARRIVE guidelines [24]. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University and carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. BALB/c nude mice (male, 4–5 weeks, 18–20 g) were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). BALB/c nude mice were kept under conditions without specific pathogens. The murine bladder cancer cell MB49 stably expressing SBSN-vector (vec), SBSN, shRNA-vector (sh-vec) and shRNA-SBSN (sh-SBSN) were constructed. BALB/c nude mice were randomly divided into two groups for subcutaneous implantation. One group was inoculated with MB49-vector cells (2×10^6) subcutaneously in the left hip and MB49-SBSN cells (2×10^6) were inoculated subcutaneously in the right hip. The other group was inoculated with MB49-shRNA-vector cells (2×10^6) subcutaneously in the left hip, and MB49-shRNA-SBSN cells (2×10^6) were injected subcutaneously in the right hip. Tumour growth was monitored every four days. The tumour volume was measured from two directions with a vernier calliper and calculated as follows: $(\text{length} \times \text{width}^2)/2$. The mice were euthanized four weeks after tumour transplantation, and the tumours were removed and weighed. The tumours were fixed in formalin and embedded in paraffin, serial sections at 4-μm, and hematoxylin and eosin (H&E) staining with Mayer hematoxylin solution, and IHC staining with anti-SBSN (ab232771) (Abcam), anti-STAT3 (#9139), anti-MMP9 (#13667), and anti-EGFR (#4267) (Cell Signalling) for analysis. For analysis of metastatic status,

the nude mice were intravenously injected with SBSN transduced, SBSN-silenced or control cells via the lateral tail vein. The lungs were collected to count surface metastatic loci under a dissecting microscope at four weeks after tumour implantation. The tumours and lungs were fixed in formalin and embedded in paraffin using the routine method. Serial 4-μm sections were cut and subjected to H&E staining with Mayer's hematoxylin solution. For the determination of SBSN expression in the serum of the mice, the mice were randomly divided into two groups for subcutaneous implantation. One group was subcutaneously inoculated with MB49-vector cells (2×10^6) and the other group was inoculated with MB49-SBSN cells (2×10^6). The blood serum from the mice was collected at five weeks after tumour transplantation, and the level of SBSN was detected by q-PCR. All data were collected by blind method.

Transwell matrix penetration assay and transwell assay

The cells (1×10^4) were seeded on the top chamber of Transwell® Permeable Supports (#3422, Corning, Painted Post, NY, USA), coated with or without the Cultrex Basement Membrane Extract (BME) (#3432–010–01, R&D Systems, Minneapolis, MN, USA), and complete medium or conditioned medium was added to the bottom of chamber. After incubating at 37 °C for 24 hours, the cells inside of the upper chamber were removed using cotton swabs. The invaded and migrated cells on the surface of the lower membrane were fixed with 4% paraformaldehyde, stained with hematoxylin, observed under a microscope, photographed, and counted.

Wound-healing assay

The cells were seeded in a 24-well plate for 24 hours and then replaced with FBS-free medium to starve the cells overnight. A scratch was formed in the cell layer with a sterile pipette tip to form a straight wound, and then FBS-containing medium or conditioned medium was added to culture the cells. Images were captured and migration was documented using an inverted microscope.

Luciferase reporter assay

Cells (7×10^4) were seeded in 24-well plates and settled for 24 hours. The indicated plasmids (SBSN, SBSN-OPT or the vector control; 200 ng) plus STAT3 luciferase reporter plasmids (50 ng, VB191007, VectorBuilder, Guangzhou, China) and pRL-TK Renilla plasmids (1 ng, E2241, Promega, Madison, WI, USA) were transfected into cells using Lipofectamine 3000 reagent (#L3000015, Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. 28 hours after transfection, luciferase and renilla signals were measured using the Dual-Luciferase Reporter Assay Kit (#E1960, Promega).

Statistical analysis

Statistical tests used for data analysis included the chi-square test and Student's two-tailed *t*-test. The survival curves were compared by Kaplan–Meier method and log-rank test. GraphPad Prism 6.0 (GraphPad Software, CA, USA) and SPSS 23.0 software (IBM SPSS Statistics, IL, USA) were used for statistical analysis. The pathway and function gene expression signatures were analysed using Gene Set Enrichment Analysis (GSEA, 4.0.3) and performed accordingly [25]. The RNA sequencing V2 profile dataset was downloaded from the Cancer Genome Atlas (TCGA). The gene sets used were downloaded from GSEA platform (www.gsea-msigdb.org). For each sample and signature, GSEA reported a signature expression score between 0 and 1 and the statistical significance (*P*-value) for signature overexpression. The data represent mean ± standard deviation (SD) and a *P*-value of <0.05 were considered statistically significant.

RESULTS

SBSN was upregulated and predicted an unfavourable prognosis in bladder cancer

The Oncomine database was used to determine the expression of SBSN in bladder cancer. Compared with normal bladder tissues (bladder mucosa, $n = 68$), the expression of SBSN in the tumour tissues (infiltrating bladder urothelial carcinoma, $n = 62$) was significantly increased ($P < 0.001$) (Supplementary Fig. 1A). To further confirm the results, we performed IHC staining to examine the expression level of SBSN in commercial bladder cancer tissue microarrays (No. *HBlaU066Su01*). Compared with paired adjacent non-tumour tissues, the expression of SBSN in the corresponding tumour tissues was significantly upregulated (Fig. 1a) and SBSN overexpression accurately located in tumour tissues but weak or little in adjacent non-tumour bladder tissues and stromal tissues (Fig. 1b). We further studied the clinical correlation between the expression level of SBSN and the clinicopathological characteristics of patients with bladder cancer, and found that the overexpression of SBSN was positively correlated with the clinical stage (Fig. 1c).

The case data of 54 patients in the tissue microarray were further analysed (Supplementary Table 1). Compared to patients with lower SBSN expression, patients with higher SBSN expression had a shorter overall survival time (Fig. 1d). The publicly available bladder cancer dataset [26] also showed that both the overall survival time ($P = 0.0015$) and the relapse-free survival time ($P = 0.039$) were significantly reduced in patients with higher expression of SBSN (Supplementary Fig. 1B). These results implied the value of SBSN as a diagnostic marker for patients with bladder cancer. Univariate and multivariate analyses were performed and the results showed that, the expression of SBSN was identified as an independent prognostic marker for bladder cancer (Supplementary Table 2). Taken together, our results suggested that SBSN could be a diagnostic marker of bladder cancer progression, and that higher SBSN expression was associated with poorer overall survival in patients with malignant bladder cancer. In addition, the expression of SBSN was examined in bladder cancer cell lines. The results showed that compared to a normal human immortalised bladder epithelial cell (SV-HUC-1), SBSN mRNA and protein expression were upregulated in eight bladder cancer cell lines (Fig. 1e), further indicating the oncogenic role of SBSN in bladder cancer.

