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Epigallocatechin gallate reverses gastric cancer by regulating the long noncoding RNA LINC00511/miR-29b/KDM2A axis

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ABSTRACT

Epigallocatechin gallate (EGCG), as one of the main ingredients of green tea, has been reported to have potential prevention on a variety of solid tumors. However, the system-wide molecular mechanisms targeted to EGCG's anti-tumor effect have not been illustrated. Here, AGS and SGC7901 GC cells were used to investigate the EGCG-mediated change of gene expression. Our data showed that EGCG retarded cell growth and promoted cell death of GC in dose-dependent manner. Analyses based on transcription, translation as well as function were performed to explore the elusive anticancer role of EGCG. Of them, cell cycle was probably implicated key pathway of EGCG. Besides, our data revealed numerous LncRNAs activated after EGCG treatment. In this study, LINC00511 was discovered to be suppressed by EGCG and highly expressed in GC cells and tissues. Knockdown of LINC00511 inhibited cell growth and promoted cell death ratio in GC. Additionally, our data suggested LINC00511 could decrease the expression of miR-29b, followed by inducing GC development. Knockdown of miR-29b recovered the effects of LINC00511 silencing. In addition, we found overexpression of KDM2A, a target of miR-29b, would rescue the level of LINC00511. All the data showed that the LINC00511/miR-29b/KDM2A axis can be used as a diagnostic and therapeutic target for GC.

1. Introduction

Gastric cancer (GC) is a high-incidence disease that negatively affects the psychology and physiology of patients [1]. Efficacious treatments efficiently improved the status of initial stage of GC patients [2]. In the last few decades, our understanding of the mechanisms underlying the progression of GC has improved. Emerging studies have shown that noncoding RNAs play a crucial role in GC development. For example, ROR was reported to act as a competing endogenous RNA(ceRNA) to regulate the level of Oct4, Sox2 and Nanog in colon cancer stem cells by sponge mir-145 [3]. The pseudogene of GBAP1 acted as ceRNA to sponge miR-22-3p [4]. However, recurrence ration is poor because of metastasis. Effective strategies can result in negative physical and psychological side effects in patients [5]. Thus, identification of novel treatment targets to address this problem is urgently needed.

Most Traditional Chinese medicines (TCMs) have been used for long history clinically in China, which are derived from medicinal herbs. Many of components included in TCMs made it become anticancer agents [6]. Green tea contains polyphenols such as epigallocatechin (EGC) and epicatechin (EC) [7,8]. EGCG is the most abundant and effective ingredient in green tea [9]. Extensive studies have shown that it has significant antioxidant, anticarcinogenic, antimicrobial and has therapeutic potential against many human diseases [10]. This component has been observed to suppress breast cancer [11–14], prostate cancer [15–18], lung cancer [19–22], pancreatic cancer [23–26], and liver cancer [27,28]. Thus, EGCG was demonstrated to be the most effective cancer chemopreventive polyphenol in green tea [29]. Previous studies have revealed that all of these anticancer effects of EGCG worked by inducing apoptosis, controlling cell proliferation, and inhibiting angiogenesis [30].

However, the key point for modern pharmacology is to investigate the role of uncovered agents [6]. Previous studies towards ECGG merely concentrated on probable routes via detecting the change of some factors involved. Here, we probed the possible mechanisms of EGCG-targeting via high-throughput full-transcriptome analysis and confirmed some dysregulated genes. We further verified the pathways of

