Investigations into the effects and related upstream/downstream regulatory mechanisms of long non-coding RNA WT1-AS in the maintenance and development of gastric cancer stem cells *in vitro* and *in vivo*

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Research

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Abstract

Background

Cancer stem cells (CSCs) are proposed to be responsible for almost all malignant phenotypes (*e.g.* heterogeneity, uncontrolled growth, metastasis, recurrence, chemoresistance) of tumors. Long non-coding RNA WT1 antisense RNA (WT1-AS) has been found to be involved in the regulation of lung cancer cell stemness. However, the roles and molecular mechanisms of WT1-AS in the maintenance and development of gastric cancer stem cells (GSCs) have not been investigated.

Methods

mRNA and protein expression was measured by RT-qPCR and western blot. CCK8 and Soft agar colony formation assays were performed to assess cell viability and colony clone formation ability. Cell cycle and apoptosis were determined by flow cytometry analysis. Cell transwell and wound healing analyses were carried out to assess cell migration ability. *In vitro* angiogenesis and 3D spheroid cultures assays were also performed. Moreover, *in vitro* experiments were carried out to explore the function of WT1-AS on tumor growth, metastasis and cell stemness. The upstream transcription factors or downstream genes of WT1-AS were screened through Bioinformatics, dual-luciferase assays and RNA-sequencing (RNA-seq) technology.

Results

Our present study demonstrated that WT1-AS knockdown or wilms tumor 1 (WT1) overexpression improved GSC proliferative and migratory capacities, promoted GSC EMT, enhanced GSC stemness, inhibited GSC apoptosis, potentiated the resistance of GSCs to 5-FU and induced HUVEC angiogenesis *in vitro*. WT1-AS loss or WT1 increase facilitated the formation of *in-vitro* 3D GSC aggregates. WT1-AS ameliorated the malignant phenotypes of GSCs by down-regulating WT *in vitro*. Additionally, WT1-AS inhibited tumor growth and metastasis, and reduced tumor stemness in GSCs-derived xenografts (*s.c.*, *i.p.*, and *i.v.*) *in vivo*. Furthermore, XBP1 was identified as an upstream regulator of WT1-AS in GSCs. RNA-seq and RT-qPCR data suggested that PSPH, GSTO2, FYN, and PHGDH might be the downstream targets of WT1-AS in GSCs.

Conclusions

Our data demonstrated that WT1-AS weakened the stem-cell like behaviors and characteristics of GSCs *in vitro* and *in vivo* by down-regulating WT1. Also, some upstream regulators and downstream targets of WT1-AS were identified in GSCs. Investigations on the molecular mechanisms underlying the complex phenotypes of GSCs might contribute to the better management of headaches in cancers.

Introduction

Gastric cancer (GC), a heterogeneous disorder with multiple phenotypes, is a serious global healthcare problem with the fifth highest incidence rate and the third highest mortality in all cancers worldwide [1, 2]. It was estimated that more than 1,000,000 new GC cases and about 783,000 GC-related deaths occurred in 2018 globally, accounting for approximately 5.7% of all new cancer cases and 8.2% of all cancer deaths [1, 2]. Despite the improvement in the diagnosis and screening strategies, many GC cases are still diagnosed at the advanced stages and patients with advanced GC have a poor prognosis [2–4]. It is widely accepted that 5-Fluorouracil (5-FU)-based combination chemotherapy is one of the standard therapeutic strategies for GC [5, 6]. However, primary or acquired drug resistance strikingly limits the therapeutic efficiencies of chemotherapeutic drugs [7]. Cancer stem cells (CSCs) are a group of cancer cell subpopulation with the features of stem cells such as differentiation and unlimited self-renewal abilities [8]. Recently, CSC hypothesis, which proposes that CSCs are mainly responsible for the malignant phenotypes (*e.g.* heterogeneity, uncontrolled growth, metastasis, recurrence, chemoresistance) of tumors, has attracted much attention from researchers [8].

Long non-coding RNAs (lncRNAs), a class of long transcripts (over 200 nucleotides in size) with little or no protein-coding potential, have emerged as crucial regulators of CSC behaviors and properties such as proliferation, epithelial-mesenchymal transition (EMT), metastasis, differentiation [9, 10]. LncRNA WT1 antisense RNA (WT1-AS), the large antisense transcript of Wilms' tumor 1 (WT1) gene, has been found to be specifically expressed in different cancers and to be involved in the regulation of tumor growth, metastasis and invasion [11]. Moreover, Du *et al.* noted that WT1-AS expression was markedly reduced in GC tissues relative to adjacent normal tissues [12]. WT1-AS overexpression suppressed GC cell cycle progression, cell proliferation, migration and invasion *in vitro* and hindered GC xenograft tumor growth and metastasis *in vivo* [12]. Additionally, Jiang *et al.* demonstrated that the enforced expression of WT1-AS negatively regulated the stemness of non-small cell lung cancer cells [13]. However, the influences of WT1-AS overexpression or loss on the properties and phenotypes of GSCs along with the molecular mechanisms of WT1-AS action have not been characterized.

WT1-AS has been reported to be a regulator of WT1 [14, 15]. For instance, Lv *et al.* demonstrated that WT1-AS up-regulation triggered a notable reduction of WT1 expression level in HepG2 hepatocellular cancer cells [16]. WT1 also has been found to be involved in the regulation of multiple pathophysiologic processes such as cell EMT, growth, differentiation, and apoptosis [17, 18]. Moreover, WT1 has been identified as an oncogene or a tumor suppressor in different cancers [18, 19]. Additionally, previous studies showed that WT1 was highly expressed in primary GC tumor tissues [20], and WT1 silence led to the reduction of cell proliferative ability and increase of cell apoptotic percentage in AZ-521 GC cells [21, 22]. Furthermore, Royer-Pokora *et al.* pointed out that Wilms tumor cells with WT1 mutation developed stem cell-like traits after long-term culture [23], suggesting the vital roles of WT1 in the development of cancer cell stemness.

In this text, we investigated whether WT1-AS could regulate the growth, metastasis and stemness of GSCs by WT1 by *in vitro* and *in vivo* experiments. Moreover, upstream transcriptional factors and downstream genes of WT1-AS were further explored in GSCs.