SBSN promoted bladder cancer aggressiveness both in vivo and in vitro

We found that SBSN expression was upregulated in infiltrating bladder urothelial carcinoma (Supplementary Fig. 1A) and higher expression of SBSN indicated a shorter relapse-free survival time (Supplementary Fig. 1B), this suggested that SBSN might be correlated with bladder cancer aggressiveness. GSEA analysis showed that the expression of SBSN in bladder cancer was positively correlated with the expression of genes related to invasiveness and metastasis (Fig. 2a). The murine bladder cancer cell MB49 was used to establish stable cell lines to overexpress and silence SBSN and the genetically modified cells were then subcutaneously injected in the nude mice, as well as the indicated control cells (Fig. 2b and Supplementary Fig. 2A). As shown in Fig. 2c, d, tumours formed by SBSN-overexpressing cells maintained a higher growth rate in terms of both size and weight, while mice inoculated with SBSN-silenced cells showed smaller and lighter tumour tissues than the control groups. Microscopic examination of tumour morphology showed that the SBSN-transduced tumour tissue boundary showed a spike-shaped structure that penetrated the surrounding muscle tissue, but the control tumour boundary was well demarcated; in contrast, silencing SBSN remarkably inhibited the local invasiveness of the tumour (Fig. 2e). To analyse the metastasis status, nude mice were injected intravenously with SBSN-overexpressing or SBSN silent cells or control cells through the lateral tail vein. The results showed that mice injected with SBSN transduced cells presented prominent lung metastases, only slight metastases were found in mice injected with SBSN-silenced cells (Fig. 2f, g). This indicates that the capability of MB49 cells to induce lung metastasis was significantly enhanced by SBSN upregulation and impaired by SBSN silence.

In vitro experiments were also performed to confirm the biological role of SBSN in bladder cancer. Two cell lines T24 and 5637 with moderate SBSN expression, stably overexpressed or

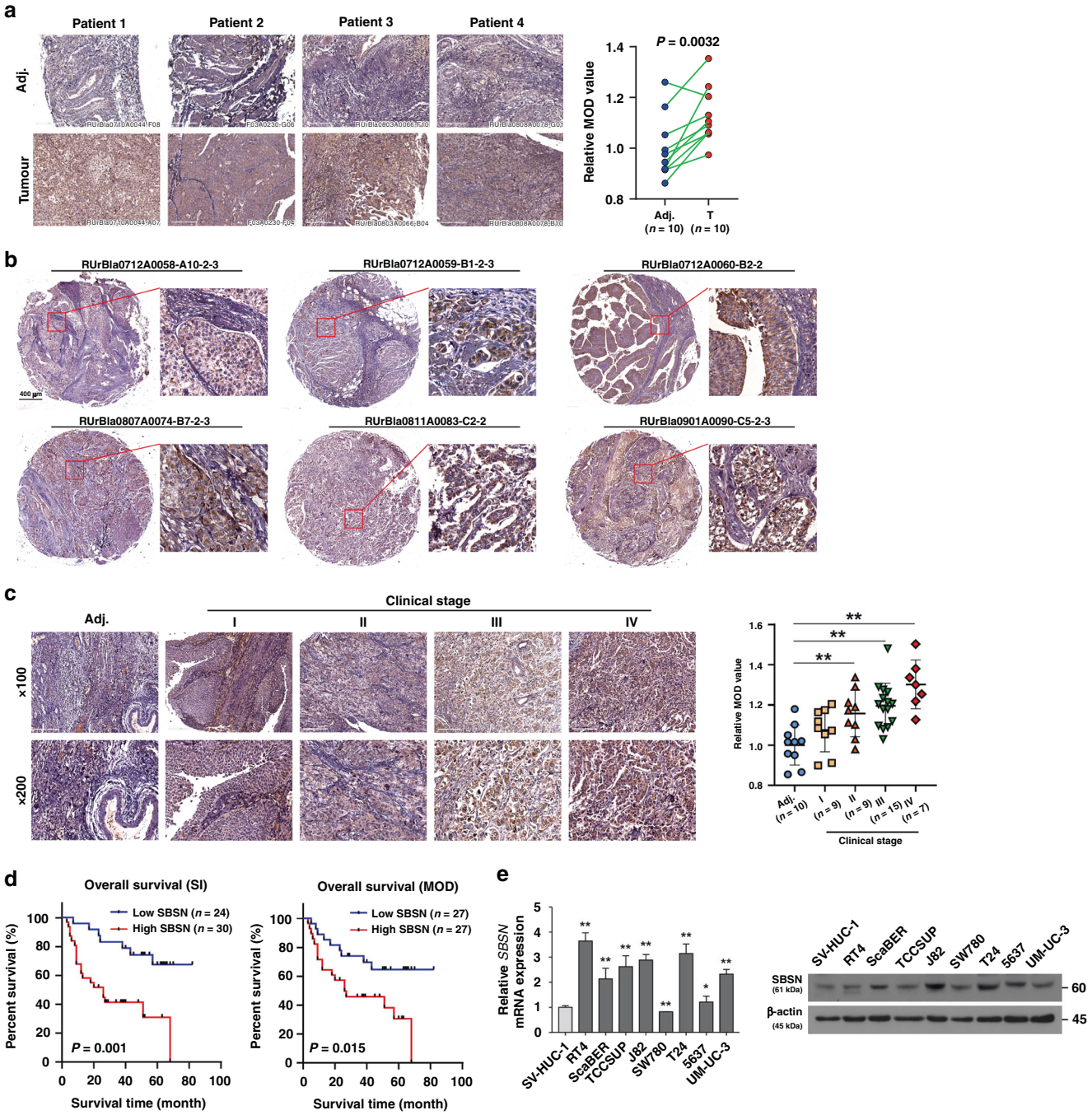

Fig. 1 SBSN is upregulated and associated with the progression of bladder cancer. a Representative images of IHC analyses of SBSN expression in formalin-fixed paraffin-embedded bladder cancer tissue (tumour; T) and the matched adjacent non-tumour tissue (Adj.) from the same patient. The average mean optical density (MOD) value was used for the quantification of IHC analysis. **b** Representative images of IHC analyses of SBSN expression in formalin-fixed paraffin-embedded bladder cancer tissue. **c** Representative images and quantification of the IHC analysis of SBSN expression in non-tumour tissues (Adj.) and bladder cancer tissues of clinical stage I, II, III and IV; Scale bar, 200 μm. MOD value was used for the quantification of IHC analysis. Excluding 14 cases with incomplete information and 2 tissues exfoliated, 40 cases were included and analysed. **d** Kaplan–Meier analyses of the overall survival of bladder cancer patients with low versus high expression of SBSN determined by SI and MOD value. Excluding 2 tissues exfoliated, 54 cases with complete survival information from the tissue array were included and analysed. The log-rank test was used to calculate the *P*-value; **e** q-PCR and western blotting analysis of SBSN expression in eight bladder cancer cell lines compared to the immortalised bladder epithelial cell line SV-HUC-1. The two-tailed *t*-test was used for statistical analysis. Error bars represent the mean ± SD of three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

silenced SBSN were constructed (Fig. 2h and Supplementary Fig. 2A). Through wound healing and Transwell assays, we found that the overexpression of SBSN enhanced, while downregulation of SBSN attenuated the motility of bladder cancer cells (Fig. 2i, j and Supplementary Fig. 2B, C). The Transwell matrix penetration assay showed that SBSN overexpression increased, while silencing SBSN expression reduced the number of invading bladder cancer cells (Fig. 2j and Supplementary Fig. 2C). Collectively, these results suggested SBSN promoted invasiveness and metastasis of bladder cancer cells both in vivo and in vitro.

