Dual-regulated mechanism of EZH2 and KDM6A on SALL4 modulates tumor progression via Wnt/β-catenin pathway in gastric cancer

Lei Ren
The Affiliated Hospital of Southwest Medical University
Hong deng
The Affiliated Hospital of Southwest Medical University
Yu Jiang
Affiliated Hospital of Southwest Medical University
Chunfeng Liu (chunfeng.liu@med.uni-muenchen.de)
The Affiliated Hospital of Southwest Medical University

Research Article

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Abstract

Background: The molecular mechanism underlying the progression of gastric cancer (GC) remains unclear, the overexpression level of SALL4 has been demonstrated in many cancers and participated in tumorigenesis, however, it is still ambiguous for the expression and function of SALL4 in GC, especially its upstream mechanistic modulators.

Method: Analysis of discrepant gene expression in GC and normal gastric tissues from The Cancer Genome Atlas (TCGA) dataset. Cultured GC cell lines were treated with siEZH2 and siKDM6A and checked for their influences on SALL4, the transduction molecules of KDM6A/EZH2-SALL4-β-catenin signaling were quantified in the GC cells.

Results: Here, we showed that only SALL4 levels of SALL family members were upregulated in nonpaired and paired GC tissues than those in corresponding normal tissues and were associated with its histological types, pathological stages, TNM stages, and overall survival from the TCGA dataset. SALL4 level was elevated in GC cells compared to normal gastric epithelial cell line (GES-1) and was correlated to cancer cell progression and invasion through the Wnt/β-catenin pathway in GC, which levels would be separately upregulated or downregulated by KDM6A or EZH2.

Conclusion: We first proposed and demonstrated that SALL4 promoted GC cell progression via the Wnt/β-catenin pathway, which was mediated by the dual regulation of EZH2 and KDM6A on SALL4. This mechanistic pathway in gastric cancer represents a novel targetable pathway.

Introduction

GC is the third most frequent cause of cancer-related death worldwide [1 2] and the fifth most common malignant cancer-diagnosed diseases globally [3,4], especially in Asia [5,6] and the prevalence rates are more dramatically increased in Eastern Asia which is the topmost one in the world [4,7]. In China, despite the continuous improvement of surgical techniques including complete lymph node dissection and totally R0 tumor resection, the endless novation of perioperative neoadjuvant chemoradiotherapy regimens, and the increasing awareness of clinical therapy compliance in GC patients, the relative 5-year survival rate of GC still remains lower than 30%[8,9]. These long-term poor prognoses of GC are unsatisfactory due to the privation of specific diagnostic biomarkers in the early stages[10,11], which caused the majority of newly diagnosed GC has reached the advanced stage when symptoms appear. The research focuses of oncologists worldwide still remain on the unvarying early-diagnosis and effective-therapy points of GC. However, the molecular mechanism underlying GC progression is unclear. Therefore, it is vital to investigate and identify the genes implicated and the genetic alterations, involved in the potential molecules associated with GC phenotype.

Spalt-like transcription factor 4 (SALL4), located on chromosome 20q13.2, encodes a zinc finger transcription factor expressed in embryonic stem cells, which has critically been involved in the regulation

of embryogenesis and organogenesis and in maintaining of pluripotency and self-renewal [12,13]. It has been reported that the strong positive SALL4 expression and H3K27 trimethylation (H3K27me3) loss in both rhabdomyosarcomatous elements and malignant neuroectodermal neoplasm by immunohistochemistry, and the highlighting potential molecular alterations such as CTNNB1 (encoded β-Catenin) mutations [14]. However, H3K27me3, as a chromatin mark associated with gene expression silencing, is catalyzed by enhancer of zeste homolog 2 (EZH2) which is the catalytic subunit of the polycomb repressive complex 2 (PRC2) [15]. From the epigenetic regulation of human genome, this trimethylation could be completely reversed by KDM6A (UTX) demethylase. Furthermore, SALL4 expression could not be activated or even silenced due to retention of the repressive H3K27me3 in the context of KDM6A depletion [16]. Nevertheless, the synergistical function of EZH2 and KDM6A on SALL4 via H3K27me3 hasn't yet been reported in gastric cancer.

In this study, we first confirmed that SALL4 promoted the progression of gastric cancer cells via Wnt/β-catenin pathway, which was mediated by the dual regulation of EZH2 and KDM6A on SALL4. This mechanistic pathway in gastric cancer represents a novel targetable pathway.

Materials And Methods

Cell culture

Gastric cancer cell lines (HGC-27, AGS, SGC-7901, MGC-803) and human normal GES-1 were all bought from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin at 37°C, 5% of CO2, and humidified incubator.

RNA extraction and Real time quantitative PCR (RT-qPCR)

RNA extraction was performed according to the protocol of RNeasy plus mini kit (Qiagen), cDNA reverse amplification was performed according to the manufacturer's instructions form the RQ1 RNase-Free DNase kit (Promega) and High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific), programed the thermal cycler using the conditions below: 25℃ (10 minutes), 37℃ (120minutes), 85℃ (5minutes), 4℃. All real-time PCR reactions were done as triplicates by using the LightCycler480, GAPDH was performed as the control. The sequences of specific primers are listed in below Table 1.

Table 1
Homo sapiens primer sequence used for QRT-PCR analysis.