Materials And Methods

Cell culture

HEK293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). SGC-7901 cells and HUVECs were obtained from China Center for Type Culture Collection (CCTCC, Wuhan, China). HEK293Tnd SGC-7901 cells were cultured in DMEM medium (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Thermo Fisher Scientific). GSCs were maintained in serum-free DMEM/F12 medium (Thermo Fisher Scientific) containing 2% B27 supplement (Thermo Fisher Scientific), 20 ng/ml epidermal growth factor (Sino Biological Co., Ltd., Beijing, China), 1% N2 supplement (Thermo Fisher Scientific), 10 ng/ml fibroblast growth factor-2 (Thermo Fisher Scientific) and 1% penicillin-streptomycin solution (Thermo Fisher Scientific). Human umbilical vein endothelial cells (HUVECs) were cultured in complete endothelial cell medium (ECM) (Sciencell Research laboratories, Carlsbad, CA, USA). All the cells were cultured in a 5% CO₂ incubator at 37°C.

Sorting and determination of GSC subpopulations by flow cytometry

CD24 and CD44 have been identified as the biomarkers of gastric cancer stem cells [24, 25]. In this text, CD24+CD44+ GSCs were sorted from SGC-7901 cells using a flow cytometry. Briefly, cells were incubated on ice for 30 min with primary antibodies against CD24 (cat. no. ab202073, Abcam, Cambridge, UK) and CD44 (cat. no. ab6124, Abcam). Next, iFluor 488 goat anti-rabbit IgG (cat. no. 16800, AAT Bioquest Inc., Sunnyvale, CA, USA) and iFluor 555 goat anti-mouse IgG (cat. no. 16739, AAT Bioquest Inc.) were added into cells post PBS buffer wash. After 30 min of dark incubation, cells were washed twice with PBS and re-suspended in PBS solution containing 2% FBS. Finally, GSC subpopulations were sorted or analyzed using the flow cytometry (BD Biosciences, San Diego, CA, USA) post filtering.

Reagents and plasmid construction

The 5-fluorouracil (5-FU) drug was purchased from MedChemExpress Inc. (Monmouth Junction, NJ, USA). The full-length fragment of WT1-AS, WT, XBP1, or TP53 was subcloned into pLenti6.3/V5-DEST lentiviral vector (cat. no. V53306, Thermo Fisher Scientific) to generate corresponding lentiviral overexpression plasmid (WT1-AS (+), WT(+), XBP1(+), or TP53(+). Also, 3 pairs of oligonucleotides targeting WT1-AS or XBP1 were constructed into pLenti6 BLOCK-iT-DEST vector (cat. no. K494400, Thermo Fisher Scientific) to generate WT1-AS(-) or XBP1(-) knockdown plasmids. The overexpression primer sequence and knockdown oligonucleotides were presented in Table 1.

Lentiviral package

Lentiviral overexpression or knockdown plasmid was transfected into HEK293T cells along with packaging plasmid mix (Novobio Biotechnology Co., Ltd., Shanghai, China) using Lipofectamine 2000 reagent (Thermo Fisher Scientific). Cell supernatants containing lentiviral particles were collected at 60 h post transfection. After centrifugation (1000 ×g, 10 min, 4°C) and filtered with 0.45 µm filters, lentiviral supernatants were concentrated in 1 ml DMEM medium by ultracentrifugation (50000 ×g, 2h, 4°C). After subpackage, lentiviral concentration solutions were stored at -80 °C. The titers of lentiviruses (10^8 transducing units/ml) were determined by double dilution method.

Established of cell lines stably transduced with lentiviral particles

GSCs at the logarithmic phase were seeded into 6 well plates and cultured in complete medium containing lentiviral particles (multiplicity of infection=0) and 8 µg/ml polybrene. At 72 h after transfection, cells were screened with µ for 3 weeks. Overexpression or knockdown effect of target genes was detected by RT-qPCR assay.

RT-qPCR assay

Total RNA was isolated from GSCs using the Trizol reagent (Thermo Fisher Scientific) according to the instructions of manufacturer. RNA was reversely transcribed into cDNA first strand using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) and oligo dT/random primers under the reaction conditions: 25°C for 5 min, 50°C for 60 min, and 70 °C for 15 min. Next, cDNA was amplified and quantified using SYBR Green I fluorescent dye (Thermo Fisher Scientific), Platinum Taq DNA Polymerase (Thermo Fisher Scientific) and specific quantitative primers. The reaction procedures were: 95 °C for 2 min, 40 cycles of 95 °C for 10s, 60 °C for 30s, and 70 °C for 45s. The quantitative primer sequences were presented in Table 1. β-actin functioned as the housekeeping gene and relative expression levels of lncRNAs and mRNAs were calculated using the $2^{-\Delta\Delta C_t}$ method.

CCK-8 assay

Cell viability was examined using the Cell Counting Kit-8 (CCK-8) kit (Beyotime Biotechnology, Shanghai, China) according to the protocols of manufacturer. Briefly, CCK-8 solution was co-incubated with treated cells at the indicated time points. After 2 h of incubation at 37°C, the optical density (OD) values were measured at 450 nm using a MK3 microplate reader (Thermo Fisher Scientific).

Soft agar colony formation assay

Cell proliferative potential was examined by soft agar colony formation assay. Firstly, the mixture solution (3 ml) containing equal volume of 1.2% agarose and 2× complete medium supplemented with 2×antibiotics and 20%FBS were added into the 6 cm culture dishes to prepare the base agar layer. Next, cells at the logarithmic phase (1000 cells/well) and the equal-volume mixtures (3 ml) of 0.7% agarose and 2× complete medium containing 2×antibiotics and 20%FBS were added into the upper layer. Next, the

culture dishes were maintained for 14 days in a 5% CO₂ incubator at 37°C. Finally, the number of colonies was counted under a microscope.

Cell cycle detection

For cell cycle determination, cells were fixed overnight at 4°C with pre-cold 70% ethanol. After low-speed centrifugation (500 ×g, 10 min, 4°C), cells were incubated for 30 min with the staining solution containing Propidium Iodide (PI, 50 µg/ml, Sigma-Aldrich), RNase A (100 µg/ml) and 0.2% Triton X-100 in the dark. Next, cell cycle distribution patterns were measured using a flow cytometry (BD Biosciences).