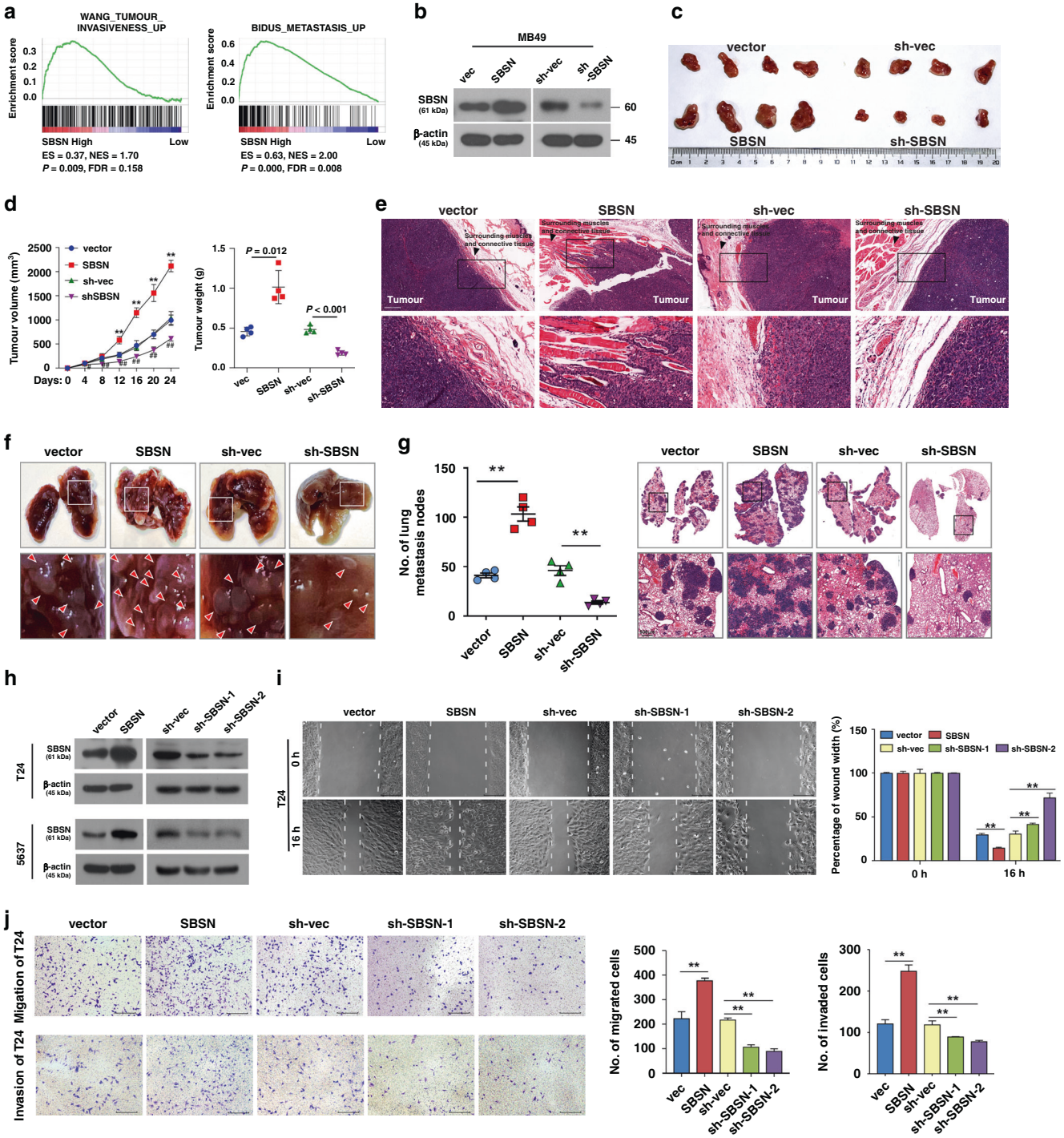

Fig. 2 SBSN enhanced bladder cancer aggressiveness. a The GSEA analysis showed that SBSN expression is positively correlated with gene signatures in tumour invasion and metastasis. **b** Western blotting analysis of the murine bladder cancer cell line MB49 stably transduced with SBSN-vector (vec), SBSN, shRNA-vector (sh-vec), or shRNA-SBSN (sh-SBSN). **c** Representative images of tumours from mice in each group ($n = 4$/group). The indicated bladder cancer cells were injected subcutaneously into BALB/c nude mice. **d** Tumour volume and weight of xenografts. **e** H&E staining of the borders of primary tumours in the indicated mice. Scale bar, 300 μm. **f** Representative bright-field images and quantification of metastases in the lungs. **g** H&E staining of lung metastases. Scale bar, 500 μm. **h** Western blotting analysis of bladder cancer cells stably transduced with SBSN-vector (vec), SBSN, shRNA-vector (sh-vec), shRNA-SBSN-1 (Ri#1), or shRNA-SBSN-2 (Ri#4). **i** Representative images (left) and quantification (right) of wound-healing assay of indicated cells. Scale bar, 100 μm. **j** Representative images (left) and quantification (right) of indicated migrated cells analysed in a Transwell assay and invaded cells analysed in a Transwell matrix penetration assay. Scale bar, 100 μm. The two-tailed *t*-test was used for statistical analysis. Error bars represent the mean ± SD of three independent experiments. #$P < 0.05$; ** or ##$P < 0.01$.