Primer name	Primer sequence 5’ to 3’
SALL4 Fwd.	CAGGCCCTGTCTAAAGCCTA
SALL4 Rev.	GGGGAAAGACTTTTCTCCCAGA
FOXM1 Fwd.	TCCCCGTGTTTCCAAGTCAG
FOXM1 Rev.	CACCCACACTCTGCTTCAGT
KDM6A Fwd.	ACAGTGCCCAGTTTTTGTTCA
KDM6A Rev.	GCAATACTTTACAGTAGACACCTCA
SOX2 Fwd	GGCGAACCATCTCTGTGGTC
SOX2 Rev	TACCAACGGTGTCAACCTGC
CREB1 Fwd	GCACAGACCACTGATGGACA
CREB1 Rev	ATGTATGGTTTGAGTGGAAAAGATT
GAPDH Fwd	GACCCCTTCATTGACCTCAACTAC
GAPDH Rev.	TCGCTCCTGGAAGATGGTGATGG

Western Blot Analysis

Cells were lysed by RIPA lysis buffer (Sigma-Aldrich Chemie GmbH, R0278), After collecting the cells, sonicating on ice and Carefully aspirate the supernatant, after detecting the total protein concentrations by BCA protein assay kit. The loading dye (4X LDS sample buffer and 10X Sample Reducing Agent) were used to dilute 20 μg of protein sample amount. 20ug protein per sample was separated in 10% SDS-PAGE and then transferred to the nitrocellulose membranes, and used ECL (Millipore) signals were to analyze the results with a 440-CF imaging system (Kodak, Rochester, NY). The primary antibodies were shown as follow: mouse monoclonal anti-β-tubulin (ab231082, Abcam); rabbit anti-SALL4 (8459, cell signaling); Rabbit anti-KDM6A (33510s, cell signaling); mouse monoclonal anti-E-Cadherin (ab231303, Abcam); rabbit anti-EZH2 (5246s, cell signaling); rabbit anti-tri-Methyl-Histone H3 (Lys27) (9733s, cell signaling); Rabbit anti-Histone3 (4499s, cell signaling); Ribbit anti-β-Catenin (8480s, cell signaling).

Wound Healing Assay

SGC-7901 and MGC-803 cells were seeded into each well of the Culture-Insert 2 Well (ibidi, No:80241, Culture-Insert 2 Well, Germany), added mitomycin C (10 ug/mL) 2 hours before removing wells. And then took images after 24 hours by microscope connect AxioCam MRm camera.

Cell growth and cell viability assays

10^4 / well of Gastric cancer cells were seeded as triplicate into 6-well plates and cultured for 72h. Cell number were counted every 12h by a hemocytometer under a light microscope. Then, cell growth curves were plotted to assess cell growth. Cell viability assays were assessed using MTT (Sigma-Aldrich) dye, absorbance at 570 nm was measured (Bio-Rad) every 12h by the spectrophotometry. For the FH535 and XAV939 (the specific inhibitors of β-catenin activity, were respectively obtained from Cell Signalling and MedChemExpress) experiments, XAV939 (10 μmol/L)[17] and FH535 (20 μmol/L)[18] were used to pretreat SGC-7901 and MGC-803 for 2 h prior to reseeding and culture.

Boyden-Chamber Assay

Transwell-12 units with 8uM pore-size membrane chamber (Corning, NY) was used for cell migration and invasion. 60ul of Matrigel (BD Biosciences, East Rutherford, NJ) was pre-coated in the upper chamber before seeding cells for 4 hours. Seeded 5x104 cells in FBS-free medium in the upper chamber, the lower chamber contained with 10% FBS medium. After Forty-eight hours after, Images were captured by an Axiovert Observer Z.1 microscope connected an AxioCam MRm camera.

Immunocytochemical staining

For immunocytochemical staining, cells were seeding on the 13 mm coverslips in 24-well plate, cells were fixed with 4% paraformaldehyde, blocked with 10% normal goat serum, then incubated with the primary antibody overnight (rabbit anti-SALL4 1:200, mouse monoclonal anti-E-Cadherin 1:300). Afterwards, incubated secondary antibody and DAPI, took out the coverslips and mounted with Fluorescence Mounting Medium. Images were token by Keyence microscope.

siRNA Transfection and overexpression of KDM6A

Cell transfection was performed according to the manufacturer's instructions from siTOOLs company, Lipofectamine™ RNAiMAX Transfection Reagent (ThermoFisher) was used with a final siRNA concentration of 1 nM, siPOOL and diluted RNAiMax were well pre-mixed at a ratio of 1:1 by vertexing and then incubated for 5 minutes at room temperature, pre-mixed by vertexing and incubated siPOOL and RNAiMax as a ratio of 1:1. After transfection for 48 hours, the silence efficacy was checked by western blotting. For the other experiments, the cells would be reseeded after siRNA transfection and grown for the corresponding treatment time for each experiment. siRNA oligonucleotides were purchased from siTOOL company shown below, KDM6A/siEZH2/siSALL4 (7403/2146/57167, siTOOLs). The KDM6A expression plasmid (EX-A3666-Lv122; GeneCopodia, Rockville, MD) was used in transfection experiments.

Bioinformatics analysis

GSE79973 dataset was analysed for expression distribution of genes in human GC and adjacent normal tissues, TCGA RNA-seq data in TPM (transcripts per million reads) format was used for differential expression analysis of the samples. RNA-seq data from TCGA GC project in HTSeq-FPKM format were used for the molecular correlation analysis, and Spearman’s correlation analysis were performed using the ggplot2 (version 3.3.3) R package.

Statistics

All data were analyzed by GraphPad Prism 9 Software (GraphPad, San Diego, California, USA) software, and the data are presented as mean ± SD from the triplicate-repeated experiments. Used the Student's t-test to compare two groups, and the Wilcoxon signed-rank test was used for the unequal variances of two group. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SALL level in gastric cancer tissue and its correlation with TNM stage from TCGA dataset.