Cell apoptotic rate detection

Cell apoptotic percentage was determined using an Annexin V-PE/7-AAD Apoptosis Detection kit (Nan Jing KeyGen Biotech Co., Ltd., Nanjing, China) following the instructions of manufacturer. Briefly, cells were re-suspended in Binding Buffer and then stained with Annexin V-PE and 7-AAD solutions for 15 min at room temperature under a dark environment. Finally, cell apoptotic patterns were analyzed using a flow cytometry (BD Biosciences).

Transwell migration assay

Cells (2×10⁴ cells/well) suspended in serum-free medium were seeded into the upper chamber of Transwell plate (8µm pore size; Costar Corning, Corning, NY, USA) and complete medium containing 10% FBS was added into the low chamber of plate. The plates were cultured in a 5%CO₂ incubator for 24 h at 37 °C. Next, cells on the top surfaces of the Transwell filters were removed. Cells on the low surfaces were fixed with 4% paraformaldehyde for 30 min and stained with 1% crystal violet solution (Sigma-Aldrich, Inc., St Louis, MO, USA) for 10 min. Finally, cells were imaged and counted using a microscope.

Wound healing assay

Cells were seeded into 24-well plates and cultured overnight at 37°C. When cells grew to the full confluency, a straight scratch wound was created using a sterile 10µl pipette tip. After washed three times with PBS solution, cells were maintained in serum-free medium at 37°C in a 5% CO₂ incubator. The wound images were captured at 0 h and 24 h post scratching using a microscope (CKX31, Olympus, Tokyo, Japan). Finally, the migratory distance of cells under the same filed were measured and statistically analyzed.

Western blot analysis

Cell lysates were prepared using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) supplemented with protease inhibitor (Sigma-Aldrich). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins (40µg/sample) were separated through SDS-PAGE and then transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). After blocked with 5% skim milk, the membranes were incubated with primary antibody against vimentin (cat. no. bs-

0756R), TWIST (cat. no. bs-2441R), CTNNB1 (beta catenin, cat. no. bs-20599R), MMP2 (cat. no. 10373-2-AP), E-cadherin (cat. no. CDH1, bs-10009R), N-cadherin (cat. no. CDH2, ab18203) and GAPDH (cat. no. bs-0755R) for 12 h at 4°C. Next, the membranes were probed with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (cat. no. D110058, Sangon Biotech Co., Ltd., Shanghai, China) for 1 h at room temperature. Finally, protein bands were developed using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific). All primary antibodies were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China).

***In vitro* angiogenesis assay**

HUVECs were plated in 24-well plates re-coated with Matrigel (Costar Corning) and pre-treated with CFDA-SE (MedChemExpress, 5 μM) for 10 min at room temperature. Next, HUVECs were cultured in condition medium containing 50% ECM complete medium (Sciencell) and 50% GSC cell supernatants (volume ratio=1:1). Six hours later, cells were imaged using Olympus IX51 fluorescence microscope (Olympus, Tokyo, Japan) and angiogenesis patterns (tube lengths) were analyzed using an Olympus Cellsens 1.5 software (Olympus).

***In vitro* 3D culture**

Cells at the logarithmic growth phase were collected and re-suspended in the complete medium containing 2.5% Matrigel (volume ratio, Costar Corning) at a density of 10^4 cells/ml. Next, cells (200 μl) were added into 96-well plates pre-coated with agarose. After low-speed centrifugation (1000 ×g, 10 min, 4°C), cells were cultured at 37°C in a 5% CO₂ incubator. Medium was replaced with fresh medium every other day. Next, these 3D multiple cell tumor spheres were imaged using a microscope after 7 days.

Promoter luciferase reporter assay

The luciferase reporter containing WT1-AS promoter sequences (-1K~+200) were constructed and corresponding WT1-AS reporter lentiviruses were generated by Novobio Biotechnology Co., Ltd. GSC cells stably transduced with XBP1(+), XBP1(-), NC lentiviruses were also infected with the WT1-AS reporter lentiviruses, followed by the detection of luciferase activities.

***In vivo* mouse xenograft experiments**

All animal experiments were performed with the approval of Experimental Animal Center of the Affiliated Hospital of Jining Medical University. A total of 45 athymic BALB/c mice (male, 6-8 weeks old) were purchased from Shanghai Laboratory Animal Research Center (Shanghai, China) and raised under the standard conditions. Mice were randomly divided into 9 groups: WT1-AS(+) (subcutaneous), WT1-AS(-) (subcutaneous), NC (subcutaneous), WT1-AS(+) (peritoneal), WT1-AS(-) (peritoneal), NC (peritoneal), WT1-AS(+) (tail vein), WT1-AS(-) (tail vein), and NC (tail vein) groups. Each group contains 5 mice.

For subcutaneous xenograft experiments, GSCs (10^6 cells) stably transduced with WT1-AS(+), WT1-AS(-), or control (NC) lentiviruses were injected into the subcutaneous tissues of mice in corresponding groups,

respectively. Tissue volume was measured 4 weeks after injection and calculated with the formula: $\text{Volume} = 0.5 \times \text{length} \times (\text{width})^2$. Tumors were resected at weeks post injection.

For peritoneal xenograft experiments, GSCs (10^6 cells) transduced with WT1-AS(+), WT1-AS(-), or control (NC) lentiviruses were intraperitoneally injected into the corresponding mice. The volumes of ascetic fluid were measured. Tumors were resected and weighed at weeks upon injection.