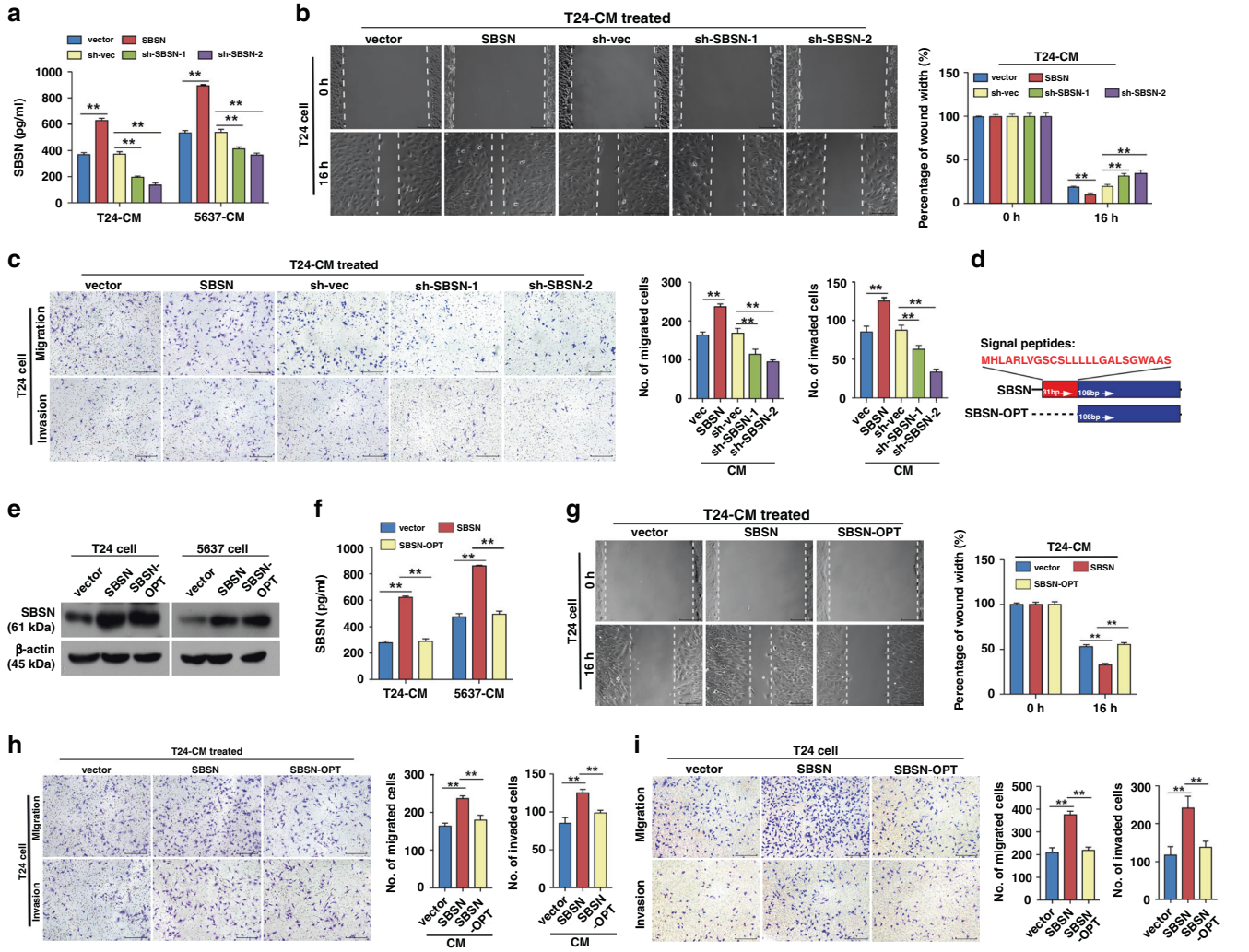

Fig. 3 Secreted SBSN enhanced bladder cancer aggressiveness. a ELISA analysis of secreted SBSN protein in indicated cell-cultured conditioned medium (CM). **b** After scratching, the indicated CM was added into the blank control cells, and the wound healing was captured and quantified. Scale bar, 100 μm. **c** Representative images (left) and quantification (right) of indicated migrated (upper) and invaded (lower) cells analysed in the Transwell assay. The CM was added to the lower part of the chamber for the Transwell assay. Scale bar, 100 μm. **d** Schematic diagram of SBSN plasmid deleted signal peptide (SBSN-OPT). **e** Western blotting analysis of SBSN expression in bladder cancer cells stably transfected with SBSN-vector (vector), SBSN, and SBSN-OPT. **f** ELISA analysis of the SBSN protein in the indicated CM. **g** Wound-healing assay of indicated CM treated blank control cells. Scale bar, 100 μm. **h** Representative images (left) and quantification (right) of migrated and invaded cells analysed in the Transwell assay; the cells were treated with indicated CM. Scale bar, 100 μm. **i** Representative images (left) and quantification (right) of migrated and invaded cells analysed in the Transwell assay. Scale bar, 100 μm. The two-tailed *t*-test was used for statistical analysis. Error bars represent the mean ± SD of three independent experiments. $^{**}P < 0.01$.

Secreted SBSN promoted the migration and invasion of bladder cancer cells

Recent studies have reported that SBSN is a secreted protein that exists in the environment of extracellular body fluids [23, 27]. We used ELISA to examine the presence of the secreted SBSN protein in the cell-cultured supernatant (Conditioned medium, CM). The results showed that the secreted SBSN was upregulated following the ectopic expression of SBSN, while secreted SBSN was decreased with SBSN silencing in bladder cancer cells (Fig. 3a). The conditioned medium containing secreted SBSN of each group was added to the control blank bladder cancer cells to perform a scratch assay. The conditioned medium with higher expression of SBSN promoted faster healing of the scratch; the conditioned medium with lower expression of SBSN promoted slower scratch healing (Fig. 3b and Supplementary Fig. 3A). Similarly, we added the conditioned medium of each group to the lower part of the Transwell chamber to induce the movement and invasion of blank control bladder cancer cells. The results show that the conditioned medium with higher expression of SBSN had a stronger ability to induce cell movement and invasion (Fig. 3c and Supplementary Fig. 3B). The above results indicated that SBSN promoted the migratory and invasive ability of bladder cancer cells.

SBSN encodes a signal peptide sequence, which may endow SBSN secretory properties and extracellular localisation ability [23]. *SignalP-5.0 Sever* was used to predict the amino acid sequence of SBSN and found that SBSN protein indeed had a signal peptide sequence (Supplementary Fig. 3C). An SBSN-OPT construct with deletion of the signal peptide sequence of SBSN was used to block its secretion (Fig. 3d) and the lentivirus infection system was used to construct a stable cell line with high-expressing SBSN without signal peptide (Fig. 3e). The western blotting results showed that the SBSN protein was stably and highly expressed in cells transduced with SBSN-OPT (Fig. 3e). ELISA results showed that the SBSN protein in the conditioned medium of SBSN-OPT transduced cells significantly decreased compared with that of the SBSN-overexpressing cells, confirming the effective blockage

of SBSN secretion by SBSN-OPT (Fig. 3f). The conditioned medium of different treatment groups was collected to treat the blank control cells. The wound healing and the Transwell assay showed that the conditioned medium of SBSN-OPT transduced cells had weaker effects on promoting the migration and invasion of bladder cancer cells, compared to the conditioned medium of cells overexpressing SBSN (Fig. 3g, h and Supplementary Fig. 3D, E). In particular, we found that SBSN-OPT transduced cells showed a declining migratory and invasive ability compared to SBSN transduced cells, indicating that SBSN secretion may display functions on the cell itself, which were similar to the autocrine process (Fig. 3i and Supplementary Fig. 3F, G). Taken together, all these results suggested secreted SBSN promoted, while deletion of the signal peptides of SBSN attenuated its promotion of migration and invasion of bladder cancer cells.