In order to explore the expression level of SALL4 gene in GC, we analyzed the TCGA dataset (GSE79973) to evaluate the distribution of gene expression in GC and its adjacent normal gastric mucosal tissues, the results showed that SALL4 gene level in GC was upregulated in comparison to the adjacent non-tumor mucosa (Fig. 1A). Based on this, we dissected the expression level of SALL family member (including SALL1, SALL2, SALL3, and SALL4) in GC. Firstly, we found only SALL4 levels in SALL family members were higher in nonpaired and paired GC tissues than those in corresponding normal tissues in TCGA dataset (Fig. 1B); simultaneously, compared to the normal tissues, only SALL4 levels were elevated not only in various pathological types (Fig. 1C) and stages of gastric cancer (Fig. 1D). Then, in respect of T, N, and M stages, which concern the tumor local extension, spread-to-nearby lymph nodes and long-distance metastasis respectively, also only the higher levels of SALL4 got involved in the different status compared to the normal stages (Fig. 1E). These results suggest that SALL4 in the SALL family should be frequently linked with GC as a research biomarker, and deserved more and more focalization.

SALL4 level in gastric cancer cells and its prognostic analysis from TCGA dataset

Encouraged by the above results, to further confirm SALL4 level in GC cells. we analyzed SALL4 expression in four GC cell lines (HGC-27, AGS, SGC-7901, and MGC803) and normal GES-1 Via real-time RT-PCR (Fig. 2A) and western blotting (Fig. 2B), we found that SALL4 levels were notably higher in AGS, SGC-7901, and MGC-803 than that in GES-1, especially in SGC-7901 and MGC-803. Owing to this, we selected these two cell lines as the subsequent experiments. Then we verified that the aggregation extent of SALL4 in the cell nucleus in SGC-7901 and MGC-803 cells was remarkably stronger than that in GES-1 cell (Fig. 2C) by immunofluorescent staining. To facilitate the succeeding study of SALL4 phenotype, here we focus on its prognostic association analysis in TCGA. We found patients with GC having SALL4-high expression illustrated a worse overall survival (OS) in the Kaplan–Meier survival analysis (Fig. 3D), which also happened in pathological stages of gastric cancer, especially in advanced gastric cancer, but not in the early-stage GC(Fig. 3E). In the further analysis when stratifying patients by the TNM stage, the OS remained significantly discrepancy. In N3 stage and T4 stage GC, patients with SALL4-high GC showed

shorter OS than those with SALL4-low GC (Fig. 2G and 2F). These results indicated that the SALL4 level was more correlated with the OS of GC patients with the highest T and N stages, implying whether SALL4 was involved in the metastasis and local invasion of gastric cancer.

Phenotypic functions of SALL4 gene in gastric cancer cell.

The high expression of SALL4 in GC cells and its prognostic survival analysis in patients with high-SALL4 levels prompted us to focalize its phenotype. We investigated the transfection efficiency of SALL4 siRNA (siSALL4) in SGC-7901 and MGC-803 cells (Fig. 3A). Then, we suppressed SALL4 expression in SGC-7901 and MGC-803 cells by using siSALL4, in contrast to control groups, silencing of the SALL4 had conspicuously repression on cancer cell growth (Fig. 3B-1# SGC-7901, 3B-2# MGC-803 cells, red vs. blue dots/lines); In addition, the migration of the cancer cells was affected after siSALL4 transfection (Fig. 3C-1# SGC-7901, 3C-2# MGC-803 cells). Furthermore, knockdown of SALL4 expression level by siSALL4 was sufficient to inhibit the invasiveness of cancer cells in a Boyden chamber assay (Fig. 3D). These results further supported why the high-SALL4 level was expressed in cancer cells compared with normal gastric mucosa cells, and also bolstered our above results that SALL4 could be involved in the metastasis and local invasion of GC patients and be closely related to the tumor-malignancy degree and long-term prognosis.

The dual regulation of EZH2 and KDM6A to SALL4 in gastric cancer cells

To seek the potential upstream regulatory mechanisms of SALL4, we searched the ARCHS4 dataset (https://maayanlab.cloud/archs4/gene/SALL4) principally invested for mining of available RNA-seq Data. According to the Z-score rating of human RNA-seq in the ARCHS4 dataset, we selected predicted and top-10 ranked upstream regulatory factors of SALL4 from the TCGA dataset, and analyzed their correlation with SALL4 mRNA expression levels in gastric cancer (Fig. 4A), FOXM1, KDM6A, SOX2, and CREB1 mRNA levels were shown positive correlations with SALL4 levels in patients with GC in the TCGA dataset (Fig. 4B). To further confirm the expression levels of SALL4, FOXM1, KDM6A, SOX2, and CREB1 in gastric cancer cells, our real-time qPCR results revealed that Only KDM6A and SALL4 were expressed at higher levels in both SGC-7901 and MGC803 cell lines than those in the GES-1 cell line (Fig. 4C).

Inspirited by these data, we focused on investigating the correlation between KDM6A and SALL4 in patients with GC. From TCGA dataset analysis, our results showed that KDM6A mRNA has higher levels in both unpaired (Fig. 4D-1#) and paired (Fig. 4D-2#) gastric cancer tissues than those in the corresponding normal gastric tissues. Furthermore, the KDM6A and SALL4 mRNA levels presented a positive correlation (Fig. 4E). To clarify KDM6A as the upstream regulator to SALL4, SALl4 expression levels were downregulated after KDM6A siRNA (siKDM6A) transfection treatment in SGC-7901 and MGC-803 analyzing by western blotting (Fig. 4F). KDM6A is the demethylase to H3K27me3, which loss conduces to the SALL4 activation[19]. Here, we observed the downregulation and upregulation levels of SALL4 in the SGC-7901 cell line after silencing and overexpressing KDM6A expressions by siKDM6A and

plasmid transfection, which contrarily induced the activation and suppression of H3K27me3 expression, separately (Fig. 4G). EZH2 is the methyltransferase for catalyzing the H3K27me3, so we hypothesized that EZH2 could also be involved in the GC progression of SALL4 regulation. To validate our hypothesis, we found, compared to the GC patients with low-EZH2 levels, GC patients with high-EZH2 levels have a conspicuously longer overall survival in TCGA dataset (Fig. 4H), which is completely opposite to the SALL4. Moreover, we found H3K27me3 inhibition and SALL4 increase in SGC-7901 after EZH2 silencing by EZH2 siRNA (siEZH2)(Fig. 4I). These results verified that KDM6A and EZH2 respectively positive- or negative-regulated SALL4 expression via H3K27me3 in human GC cell lines, respectively.