For lung tumor metastasis models, GSCs (10^6 cells) stably transduced with WT1-AS(+), WT1-AS(-), or control lentiviruses were administrated into corresponding mice via the tail vein. Lung tissues were obtained at

Partial tumor tissues were fixed with 10% formalin, embedded with paraffin and cut into 5 μm sections, followed by hematoxylin-eosin (HE) staining and Ki-67 immunohistochemistry (IHC), and cell subpopulation analysis. HE analysis was carried out using the Hematoxylin and Eosin Staining Kit (Beyotime) according to the manufacturer's instructions. Briefly, tumor sections were deparaffinized with xylene, rehydrated with different concentrations of ethanol, and stained with hematoxylin and eosin. After dehydrated, permeabilized and sealed, the sections were imaged under a microscope. For IHC analysis, tumor sections were deparaffinized and rehydrated. After the removal of endogenous peroxidase and treatment of antigen retrieval, sections were blocked with 10% normal goat serum for 30 min at room temperature and incubated overnight at 4°C with primary antibody against Ki67 and then probed with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Next, the sections were incubated with 3,3'-diaminobenzidine (DAB) solution and counterstained with hematoxylin. After the routine treatment of dehydration, clearing and mounting, the sections were imaged. Cell subpopulation analysis was performed with the experimental procedures similar with cell sorting except cell sorting procedures.

RNA-seq

RNA was isolated from GSCs stably transduced with lentiviruses using Trizol Reagent (Thermo Fisher Scientific), followed by the measurement of RNA concentration, purity and integrality. After quality control, RNA was purified, fragmented, and transformed into cDNA library. Next, cDNA library was enriched, quantified by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and sequenced using the Illumina HiSeq 2500 instrument (Illumina).

Raw data obtained after RNA-seq were processed into clean data by removing the sequences with 3' adapters or reads with the average quality score < Q20. Next, filtered clean data were aligned to the reference genome (Homo_sapiens.GRCh38.dna.primary_assembly.fa) using the HISAT2 software (<http://ccb.jhu.edu/software/hisat2/index.shtml>). Read counts were normalized using the FPKM (fragments per kilo bases per million fragments) method. Gene were regarded as differentially expressed when they satisfied the conditions: $|\log_2 \text{FoldChange}| > 1$ and $P \text{ value} < 0.05$. Cluster analysis for differentially expressed genes were performed using the long distance hierarchical clustering method (Complete Linkage) and Euclidean distance metric (R language: Pheatmap software package) according

the expression levels of the same genes in different samples and expression patterns of different genes in the same sample. Expression trends of genes in the same cluster were similar. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed using KAAS software. The top 5 KEGG pathways were picked out according to the P values (the 5 minimum P values).

Clinical samples

GC tumors and adjacent normal tissues were collected from 3 cases of GC patients underwent surgery sections during May 2017 to July 2018. Our study was conducted with the approval of the Medical Ethics Committee of the Affiliated Hospital of Jining Medical University, and the written informed consents from all participants.

Statistics Analysis

Data (*in vitro* and *in vivo* experiments) analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc., San Diego, CA, USA) with the results presenting as mean $\pm$ standard deviation. Difference between groups was analyzed using paired or unpaired *T* test. Difference among groups was analyzed using two-way ANOVA along with Sidak post-hoc test or one-way ANOVA along with Tukey's post-hoc test. $P < 0.05$ was regarded a significant difference.

Results

Expression analysis of WT1-AS or WT1 in GSCs stably transduced with corresponding overexpression or knockdown lentiviruses.

To investigate the effects of WT1-AS overexpression or knockdown on phenotypic and biological characteristics of GSCs, lentiviruses that can generate the full-length fragment of WT1-AS (WT1-AS(+)) and lentiviruses carrying the knockdown sequences of WT1-AS (WT1-AS(-)#1, WT1-AS(-)#2, and WT1-AS(-)#3) along with corresponding control lentiviruses (NC) were generated. Next, GSC cell lines stably transduced with these lentiviruses were established. RT-qPCR assay validated that WT1-AS expression level was remarkably up-regulated in GSCs stably transduced with WT1-AS(+) lentiviruses compared to control group (Fig. 1A). Conversely, a notable down-regulation of WT1-AS expression level was observed in GSCs stably transduced with WT1-AS(-)#1, WT1-AS(-)#2, or WT1-AS(-)#3 lentiviruses than that in control group (Fig. 1B). In view of the best knockdown efficiency of WT1-AS(-)#1, GSCs stably transduced with WT1-AS (-)#1 lentiviruses were selected in the subsequent experiments. Also, our data revealed that the transduction of WT1(+) lentiviruses led to the dramatic up-regulation of WT1 mRNA level in GSCs relative to control group (Fig. 1C).

WT1-AS overexpression inhibited cell proliferation, facilitated cell apoptosis and reduced 5-FU resistance by down-regulating WT1 in GSCs.

CCK-8 assay showed that the enforced expression of WT1-AS notably weakened cell viability in GSCs (Fig. 2A). Soft agar colony formation assay also disclosed that WT1-AS1 overexpression led to the

obvious decrease of cell colony number in GSCs (Fig. 2B). Moreover, WT1-AS hindered cell cycle progression in GSCs, as evidenced by the notable reduction of cell percentage in G2 and S phases in GSCs stably transduced with WT1-AS(+) lentiviruses compared with control group (Fig. 2C). These outcomes showed that WT1-AS overexpression weakened the proliferative activity of GSCs. Also, a noticeable increase in cell apoptotic rate was observed in WT1-AS-overexpressed GSCs compared to NC group (Fig. 2D). Moreover, WT1-AS overexpression hindered cell cycle progression (Fig. 2E) and induced cell apoptosis (Fig. 2F) in 5-FU-treated cells, suggesting that WT1-AS weakened the resistance of GSCs to 5-FU. Conversely, WT1-AS knockdown led to the increase of cell viability (Fig. 2A), cell colony number (Fig. 2B) and cell percentage in G2 and S phases (Fig. 2C), and reduction of cell apoptotic rate (Fig. 2D) in GSCs. WT1-AS loss enhanced the resistance of GSCs to 5-FU (Fig. 2E and 2F). Moreover, our data disclosed that WT1 overexpression facilitated cell proliferation, curbed cell apoptosis and potentiated 5-FU resistance in GSCs (Fig. 2A-2F). Additionally, WT1 up-regulation weakened the effects of WT1-AS on cell proliferation, apoptosis and 5-FU resistance in GSCs (Fig. 2A-2F).

WT1-AS hindered cell migration and EMT by down-regulating WT1 in GSCs.