SBSN drove bladder cancer metastasis by activating EGFR/SRC/STAT3 signalling

We further explored the underlying mechanism of SBSN driving bladder cancer metastasis. GSEA analysis revealed that the expression of SBSN was positively correlated with the SRC/STAT3 signalling-activated gene signatures [28, 29] (Fig. 4a). A luciferase reporter assay revealed that overexpression of SBSN enhanced, but downregulation of SBSN attenuated, the transcriptional activity of STAT3 (Fig. 4b). The immunofluorescence staining and subcellular fractionation assays showed that overexpressing SBSN led to stronger nuclear signals for STAT3, while silencing SBSN resulted in a significant cytoplasmic location of STAT3 (Fig. 4c and Supplementary Fig. 4). Consistently, the q-PCR results revealed the expression of multiple STAT3 downstream genes was enhanced by SBSN overexpression, indicating the promotion of transcriptional activity of STAT3 (Fig. 4d). Western blotting showed that SBSN enhanced the phosphorylation of SRC kinase at the activation site (Tyrosine 416) and inhibited the phosphorylation at the inhibition site (Tyrosine 527); improved the phosphorylation of STAT3 at the activation site (Tyrosine 705), but had no effect on the total expression of SRC and STAT3 (Fig. 4e), which indicated that SBSN improved the transcriptional activity of STAT3 and activated the SRC/STAT3 signalling pathway. To further confirm the role of STAT3, the cells were then treated with a STAT3 inhibitor (STATTIC, 5 μM). The results of the luciferase reporter assay showed that SBSN-enhanced STAT3 activation, consistent with the phosphorylation of SRC and STAT3, was inhibited by STAT3 inhibition (Fig. 4f, g).

EGFR has been reported to be a canonical receptor that is upstream of SRC/STAT3 and activates SRC/STAT3 signalling [30, 31]. The results of western blotting showed that SBSN enhanced the expression of phosphorylated EGFR, but not the overall total expression of EGFR (Fig. 4e), indicating activation of EGFR signalling. Cells treated with the STAT3 inhibitor did not show any change in EGFR phosphorylation and expression (Fig. 4g), while cells treated with an EGFR-specific inhibitor (Erlotinib, 1 μg/mL) showed remarkable suppression of SBSN-induced STAT3 transcriptional activation (Fig. 4f), as well as SBSN-enhanced phosphorylation of EGFR, SRC, and STAT3 (Fig. 4g). The Transwell assay further confirmed that the effects of SBSN on improving bladder cancer cell movement and invasion were significantly inhibited by STAT3 or EGFR inhibition (Fig. 4h).

Secreted SBSN activated the EGFR/SRC/STAT3 signalling pathway

As we confirmed secreted SBSN could promote migratory and invasive capability of bladder cancer cells, we then explored the pathways involved in the transmission of autocrine signalling of secreted SBSN. The results of luciferase reporter assay showed that the conditioned medium of SBSN-overexpressing cells had a strong effect on activating STAT3 signalling of the recipient blank cells (Fig. 5a). Whereas, compared with the conditioned medium of SBSN transduced cells, the conditioned medium of SBSN-OPT transduced cells displayed a weaker promotion of STAT3 activation of the recipient blank cells (Fig. 5b); similar results were obtained for SBSN and SBSN-OPT transduced cells, in which the STAT3 transcriptional activity in SBSN-OPT transduced cells was significantly weakened (Fig. 5c). The results of western blotting showed that compared to SBSN transduced cells, EGFR, SRC, and STAT3 phosphorylation in SBSN-OPT transduced cells decreased significantly (Fig. 5d). The Transwell assay showed that the conditioned medium from SBSN-transduced cells induced the migration and invasion of bladder cancer cells, which was significantly suppressed by STAT3 inhibitor (STATTIC) or EGFR inhibitor (Erlotinib) (Fig. 5e and Supplementary Fig. 5). EGFR, as a membrane receptor, receives and transmits extracellular signals to cells. We hypothesised that EGFR could act as a relay station for secreted SBSN to transmit signals to cells. The results of the co-immunoprecipitation and cell immunofluorescence staining assay both showed that EGFR and SBSN indeed performed mutual interaction (Fig. 5f, g). Further, the results of cell immunofluorescence experiments detected by confocal microscopy showed that EGFR and SBSN were co-localised on the cell membrane (Fig. 5h). Therefore, we preliminarily confirmed that the secreted SBSN had a strong ability to activate the EGFR/SRC/STAT3 signalling pathway to promote the invasion and movement of bladder cancer cells.

SBSN activated EGFR/SRC/STAT3 signalling in vivo

Regulation of the EGFR/STAT3 signal by SBSN was also confirmed in the subcutaneous mouse model. The results of IHC showed that phosphorylation of EGFR, expression of nuclear STAT3 and matrix metallopeptidase 9 (MMP9, a marker of tumour invasion and meanwhile the downstream gene regulated by STAT3), were all upregulated in the SBSN-overexpressing group, while down-regulated by SBSN silencing (Fig. 6a). Further, the nuclear expression levels of STAT3 were further investigated in the metastatic tumour nodes from the lungs of the mice. Consistently, the results showed expression of nuclear STAT3 was remarkably enhanced in SBSN-overexpressing metastatic tumours, but reduced in SBSN-down expressing metastatic tumours (Fig. 6b). To confirm the secretion of SBSN in vivo, the blood serum from the mice was obtained to examine the secretion levels of SBSN. As expected, upregulating SBSN promoted the secretion in the serum of the mice (Fig. 6c). Collectively, our findings indicated that secreted SBSN interacted and enhanced phosphorylation of EGFR to promote activation of SRC/STAT3 signalling and promotion of invasion and metastasis of bladder cancer (Fig. 6d).

DISCUSSION

The identification of biomarkers of bladder cancer metastasis is required to improve the early diagnostic efficiency and to predict the prognosis of bladder cancer patients. Over several decades, extensive potential molecular marker candidates have been explored for bladder cancer diagnosis, including NMP22, BTA, DAPK, RARB, and E-cadherin, which have been confirmed to be abnormally expressed in the tissues of patients with bladder cancer. Among these, NMP22 and BTA have been approved by the FDA for clinical use, with high sensitivity and specificity, greatly improving the early detection rate of bladder cancer [32–34]. SBSN may act as an oncogenic gene to promote malignant progression and has been proposed as a prognostic biomarker in various tumours [23]. In the present study, we showed that SBSN is overexpressed in bladder cancer and is independently associated with poor survival outcomes, suggesting the diagnostic value of SBSN for bladder cancer. Furthermore, we provided evidence indicating that upregulation of SBSN in bladder cancer promotes cell invasion, motility, and lung metastasis in both in vivo and in vitro, further confirming the oncogenic function of SBSN in bladder cancer metastasis.