SALL4 promoted the tumorigenicity of human gastric cancer cells via Wnt/β-catenin signaling pathway

It had been reported that SALL4 modulated the tumorigenicity of breast cancer cells[20], cervical cancer cells[21] and esophageal squamous cell carcinoma[22], and induced acute myeloid leukemia in transgenic mice via activation of the Wnt/β-catenin pathway[23]. To indagate whether the function of the Wnt/β-catenin pathway was mediated by SALL4 in GC cells and the potential upstream regulatory mechanisms involving EZH2 and KDM6A.

Here, as shown in Fig. 5A, the positive correlation between SALL4 and CTNNB1 (encoded β-catenin) was excavated in patients with GC from TCGA dataset (Fig. 5A). And then, our western blotting results detected the downregulation level of β-catenin after siSALL4 transfection in SGC-7901 and MGC-803 (Fig. 5B), which was consistent with the correlation result from the TCGA in human GC patients. Besides, we found that β-catenin levels were dominantly decreased by knockdown of EZH2 and KDM6A upstream of SALL4 (Fig. 5C). Hereby, we proposed that SALL4 modulated the evolution of GC cells that mediated by the upstream modulator including EZH2 and KDM6A via the activation of the Wnt/β-catenin pathway (Fig. 5D). To verify the pertinence between the tumorigenesis and β-catenin in GC, the specific inhibitors of Wnt/β-catenin (FH535 and XAV939) were used, which could stably accelerate the inhibition of β-catenin[18,21,24,25]. After validating the repressed efficacy of FH535 and XAV939 in SGC-7901 (Fig. 5E) and MGC-803 (Fig. 5H), we found the cell growth and viability of GC were significantly inhibited by FH535 and XAV939 (Fig. 5F, 5G, 5I, 5J). These results indicated that KDM6A/EZH2-H3K27me3-SALL4-β-catenin might be the modulatory pathway for regulating the progression of gastric cancer cell lines.

Discussion

SALL4 level has been observed as the silence state in completely differentiated cells while its overexpression was reported in a variety of cancers, such as lung cancer [26], central nervous system tumor [27], liver cancer [28], colorectal cancer (CRC) [29], leukemia [30], breast cancer (BC) [31], glioma [32], endometrial cancer [33], and gastric cancer [12], the majority of which have revealed the role of SALL4 in carcinogenesis, including cell invasion and migration, cell proliferation and apoptosis, and cell stemness. This unique carcinogenesis function demonstrates that SALL4 should be a potential correlation between pluripotency and cancer [34]. However, it is still ambiguous for the expression and function of SALL4 in GC.

The ultimate clinical prognoses of GC vary by patient, simultaneously depending on the conventional pathological prognostic indicator that is TNM staging. Yang[35] et al reported that SALL4 correlates with clinicopathological features associated with cancer progression in GC. Our study investigated that only SALL4 of the SALL family is upregulated in nonpaired and paired GC patients compared to the corresponding normal gastric tissues and is related to the TNM stage and overall survival from the TCGA dataset, especially in the T stage (local invasion) and N stage (lymph node metastasis), which suggested that SALL4 is the potentially metastatic indicator in GC [36]. In the present study, our results also revealed that SALL4 is upregulated in gastric cancer cells relative to normal gastric epithelium cell lines and facilitates cell proliferation and cell invasion of GC in vitro.

Although EZH2 and KDM6A are respectively methyltransferases and demethylases of histone H3 at lysine 27, there are no available reports on how to associate the functional role of EZH2/ KDM6A and SALL4 in GC. It has been reported that H3K27me3 loss is also commonly associated with SALL4 activation [19], moreover, the H3K27me3 marks are present in so-called bivalent domains of the promoter regions of SALL4 [37]. Our results revealed that KDM6A could activate SALL4 expression through the H3K27me3 loss of KDM6A demethylation which would be inverted by KDM6A knockdown, while EZH2 inhibited SALL4 expression by the mobilization of H3K27me3. The H3K27me3 modified gene is especially interrelated with gastric cancer susceptibility and synergistically modulated by the two opposite-function enzymes (EZH2 and KDM6A) [38]. We also revealed that KDM6A and EZH2 exhibited the propensity to inversely regulate SALL4 through H3K27me3 in GC. The activation of the Wnt/β-catenin pathway by SALL4 directly binding to CTNNB1 (encoded β-catenin) potently promotes cervical cancer development and progression [21]. It is reported that the survival and progression of SALL4 upregulation in GC are involved in a putative WNT pathway[35]. Our results uncovered a positive correlation between CTNNB1 and SALL4 in GC cells, which are both positively regulated by KDM6A and negatively regulated by EZH2. Moreover, the growth and viability of GC cells were also distinctly decreased after the treatment with β-catenin inhibitors (FH535 and XAV939).

In summary, we first proposed and demonstrated that SALL4 enhanced cell proliferation and growth in GC cells via the Wnt/β-catenin pathway, which was mediated by the dual regulation of EZH2 and KDM6A on SALL4.