Next, Transwell migration assay disclosed that enforced expression of WT1-AS triggered the notable reduction of cell migratory potential in GSCs (Fig. 3A). The wound healing assay also presented that WT1-AS overexpression hindered cell migration in GSCs (Fig. 3B). RT-qPCR and western blot assays demonstrated that WT1-AS up-regulation led to the notable reduction in mRNA and protein expression levels of vimentin, twist1 and cttnb1 in GSCs (Fig. 3C and 3D). These outcomes suggested that WT1-AS overexpression inhibited cell migration and EMT in GSCs. Conversely, WT1-AS depletion facilitated cell migration and induced the expression of vimentin, twist1 and cttnb1 in GSCs (Fig. 3A-3D). In addition, increased cell migratory capacity and elevated expression of vimentin, twist1 and cttnb1 was observed in GSCs stably transduced with WT1(+) lentiviruses compared with control group (Fig. 3A-3D). Furthermore, WT1 overexpression abrogated the detrimental effects of WT1-AS on cell migration and EMT in GSCs (Fig. 3A-3D).

WT1-AS overexpression inhibited angiogenesis, reduced GSC stemness, and hindered the formation of *in-vitro* 3D GSC aggregates by down-regulating WT1.

Next, our data further revealed that the addition of supernatants of GSCs transduced with WT1-AS(+) lentiviruses led to the reduction in the angiogenesis activity of HUVECs *in vitro*, suggesting that WT1-AS overexpression suppressed angiogenesis (Fig. 4A). Moreover, WT1-AS overexpression notably reduced the percentage of CD44+ GSCs relative to control group (Fig. 4B). Additionally, our outcomes revealed that WT1-AS loss or WT1 overexpression in GSCs promoted angiogenesis *in vitro* and increased CD44+ stem-like subpopulation (Fig. 4A and 4B). Furthermore, WT1 increase markedly alleviated the detrimental effects of WT1-AS on angiogenesis and attenuated WT1-AS-mediated loss of CD44+ subpopulation (Fig. 4A and 4B). Next, 3D cell culture experiments were performed to mimic the physiological microenvironment of GSCs in view of the strong dependence of stem cells on their environment. Results showed that WT1-AS overexpression triggered the notable reduction in the size of 3D spheroid cultures

derived from GSCs (Fig. 4C). Conversely, WT1-AS loss promoted the formation of *in-vitro* 3D GSC aggregates (Fig. 4C). Also, a marked increase in the size of 3D spheroids was observed in WT1-overexpressed GSCs compared to control group (Fig. 4C). Moreover, WT1 overexpression weakened the inhibitory effects of WT1-AS on the formation of 3D spheroid cultures (Fig. 4C), suggesting that WT1-AS hindered the development of GSCs by down-regulating WT1 in 3D cell culture model.

WT1-AS inhibited tumor growth and reduced cell stemness in subcutaneous xenograft tumors derived from GSCs.

Next, the effects of WT1-AS overexpression or knockdown on the growth and stemness of GSCs-derived xenograft tumors were further investigated *in vivo*. Subcutaneous xenograft experiments demonstrated that enforced expression of WT1-AS led to the notable reduction in the volume of xenograft tumors (Fig. 5A). Also, Ki67-positive cell percentage was markedly decreased in xenograft tumors of WT1-AS(+) group compared to NC group (Fig. 5B). Moreover, HE staining analyses presented that WT1-AS overexpression led to the notable reduction in the blue staining area/intensity and abnormality in the morphology of cells and cell nucleus (Fig. 5B). These data suggested that WT1-AS1 inhibited DNA replication and hindered the development of subcutaneous xenograft tumors. Additionally, a noticeable reduction in the percentage of CD44 positive population was observed in subcutaneous xenograft tumors of WT1-AS(+) group versus NC group (Fig. 5C). Conversely, WT1-AS knockdown induced the tumor growth and reduced CD44+ subpopulation in GSCs-derived subcutaneous xenograft tumors (Fig. 5A-5C).

WT1-AS inhibited tumor growth and metastasis, and reduced tumor stemness in xenografts derived from GSCs.

Additionally, abdominal xenograft experiments demonstrated that enforced expression of WT1-AS led to the notable reduction of ascites volume (Fig. 6A) and abdominal xenograft tumor weight (Fig. 6B). Also, a conspicuous down-regulation in the cell percentage of Ki67+ (Fig. 6C) and CD44+ (Fig. 6D) was observed in abdominal xenograft tumors of WT1-AS(+) group than that in NC group. HE staining analyses also revealed that cell nuclear (blue) staining areas were reduced and cell injury was mitigated in WT1-AS-overexpressed abdominal xenograft tumors relative to NC group (Fig. 6C). These data demonstrated that WT1-AS hindered the development of GSCs-derived abdominal xenografts. Inversely, WT1-AS depletion promoted the development of GSCs-derived abdominal xenograft tumors. To further investigate the effects of WT1-AS overexpression or knockdown on lung metastasis of GSC-derived xenograft tumors, GSCs stably transduced with NC, WT1-AS(+) or WT1-AS(-) lentiviruses were injected into mice via tail vein. IHC and flow cytometry analyses respectively showed that the positive cell percentages of Ki67 and CD44 were notably reduced in the lung tissues of WT1-AS(+) group, but markedly increased in WT1-AS(-) group (Fig. 6E and 6F). HE staining data also revealed that WT1-AS overexpression inhibited the lung metastasis of GSCs, as evidenced by the reduction in the blue staining area/intensity and alleviation of lung tissue lesions (Fig. 6E). Conversely, WT1-AS loss induced the lung metastasis of GSCs (Fig. 6E and 6F). These results suggested that WT1-AS inhibited tumor growth and metastasis, and reduced tumor stemness in xenografts derived GSCs (*i.p.* and *i.v.*).