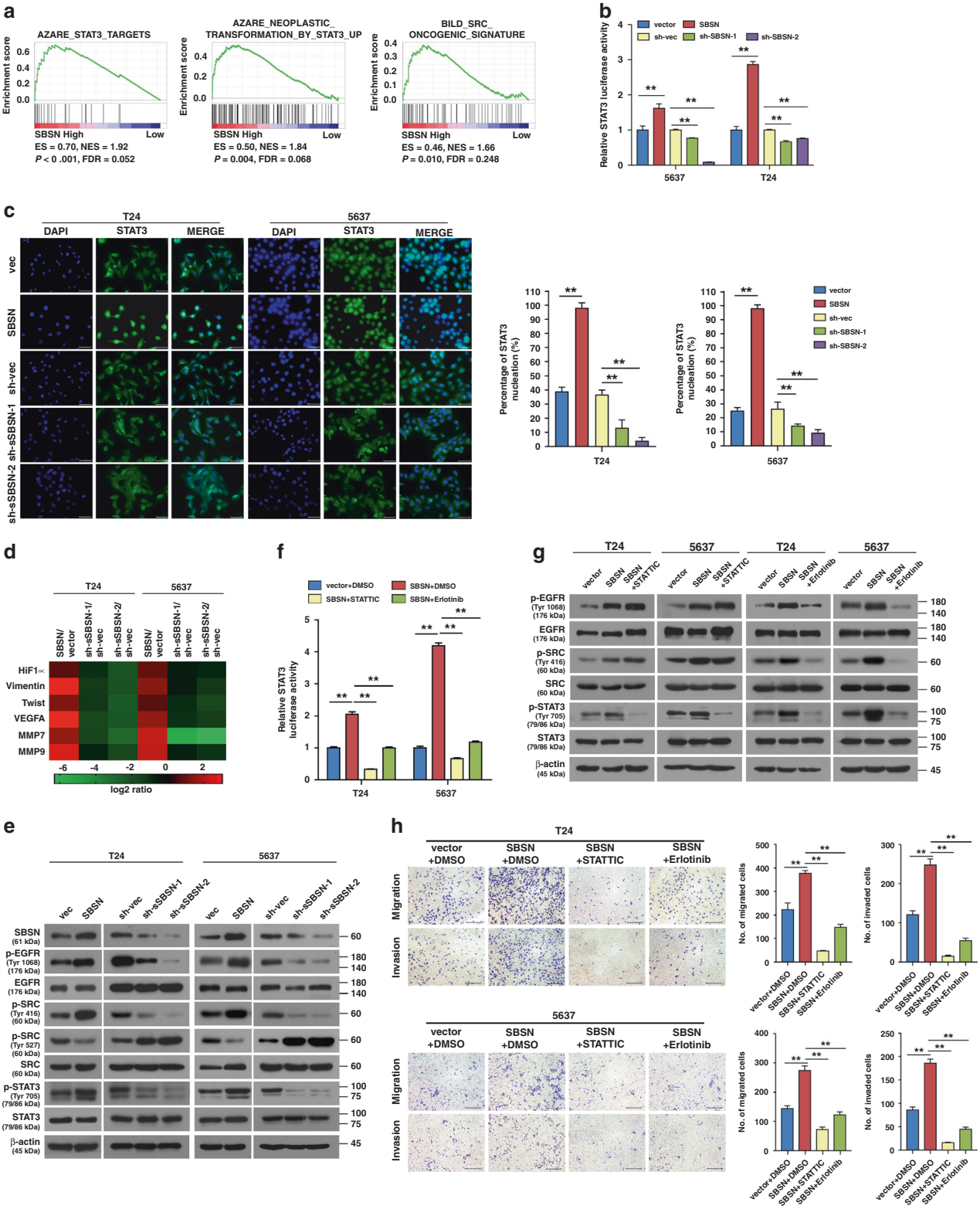

Fig. 4 SBSN activated EGFR/SRC/STAT3 signalling pathway. a GSEA analysis showed that SBSN expression correlated with gene signatures of STAT3 and SRC pathway. The gene signature sets were downloaded from GSEA platform (www.gsea-msigdb.org/). **b** STAT3 luciferase reporter activity was analysed in the indicated cells. **c** Immunofluorescence staining analysis of subcellular localisation of STAT3 in indicated cells. Scale bar, 50 μm. The histogram showed the relative quantification of STAT3 nuclear expression. **d** q-PCR analysis of STAT3 regulated genes in indicated cells. **e** Western blotting analysis of EGFR/SRC/STAT3 pathway-related proteins in indicated cells. **f** The activity of the STAT3 luciferase reporter was analysed in indicated cells. **g** Western blotting analysis of EGFR/SRC/STAT3 pathway-related regulatory proteins in indicated cells. **h** Representative images and quantification of indicated migrated and invaded cells in Transwell assay. Scale bar, 100 μm. **f**–**h** STATTIC (5 μM, 24 hours) or Erlotinib (1 μg/mL, 24 hours) was used. The two-tailed *t*-test was used for statistical analysis. Error bars represent the mean ± SD of three independent experiments. $^{**}P < 0.01$.