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Figures

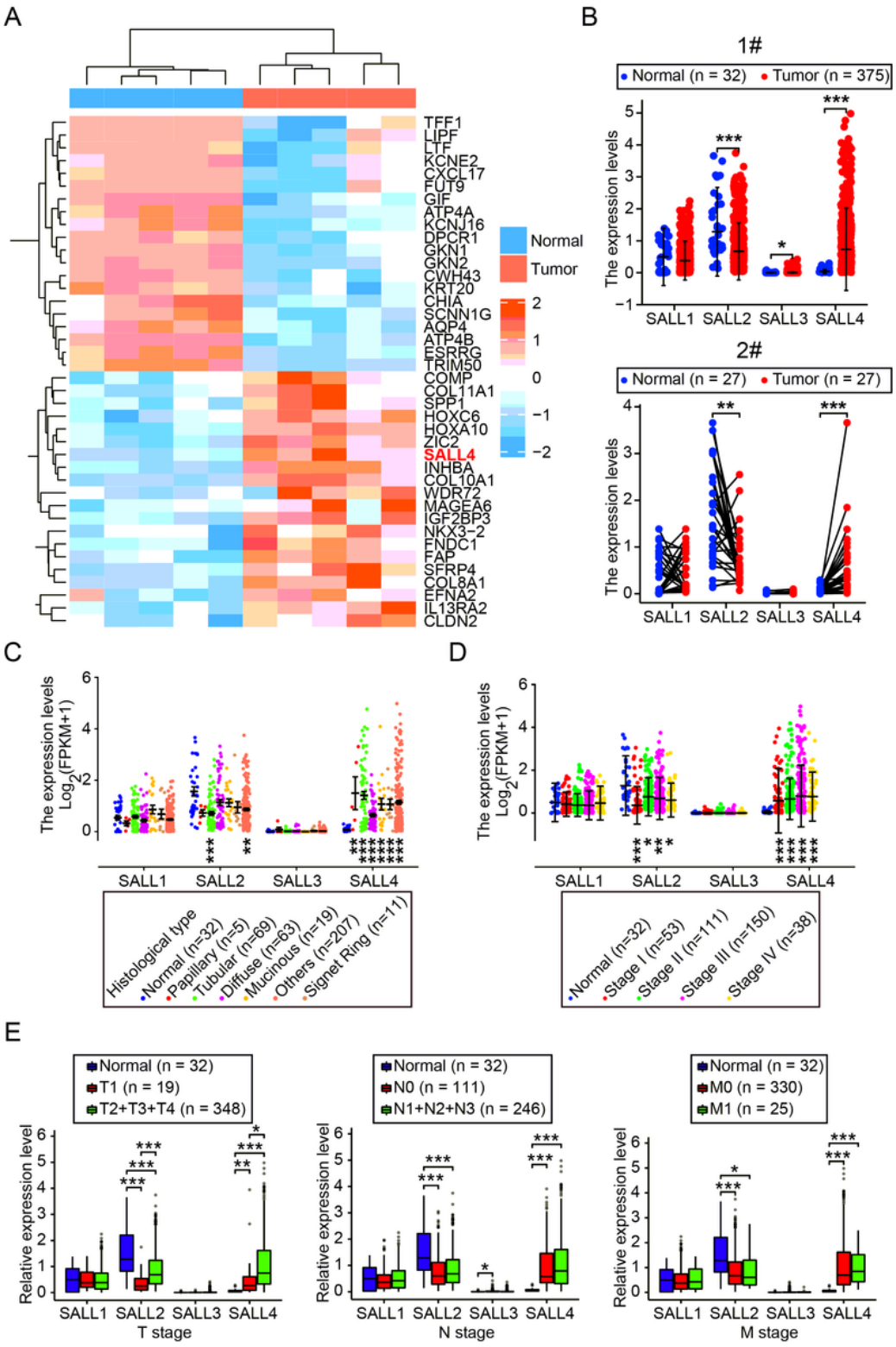

Figure 1

Association of the SALL family member with human gastric cancer. A. Gene mRNA expression in gastric cancer patients compared to the corresponding adjacent normal tumor tissue from The Cancer Genome Atlas (TCGA). **B**. Dot comparison of mRNA expression of SALL family member (SALL1, SALL2, SALL3, and SALL4) in nonpaired (1#) and paired GC (2#) tissues than those in corresponding normal tissues. **C and D**. Dot comparison of mRNA expression of SALL in gastric cancer was associated with pathological

types (C) and stages (D). **E**. Dot comparison of mRNA expression of SALL in gastric cancer involved in the T and N stage of the TNM status. P-values were computed by Wilcoxon rank-sum test.