Identification of upstream transcription factors of WT1-AS

Next, twenty-four transcription factors (general transcription factor Ili (GTF2I), signal transducer and activator of transcription 4 (STAT4), ETS proto-oncogene 1 (ETS1), GATA binding protein 1 (GATA1), paired box 5 (PAX5), nuclear receptor subfamily 3 group C member 1 (NR3C1), tumor protein p53 (TP53), nuclear factor kappa B subunit 1 (NFKB1), retinoid X receptor alpha (RXRA), transcription factor AP-2 alpha (TFAP2A), MYB proto-oncogene (MYB), SP1, E2F transcription factor 1 (E2F1), hypoxia inducible factor 1 alpha subunit (HIF1A), YY1, peroxisome proliferator activated receptor alpha (PPARA), upstream transcription factor 1 (USF1), ETS proto-oncogene 2 (ETS2), ETS transcription factor ELK1 (ELK1), androgen receptor (AR), estrogen receptor 1 (ESR1), X-box binding protein 1 (VDR), X-box binding protein 1 (XBP1), and MYC with a possibility to bind with the promoter region of WT1-AS were screened out by the prediction website (<http://alggen.lsi.upc.es/>) (Ensemble regulatory elements: ENSR00001569407; TSS distance (bp): -318 bp; Size: 801 bp). Next, the expression analyses for these 24 genes were performed by RT-qPCR assay in GCs, GSCs, GC tumors and peritumoral tissues. Among genes with the same change trends in the groups of GC tumors versus peritumoral tissues and GSCs vs GCs, TP53 and XBP1 were selected for further investigations in view of their vital roles in the development and progression of stem cells [26-28]. Our outcomes revealed that TP53 expression was notably downregulated in GSCs versus GCs (Fig. 7A) and GC tumor tissues versus adjacent normal tissues (Fig. 7B). RT-qPCR assay demonstrated that TP53 or XBP1 expression level was remarkably increased in GSCs infected with TP53(+) or XBP1(+) lentiviruses, respectively (Fig. 7C and 7D). However, TP53 up-regulation had no effect on WT1-AS expression in GSCs (Fig. 7C). XBP1 overexpression triggered the notable down-regulation of WT1-AS expression level in GSCs (Fig. 7D). Moreover, our data showed that XBP1 mRNA level was noticeably down-regulated and WT1-AS level was markedly elevated in GSCs infected with XBP1(-)#1, XBP1(-)#2 or XBP1(-)#3 lentiviruses carrying corresponding XBP1 knockdown fragment compared to NC group (Fig. 7E), suggesting that XBP1 knockdown facilitated WT1-AS expression in GSCs. The XBP1(-)#2 was selected in subsequent experiments due to its strongest knockdown efficiency. Additionally, XBP1 overexpression noticeably reduced the luciferase activity of WT1-AS promoter-driven luciferase reporter, while XBP1 loss conspicuously increased the activity of WT1-AS promoter in GSCs (Fig. 7F). These data suggested that XBP1 could negatively regulate WT1-AS expression by binding with the promoter region of WT1-AS.

Identification of downstream genes of WT1-AS by RNA-seq technology

Next, RNA-seq technology was employed to search for potential downstream genes regulated by WT1-AS in GSCs stably transduced with NC, WT1-AS(+) (AS_OV), WT1-AS(-) (AS_KD), or WT1-AS(+) + WT1(+) lentiviruses (ASWT_OV). RNA-seq outcomes revealed that 35 genes were differentially expressed in ASWT_OV versus AS_OV group (Table 2). Also, 587 or 332 differentially expressed genes were identified in AS_OV versus NC group or AS_KD versus NC group (Table 2). The heat map of all differentially expressed genes was presented in Fig. 8A and corresponding gene information was displayed in Table 3. To identify potential biological relationships among genes, cluster analysis for all differentially expressed genes (n=842) in AS_OV versus NC, AS_KD versus NC and ASWT_OV versus AS_OV groups was

performed according to the co-expression patterns of genes. As presented in Fig. 8B and Table 4, these differentially expressed genes (n=842) were grouped into 9 different clusters. Cluster analyses suggested that genes (n=516) belonging to cluster-2 and cluster-7 could be regulated by WT1-AS rather than WT1 in GSCs. Among the differentially expressed genes in the cluster-2 and cluster-7, 36 genes were found to be involved in the regulation of the top 5 KEGG enrichment pathways (Table 5). Among these 36 genes, 10 genes (phosphoserine phosphatase (PSPH), phosphoglycerate dehydrogenase (PHGDH), glutathione S-transferase omega 2 (GSTO2), calcium channel $\alpha 2\delta 1$ subunit (CACNA2D1), FYN, monoamine oxidase B (MAOB), aminopeptidase-N (ANPEP), Integrin alpha 2 (ITGA2), cathepsin S (CTSS), and protein kinase C alpha (PRKCA) with potential oncogenic effects were selected for further investigations. RT-qPCR outcomes showed that the expression levels of PSPH, GSTO2, FYN, and PHGDH were notably reduced in WT1-AS-overexpressed GSCs, but were markedly up-regulated in WT1-AS-depleted GSCs (Fig. 8C). Moreover, WT1 overexpression did not influence the expression of PSPH, GSTO2, FYN, and PHGDH in WT1-AS-overexpressed GSCs (Fig. 8C). These outcomes suggested that PSPH, GSTO2, FYN, and PHGDH could be regulated by WT1-AS, but not WT1 in GSCs. In other words, PSPH, GSTO2, FYN, and PHGDH might be the downstream targets of WT1-AS.

Discussion

In this text, we demonstrated that WT1-AS knockdown or WT1 overexpression in CD24⁺/CD44⁺ GSCs enhanced GSC cell proliferative and migratory capacities, promoted GSC cell EMT, increased CD44⁺ stem-like cell subpopulation, induced HUVEC angiogenesis, inhibited GSC cell apoptosis, and enhanced the resistance of GSCs to 5-FU *in vitro*. Moreover, 3D cell culture experiments demonstrated that WT1-AS loss or WT1 increase facilitated the formation of *in-vitro* 3D GSC aggregates. Moreover, our data presented that WT1-AS weakened the malignant behaviors and properties of GSCs by down-regulating WT *in vitro*. In addition, our *in vivo* experiments demonstrated that WT1-AS inhibited the growth and metastasis of GSCs-derived xenograft tumors and reduced CD44⁺ stem-like cell subpopulation in xenografts. These data suggested that WT1-AS functioned as a negative regulator in the maintenance and reprogramming of GSC stemness properties.