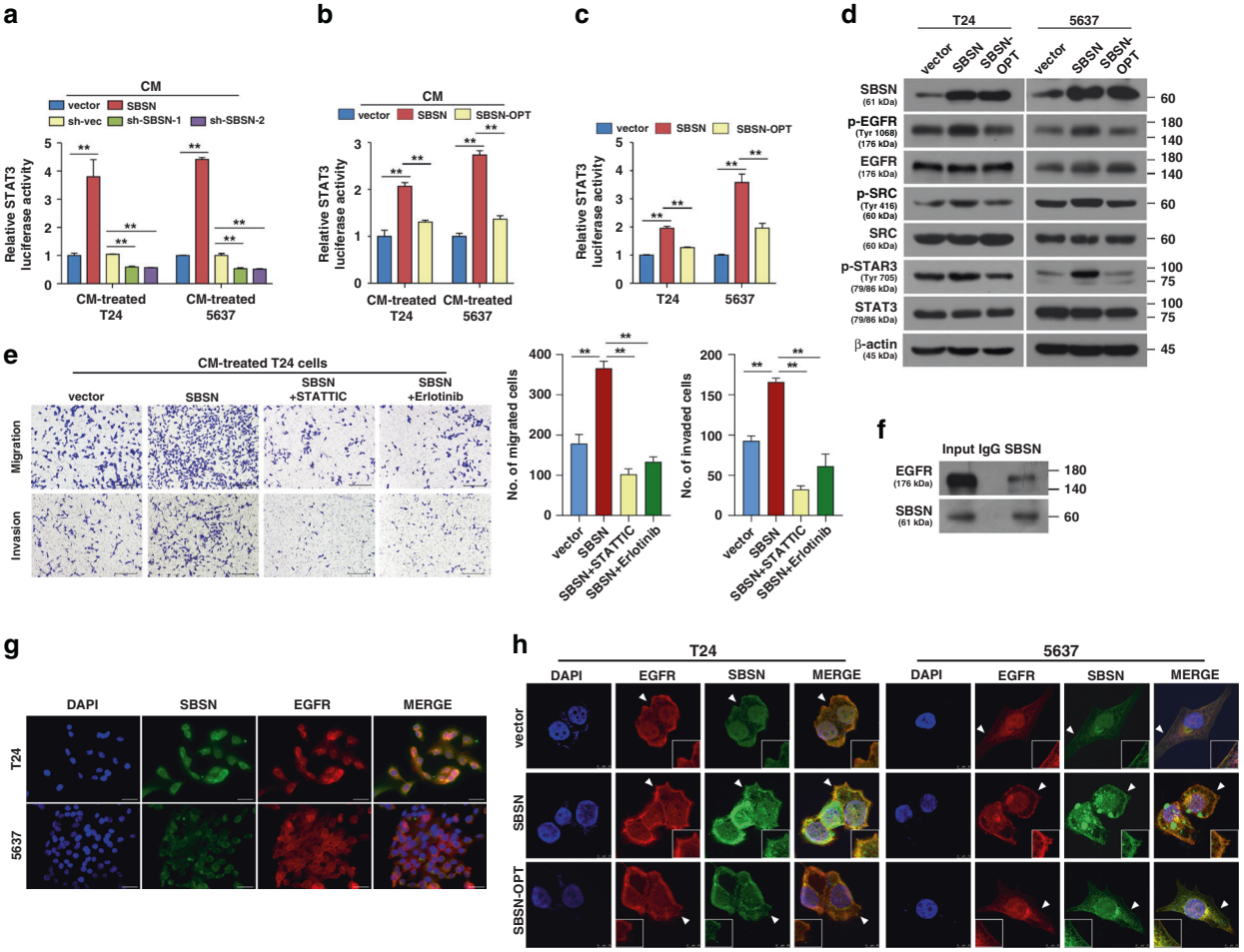

Fig. 5 Secreted SBSN activated EGFR/SRC/STAT3 signalling pathway. a, **b** STAT3 luciferase reporter activity was analysed in the blank control cells treated with the conditioned medium (CM) of indicated cells. **c** STAT3 luciferase reporter activity was analysed in indicated cells. **d** Western blotting analysis of EGFR/SRC/STAT3 pathway-related proteins in the indicated cells. **e** Representative images and quantification of the indicated migrated and invaded cells in Transwell assay. Scale bar, 100 μm. STATTIC (5 μM, 24 hours) or Erlotinib (1 μg/mL, 24 hours) was used. **f** Immunoprecipitation analysis showing SBSN interacted with EGFR. Antibodies against SBSN was used to perform co-immunoprecipitation (co-IP). **g** Immunofluorescence staining analysis showing SBSN co-localised with EGFR in cells. Scale bar, 50 μm. **h** Confocal microscopy analysis of immunofluorescence staining of the subcellular localisation SBSN and EGFR. Scale bar, 10 μm. The two-tailed *t*-test was used for statistical analysis. Error bars represent the mean ± SD of three independent experiments. $^{**}P < 0.01$.

It has been widely documented that the initiation and development of tumour metastasis involves changes in the expression of multiple genes and altered activity of different signalling pathways. Excessive activation of EGFR activates multiple downstream pathways including Ras/ERK and phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway signalling in response to extracellular ligands, followed with promotion of tumour cell reproduction, angiogenesis, and metastasis, and attenuates cell apoptosis, which may lead to drug resistance [35–37]. Therefore, many EGFR-TKIs, such as gefitinib, erlotinib, lapatinib and cetuximab, which have been designed to selectively block the EGFR signal transduction system, have been applied to treat malignancies and have achieved favourable outcomes [38–42]. In a study of bladder cancer, Michael et al. [43] identified excessive EGFR expression in 95% of bladder cancer cases with substantial squamous differentiation without the presence of activating EGFR mutations, in which the cancer cells were sensitive to the EGFR-TKIs erlotinib and gefitinib. Rebouissou et al. [44] suggested the basal-like bladder cancer subgroup was sensitive to anti-EGFR treatment and enhanced activation of EGFR signalling correlated with increased anti-EGFR drug sensitivity. However, clinical studies have found that about 30% of bladder cancer patients still progress after receiving treatment [45] mainly due to overactivation of multiple signalling pathways conducive to EGFR inhibitor resistance, while tumour heterogeneity allows tumour cells to activate different mechanisms to escape EGFR therapy [16]. Rose et al. [43] demonstrated that abnormal activation of EGFR led to activation of the PI3K/AKT pathway and increased the vascular endothelial growth factor (VEGF) secretion by tumour or stromal cells, induced tumour cell proliferation, leading to erlotinib and gefitinib resistance of bladder cancer cells. EGFR was found to interact with E-cadherin, following the activation of PI3K/mechanistic target of rapamycin (mTOR), EGFR/focal adhesion kinase (FAK) signalling, and mitogen-activated protein kinase (MAPK)/AKT signalling, resulting in promotion of epithelial-mesenchymal transition, suppression of differentiation and promotion of the progression of bladder cancer [46]. Collectively, the key components in EGFR signalling pathway all present as attractive targets for therapeutic interventions in tumour metastasis. In our study, we found that SBSN interacts with and activates EGFR, which in turn activates SRC/STAT3 signalling and promotes the synthesis and secretion of effector molecules resulting in enhancement of tumour cell migration and invasion, ultimately leading to bladder cancer aggressiveness and disease progression.

The SRC protooncogene is a nonreceptor protein tyrosine kinase and a downstream molecule of EGFR, which transmits