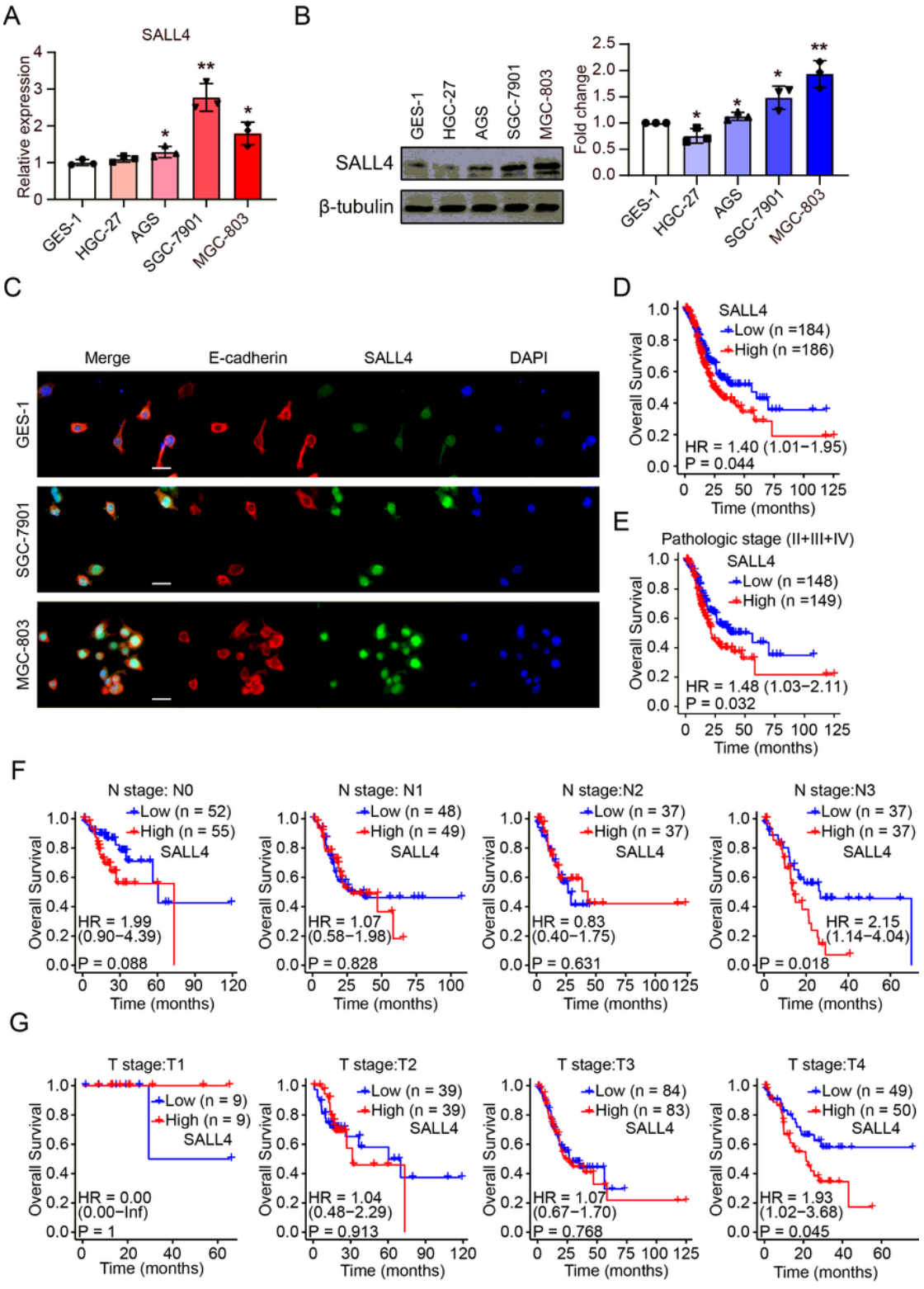

Figure 2

SALL4 expression in human gastric cancer cell line and its prognostic analysis in overall survival of gastric cancer in TCGA dataset. A and B. The mRNA and protein expression level of SALL4 in 4 GC cell

lines (HGC-27, AGS, SGC-7901, and MGC-803) and GES-1 by real-time RT-PCR and western blotting. **C**. Immunofluorescence analyses of SALL4 level in human SGC-7901 and MGC803 GC cells, Scale bar: 50μm. **D and E**. Kaplan–Meier survival curves of the SALL4-high and SALL4-low expression in GC patients (D) and the advanced GC (II + III + IV) (E). **F and G**. Kaplan–Meier survival curves for all N stages (F) and N stages (G) in patients with GC.