It is well known to us that transcription factors can regulate the expression of lncRNAs [29]. Thus, transcription factors that had the likelihood to bind with the promoter regions of WT1-AS were predicted. Among these transcription factors, TP53 [26, 30] and XBP1 [27, 28, 31] were found to be implicated in the formation and development of cancer stem cells. Our experiments demonstrated that TP53 increase did not influence WT1-AS expression in GSCs. However, XBP1 could negatively regulate WT1-AS expression by binding with promoter region of WT1-AS in GSCs.

Recently, RNA-seq technology has been widely used to decipher the molecular mechanisms of lncRNAs in physiological and pathological conditions [32]. Also, a growing body of evidences shows that lncRNAs can exert their functions by regulating the expression of coding genes [33]. Hence, downstream genes (excluding WT1) that could be regulated by WT1-AS were investigated by RNA-seq. Combined with the KEGG enrichment data and cluster analyzes, 10 genes (PSPH [34, 35], PHGDH [36, 37], GSTO2 [38],

CACNA2D1 [39, 40], FYN [41], MAOB [42, 43], ANPEP [44, 45], ITGA2 [46, 47], CTSS [48] and PRKCA [49] were selected for further explorations in view of their close association with cancer progression and prognosis. Our RT-qPCR assay validated that PSPH, GSTO2, FYN, and PHGDH could be regulated by WT1-AS, but not WT1 in GSCs. In other words, PSPH, GSTO2, FYN, and PHGDH were the downstream targets of WT1-AS. A recent study pointed out that enforced expression of FYN promoted cell migration, invasion and EMT *in vitro* and induced GC lung metastasis *in vivo* [50]. Additionally, Guo *et al.* demonstrated that FYN expression was positively regulated by 5'-Nucleotidase Domain Containing 2 (NT5DC2), while NT5DC2 depletion markedly weakened GSC cell viability and tumorsphere formation potential *in vitro* and hampered the growth of GSCs-derived xenograft tumors *in vivo* [51], suggesting that FYN might function as a positive regulator in the development of GSCs. Our RNA-seq and RT-qPCR assay validated that FYN expression was notably reduced in WT1-AS-overexpressed GSCs, but markedly increased in WT1-AS-depleted GSCs. These data suggested that WT1-AS might hindered the development of GSCs by down-regulating FYN.

PHGDH also has been found to be highly expressed in GC tissues and GC patients with high PHGDH expression has a poor prognosis [52]. Moreover, PHGDH depletion weakened 5-FU resistance in GCs [53]. Additionally, Sharif *et al.* demonstrated that PHGDH was required to maintain the self-renewal activity, stemness and pluripotency in embryonal cancer stem-like cells [54]. Our data showed that WT1-AS overexpression led to the loss of stem-like features and down-regulation of PHGDH level in GSCs. Combined with the prior report [54], we supposed that WT1-AS might negatively regulated the self-renewal, tumorigenesis and metastasis of GSCs by silencing PHGDH.

Conclusions

Taken together, our data demonstrated that WT1-AS weakened the stem-cell like behaviors and characteristics of GSCs *in vitro* and *in vivo* by down-regulating WT1, elucidating the vital roles of WT1-AS in the maintenance and development of GSCs and revealing a mechanism governing WT1-AS-induced loss of GSC stem-like phenotypes. Moreover, XBP1 as an upstream regulator of WT1-AS was identified in GSCs. Additionally, our data and previous studies suggested that FYN and PHGDH might be the potential downstream targets of WT1-AS. Furthermore, multitudinous downstream genes that could be regulated by WT1-AS or WT1 were identified by RNA-seq technology in GSCs. An in-depth understanding on the molecular mechanisms underlying the complex phenotypes of GSCs might contribute to the better management of intra-tumoral heterogeneity, drug resistance, tumor development and recurrence.

Abbreviations

Cancer stem cells (CSCs), long non-coding RNA WT1 antisense RNA (WT1-AS), gastric cancer stem cells (GSCs), wilms tumor 1 (WT1), gastric cancer (GC), 5-Fluorouracil (5-FU), epithelial-mesenchymal transition (EMT), hematoxylin-eosin (HE), immunohistochemistry (IHC), diaminobenzidine (DAB), general transcription factor Ili (GTF2I), signal transducer and activator of transcription 4 (STAT4), ETS proto-oncogene 1 (ETS1), GATA binding protein 1 (GATA1), paired box 5 (PAX5), [nuclear receptor subfamily 3](#)

group C member 1 (NR3C1), tumor protein p53 (TP53), nuclear factor kappa B subunit 1 (NFKB1), retinoid X receptor alpha (RXRA), transcription factor AP-2 alpha (TFAP2A), MYB proto-oncogene (MYB), SP1, E2F transcription factor 1 (E2F1), hypoxia inducible factor 1 alpha subunit (HIF1A), peroxisome proliferator activated receptor alpha (PPARA), upstream transcription factor 1 (USF1), ETS proto-oncogene 2 (ETS2), ETS transcription factor ELK1 (ELK1), androgen receptor (AR), estrogen receptor 1 (ESR1), X-box binding protein 1 (VDR), X-box binding protein 1 (XBP1), phosphoserine phosphatase (PSPH), phosphoglycerate dehydrogenase (PHGDH), glutathione S-transferase omega 2 (GSTO2), calcium channel $\alpha 2\delta 1$ subunit (CACNA2D1), monoamine oxidase B (MAOB), aminopeptidase-N (ANPEP), Integrin alpha 2 (ITGA2), cathepsin S (CTSS), protein kinase C alpha (PRKCA), 5'-Nucleotidase Domain Containing 2 (NT5DC2)

Declarations

Ethics approval and consent to participate

All animal experiments were performed with the approval of Experimental Animal Center of the Affiliated Hospital of Jining Medical University and the procedures for Care and Use of Laboratory Animals in cancer research.

Consent for publication

Informed consent for publication was obtained from all participants.

Authors' contributions

Xiaobei Zhang designed and performed the experiments, wrote the manuscript. Meng Jin, Shiqi Liu, Mingde Zang, Lei Hu contributed to experimental work and data analysis. Tao Du and Baogui Zhang designed the experiments and revised the manuscript. All authors have read and approved the final manuscript. All authors have read and approved the final manuscript.

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Data Availability Statement

The data and material displayed in this manuscript is available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors have no conflict of interest to declare.