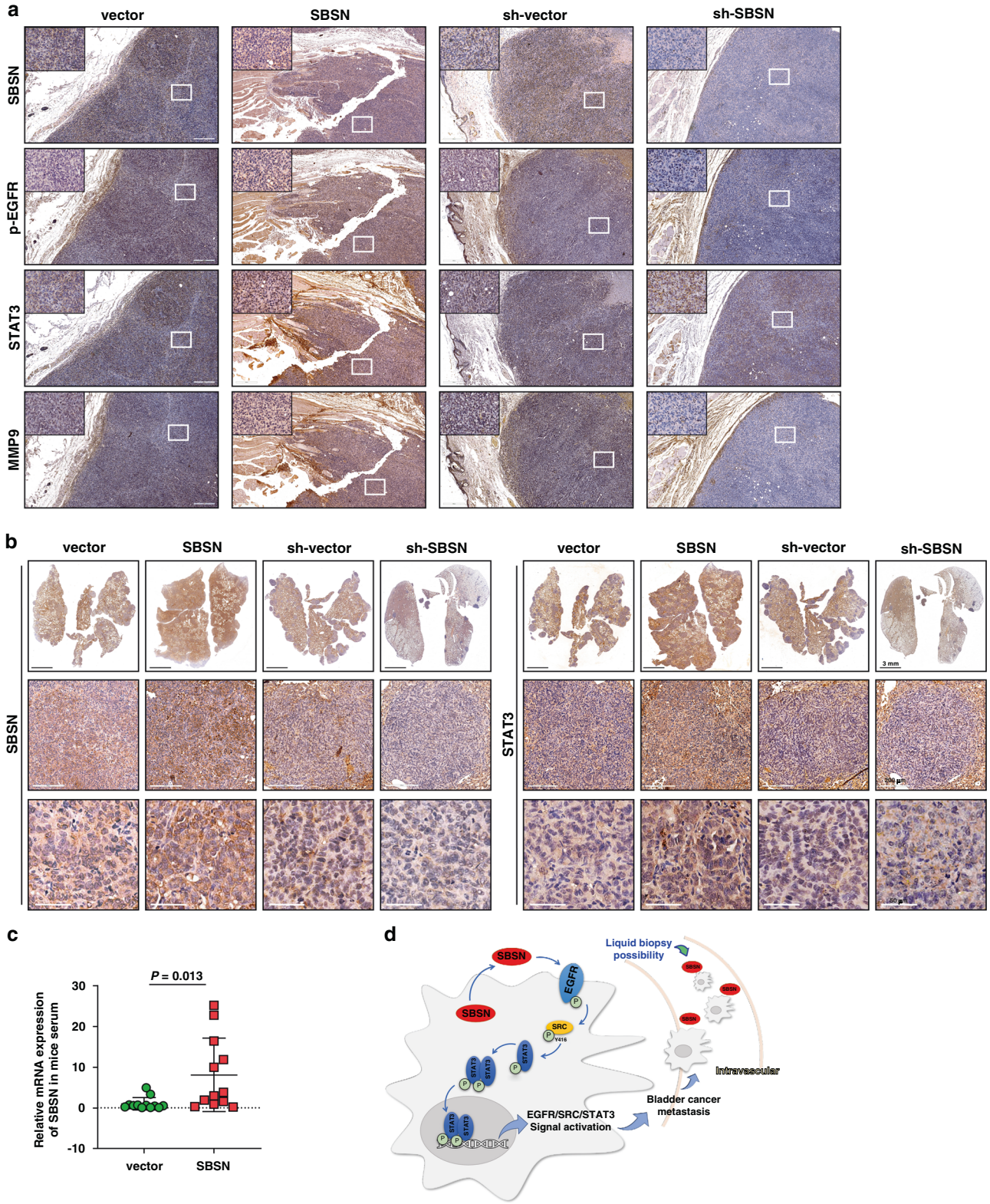

Fig. 6 SBSN activated EGFR/STAT3 signalling pathway in vivo. a Representative images of IHC staining (SBSN, p-EGFR, STAT3, MMP9) of primary tumours from indicated groups. Scale bar, 300 μm. **b** Representative images of IHC staining (SBSN, STAT3) from the metastatic tumour node of the indicated mice lung. **c** q-PCR analysis of SBSN expression in the serum of the mice. The two-tailed *t*-test was used for statistical analysis. Error bars represent the mean ± SD of three independent experiments. **d** Proposed model of SBSN activation of EGFR/SRC/STAT3 and bladder cancer metastasis.

signals promoting cell survival and mitogenesis [47, 48]. Aberrant activation of SRC signalling leads to enhanced migratory and invasive capabilities in many human tumours and plays a critical role in tumour metastasis [49, 50]. Increasing evidence has shown that SRC exerts a profound effect on in tumour invasion and metastasis, and inhibitors targeting SRC are considered promising drugs for tumour therapy. Four SRC inhibitors, bosutinib, dasatinib, ponatinib, and vandetanib have been developed and approved by the FDA for the treatment of cancer patients [51]. In turn active phosphorylated SRC phosphorylates STAT3 at Tyr705, which results in dimerisation and translocation of STAT3 to the nucleus, and subsequently induces transcription of a broad panel of genes involved in cell proliferation and survival, angiogenesis, and immunosuppressive growth factors, such as Cyclin D, Bcl-xL, MMPs, and VEGFA, all of which have been confirmed to be closely related to the progression of various malignancies [14, 52]. Phosphorylated STAT3 activates EGFR signal transduction from the cell surface to the nucleus, and enhances adhesion and invasion of cells to local tissues [15]. STAT3 activation contributes to resistance, of EGFR inhibitor and targeting STAT3 may be effective in restoring drug sensitivity in the context of EGFR inhibitor resistance [16, 53].

Collectively, EGFR/SRC/STAT3 signal might present promising value as the therapeutic target of cancers. In this study, we found that SBSN increased the phosphorylation of SRC at Tyr416 and dephosphorylation at Tyr527 via activation of the EGFR in bladder cancer. EGFR activation then induced the phosphorylation and nuclear translocation of STAT3, resulting in bladder cancer invasion and metastasis; whereas, silencing SBSN led to the inhibition of EGFR/SRC/STAT3 signalling activity, which attenuated bladder cancer progression. Combined with the EGFR/SRC/STAT3 signal targeting strategy, SBSN could serve as a valuable target for invasive bladder cancer.

Currently, the clinical diagnosis of advanced metastatic bladder cancer is of low efficiency. As the most common symptom of bladder cancer, the positive predictive value of haematuria for bladder cancer is only 8% [54]. Cystoscopy is a relatively effective but invasive tool for detecting bladder cancers [55, 56]. The sensitivity of cystoscopy for in situ carcinoma is low and tumours may be missed as the effectiveness is operator-dependent [55]. Complications, including pain during urination, urinary frequency, visible haematuria and infection, are experienced frequently after cystoscopy [57]. Despite the exploration of urinary biomarkers for the early and non-invasive detection of bladder cancer, no biomarker is presently being used in clinical practice. Our study confirmed that SBSN could be secreted by tumour cells, which is consistent with recent studies of SBSN [23, 58]. We determined that the secreted SBSN protein represents notable activation of the EGFR/SRC/STAT3 pathway, and enhances the movement and invasion of bladder cancer cells, indicating that the secreted SBSN plays essential roles in promotion of bladder cancer metastasis. Based on our study, SBSN expression and its secretion, which were upregulated in bladder cancer, were associated with poor clinicopathological characteristics of bladder cancer patients, which could have great potential to be used for diagnostic improvement. The latter would be a promising investigation in our further study.

In conclusion, our present study demonstrated that SBSN expression, as well as secreted SBSN in the bladder, significantly enhanced invasion and metastasis of bladder cancer through interaction with EGFR and subsequently activation of EGFR/SRC/STAT3 signalling. We propose that SBSN could act as a novel potential prognostic biomarker and therapeutic target in bladder cancer.

DATA AVAILABILITY

The data that support the findings of this work are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

LJ and JY developed the original idea, designed the study, analysed data, and wrote the manuscript. ZZ, ZZ and HC contributed to the development of the protocol and performed most of the experiments and data analysis. WB, XK, PZ, ZG, XX, CY and JP contributed to the in vitro biological experiments and data analysis., ZZ, ZZ, HC, DL and XC performed the in vivo experiments and data analysis. RT, ZF, and LZ contributed to clinical data collection and statistical analysis. LW provided the bioinformatics analysis.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Investigation has been conducted in accordance with the ethical standards according to the Declaration of Helsinki and national and international guidelines and has been approved by the authors' Institutional Review Board.

CONSENT FOR PUBLICATION

All informed consents were obtained. This report does not contain any individual person's information.

ADDITIONAL INFORMATION

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