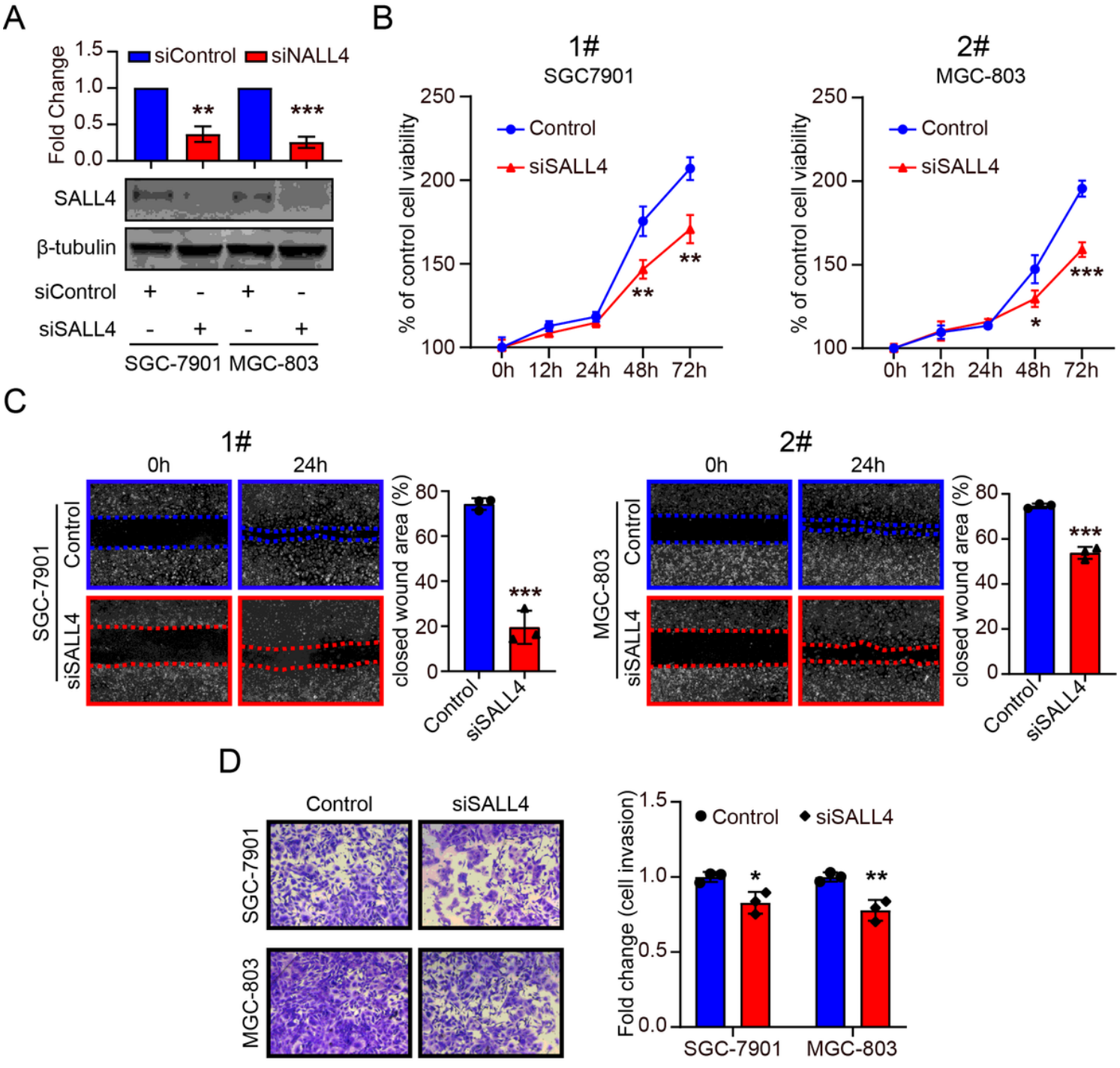

Figure 3

SALL4 downregulated expression inhibited the phenotypic features of SGC-7901 and MGC-803 cells. A. siSALL4 transfection efficacy was analyzed in SGC-7901 and MGC803 cells by western blotting. B. Cell

viability of SGC-7901 and MGC-803 cells were determined by MTT assay. data from 3 wells were calculated as mean ± SD compared to the control group, *P<0.05. C and D. Wound healing and Boyden-chamber assay respectively analyzed the migratory (C) and invasive (D) ability of SGC-7901 and MGC-803 cells after siSALL4 treatment.