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Tables

Due to technical limitations, Tables 1-5 are only available as a download in the supplemental files section

Figures

Figure 1

Expression analysis of WT1-AS and WT1 in GSCs stably transduced with lentiviruses. (A) Expression level of WT1-AS was measured by RT-qPCR assay in GSCs stably transduced with NC or WT1-AS(+) lentiviruses. (B) Expression level of WT1-AS was determined by RT-qPCR assay in GSCs stably transduced with NC, WT1-AS(-)#1, WT1-AS(-)#2, or WT1-AS(-)#3 lentiviruses. (C) WT1 mRNA level was detected by RT-qPCR assay in GSCs stably transduced with NC or WT1(+) lentiviruses. pLenti6.³/V5-DEST empty vector and packaging plasmid mix were co-transfected into HEK293T cells to generate NC control lentiviruses. ***P < 0.001. *: compared to NC group.

Figure 2

WT1-AS overexpression suppressed cell proliferation, facilitated cell apoptosis and reduced 5-FU resistance by down-regulating WT1 in GSCs. (A) Cell viability was measured by CCK-8 assay. (B) Cell proliferation activity was estimated by soft agar colony formation assay. (C and D) Cell cycle distribution patterns and cell apoptotic rate were analyzed using a flow cytometry. (E and F) GSCs cells were treated with 5-FU (25μM) for 7 days, followed by the measurement of cell apoptotic rate and cell cycle

distribution patterns. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. *: compared to NC group. ## $P < 0.01$. ### $P < 0.001$. #: compared to WT1-AS(+) group.

Figure 3

WT1-AS suppressed cell migration and EMT by down-regulating WT1 in GSCs. (A and B) Cell migratory ability was assessed by Transwell migration assay (A) and wound healing assay (B). (C and D) The mRNA and protein expression levels of vimentin, twist1, ctnnb1, MMP2, CDH1 and CDH2 were measured by RT-qPCR and western blot assays, respectively. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. *: compared to NC group. ## $P < 0.01$. ### $P < 0.001$. #: compared to WT1-AS(+) group.

Figure 4

WT1-AS overexpression inhibited angiogenesis, reduced GSC stemness, and hampered the formation of in-vitro 3D GSC aggregates by down-regulating WT1. (A) HUVECs were cultured in condition medium containing the supernatants of GSCs stably transduced with different lentiviruses, followed by the assessment of angiogenesis potential via tube length. (B) The percentage of CD44⁺ subpopulation was determined by flow cytometry in GSCs stably transduced with NC, WT1(+), WT1-AS(+), WT1-AS(-), or WT1-AS(+)+WT1(+) lentiviruses. (C) The effects of WT1-AS or/and WT on the development of GSCs were explored in 3D cell culture system. $^{**}P < 0.01$. $^{***}P < 0.001$. *: compared to NC group. $^{#}P < 0.05$. $^{###}P < 0.001$. #: compared to WT1-AS(+) group.

A

B

C

Figure 5

WT1-AS inhibited tumor growth and reduced cell stemness in subcutaneous xenograft tumors derived from GSCs. (A-C) GSCs stably transduced with NC, WT1-AS(+) or WT1-AS(-) lentiviruses were injected into the subcutaneous tissues of mice, followed by tumor volume measurement (A), Ki67 IHC analysis (B), HE analysis (B) and CD44+ cell proportion detection (C). **P < 0.01. ***P < 0.001. *: compared to NC group.

Figure 6

WT1-AS inhibited tumor growth and metastasis, and reduced tumor stemness in xenografts derived from GSCs (i.p. and i.v.). (A-E) GSCs stably transduced with NC, WT1-AS(+) or WT1-AS(-) lentiviruses were intraperitoneally administrated into mice, followed by ascites volume (A), abdominal xenograft tumor weight (B), Ki67 IHC analysis (C), HE analysis (C) and CD44+ cell proportion detection (D). (E and F) GSCs stably transduced with NC, WT1-AS(+) or WT1-AS(-) lentiviruses were administrated into mice via tail vein. (E) Ki67 expression patterns in lungs of mice were measured by IHC assay. (E) The pathological

alterations of lungs were examined by HE analysis. (F) The proportion of CD44+ cell population was measured using a flow cytometry. **P < 0.01. ***P < 0.001. *: compared to NC group.

Figure 7

Identification of upstream transcription factors of WT1-AS. (A and B) The mRNA expression levels of 24 transcription factors were measured by RT-qPCR assay in GSCs (Fig. 7A), GCs (Fig. 7A), GC tumors (n=3) (Fig. 7B) and peritumoral tissues (n=3) (Fig. 7B). (C) GSCs were infected with NC or TP53(+) lentiviruses,

followed by the examination of TP53 mRNA level and WT1-AS level via RT-qPCR assay. (D) GSCs were infected with NC or XBP1(+) lentiviruses, followed by the examination of XBP1 mRNA level and WT1-AS level via RT-qPCR assay. (E) XBP1 mRNA level and WT1-AS level were determined by RT-qPCR assay in GSCs infected with NC, XBP1(-)#1, XBP1(-)#2, or XBP1(-)#3 lentiviruses. (F) GSCs were co-infected with WT1-AS promoter-driven luciferase reporter and NC, XBP1(+) or XBP1(-) lentiviruses, followed by the detection of luciferase activities. (A)-* versus GCs. (B)-*: versus adjacent normal tissues. (C-F)-*: versus NC group.

Figure 8

Identification of downstream genes of WT1-AS by RNA-seq technology (A) The heat map of all differentially expressed genes in ASWT_OV versus AS_OV, AS_OV versus NC and AS_KD versus NC groups. (B) Cluster analyses for all differentially expressed genes according to the expression trends of

genes. (C) The mRNA levels of PSPH, GSTO2, CACNA2D1, FYN, MAOB, ANPEP, ITGA2, CTSS, PRKCA and PHGDH were measured by RT-qPCR assay in GSCs stably transduced with NC, WT1-AS(+), WT1-AS(-), or WT1-AS(+)+WT1(+) lentiviruses. **P < 0.01. ***P < 0.001. *: compared to NC group. ###P < 0.001. #: compared to WT1-AS(+) group.

Supplementary Files

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