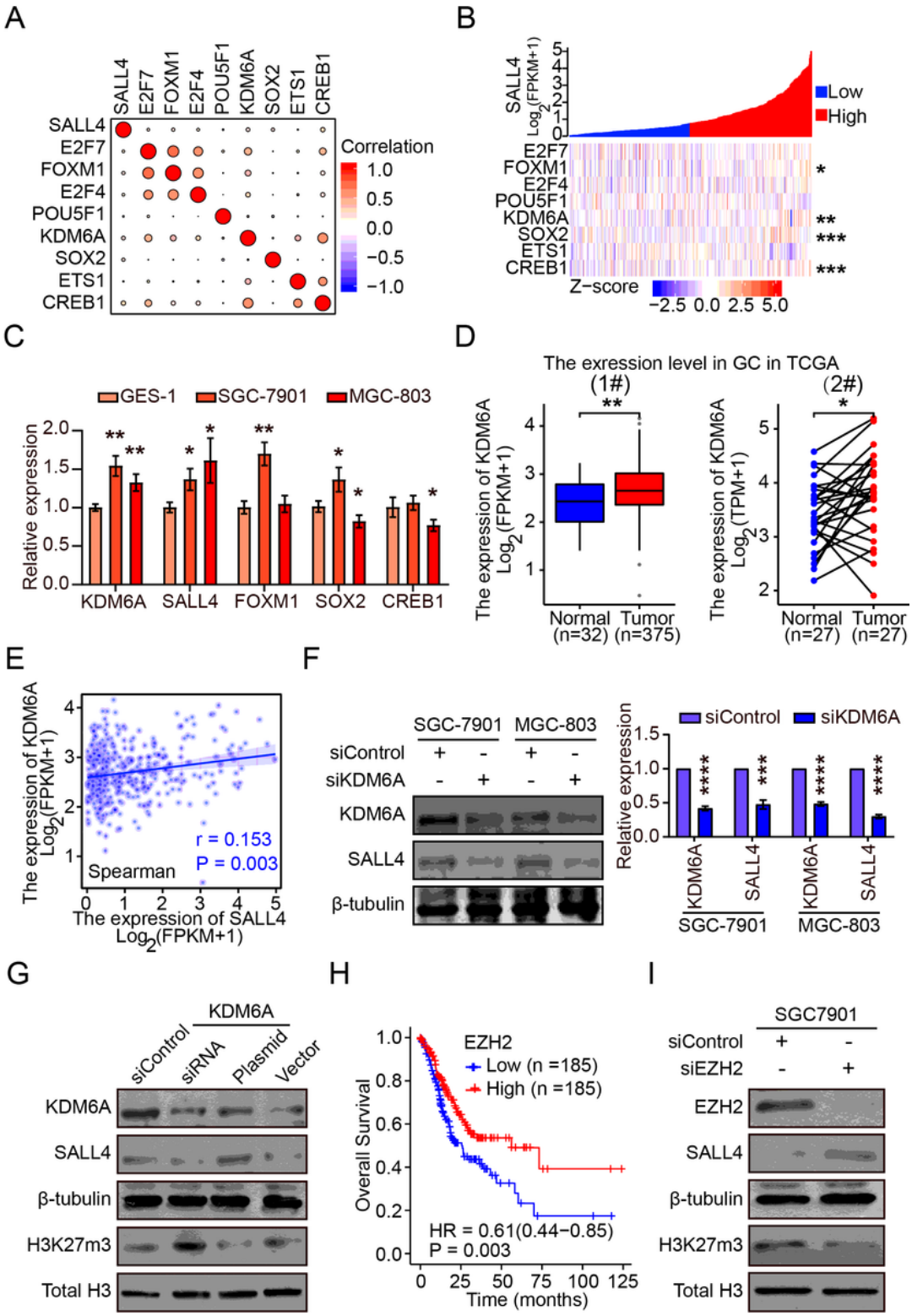

Figure 4

The upstream modulation of EZH2 and KDM6A to SALL4 in GC cell lines. A and B. Comparison of mRNA correlation (A) and co-expression (B) in human gastric cancer in TCGA. P-values were computed by Spearman. **C**. mRNA levels of four genes (KDAM6, FOXM1, SOX2, and CREB1) are notably associated with SALL4 in the GC cell line. **D**. KDM6A expression levels in nonpaired (1#) and paired GC (2#) tissues, and in the corresponding normal tissues from TCGA. **E**. Comparison of mRNA co-expression of SALL4 and KDM6A in human GC tissues. P-values were computed by Spearman. **F**. SALL4 levels after siKDM6A transfection in SGC-7901 and MGC-803 by western blotting. **G**. The levels of KDM6A, SALL4, H3K27me3 proteins in KDM6A-downregulating and -upregulating SGC-7901 cells by western blotting. **H**. Kaplan–Meier survival curves for EZH2-high and EZH2-low expression level in patients with GC. **I**. The levels of EZH2, SALL4, H3K27me3 proteins after siEZH2 transfection in SGC-7901 cells by western blotting. P-value was computed using unpaired t-test. (n = 3 independent experiments).

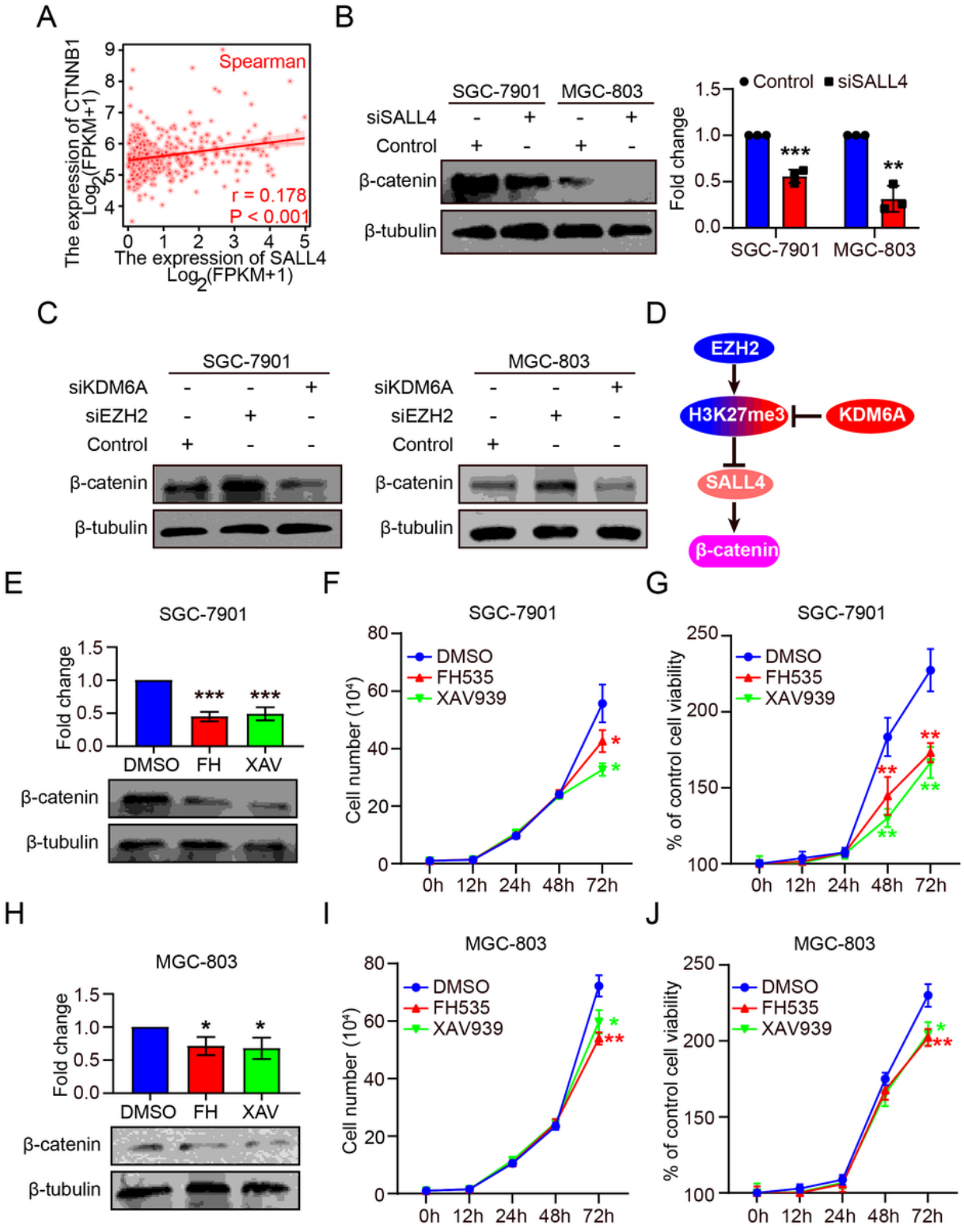

Figure 5

SALL4 upregulated the activity the Wnt/β-catenin signaling pathway in GC cells.

A. Comparison of mRNA co-expression of SALL4 and CTNNB1 in human GC tissues. P-values were computed by Spearman. **B**. Western blotting was used to detect β-catenin level after siSALL4 transfection in SGC-7901 and MGC-803, and the quantitative analysis from 3 independent experiments is shown. **C**.

The β-catenin level was detected after siKDM6A or siEZH2 transfection in SGC-7901 and MGC-803. **D**. The dual-regulated schematic illustration of KDM6A/EZH2-H3K27me3-SALL4-β-catenin. **E-J**. The inhibition efficiency of FH535 (FH) and XAV-939 (XAV) was respectively checked in SGC-7901 (E) and MGC-803 (H) by western blotting, meanwhile, the growth and viability were evaluated by the cell growth curve and MTT assay after pretreating with FH and XAV in SGC-7901 (F and G) and MGC-803 (I and J). Data were shown as the mean ± SD from three separate experiments.