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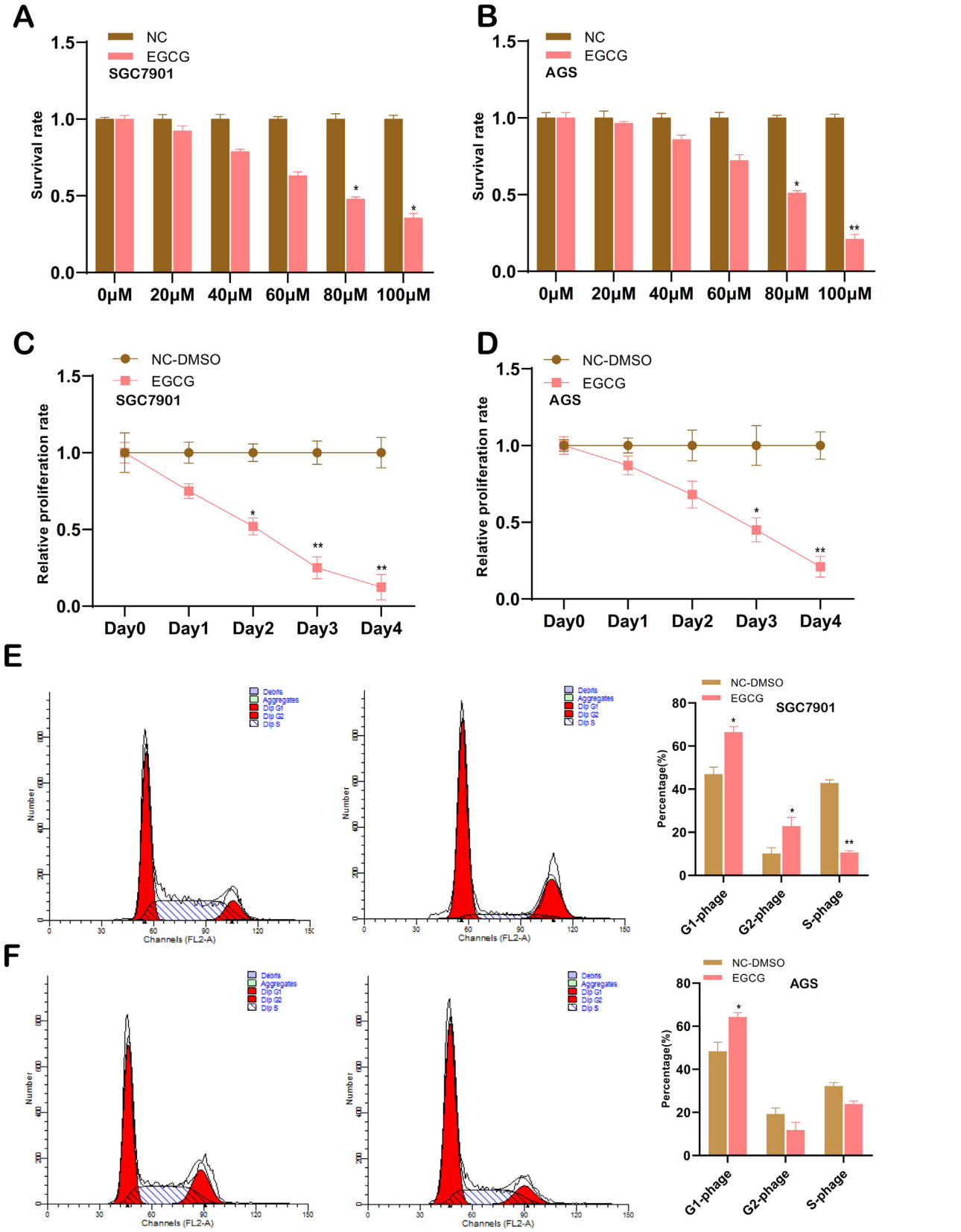

Fig. 1. Effects of EGCG on the proliferation ability of GC cells. (A, B) Effect of EGCG on the survival rate of SGC7901 and AGS cells based on dose-dependent assay. (C, D) CCK-8 assay determined the effect of EGCG on cell growth of SGC7901 and AGS based on time-dependent assay. (E, F) Statistical analysis of EGCG-mediated effects on cell cycle process of SGC7901 and AGS. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

these genes with interaction of EGCG in AGS and SGC7901 cells. All the data showed various gene and lncRNAs level could be changed by EGCG, thus resulting in arrested cell progression and induced cell death.

2. Materials and methods

2.1. Cell culture and drugs

AGS and SGC7901 were obtained from ATCC (HTB-22™, VA, USA) and then cultured in DMEM medium (BI, Israel) with 10% FBS (Gibco, USA) at 37 °C with 5% CO_2. EGCG (purity > 98%) was obtained from Weikeqi Biotechnology Co. Ltd. (Sichuan, China).

2.2. RNA sequencing

mirVana PARIS Kit (Thermo Fisher Scientific) was used to extract total RNA as manufacturer described. Purity of extracted RNA was measured by Bioanalyzer and amplified polyA by NEBNext(r) Poly(A) mRNA Magnetic Isolation Module. NEBNext(r) Ultra Directional RNA Kits (New England Biolabs, Inc.) was used to synthesize mRNA libraries for sequencing. During this step, actinomycin D was added and then run on a Bionanalyzer. Library Quantification kits was applied to measure the relative concentration of enriched RNA, followed by sequencing by Illumina HiSeq 2500 across 5 lanes with stranded paired-end 100 base pair reads.

2.3. Data and function analysis

Reducing poor sequence from originally reads by Trim_galore (v0.3.7, Babraham Bioinformatics) as described before and then aligned the remaining sequences with reference genome (hg19, UCSC) by STAR_2.4.0j. The candidates with error detection rate (FDR) were chosen for differential expression analysis. R software was conducted for GO and KEGG analysis. *p*-Value was calculated as Benjamini-Hochberg method mentioned.

2.4. qPCR analysis

Cells with treatment or non-treatment were collected, centrifuged and then kept pellets in liquid nitrogen. Overall RNA was collected by PureLink RNA Mini Kit (Thermo Fisher Scientific), and then subjected to synthesize cDNA by High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

qPCR system was performed as follows: reactions with PowerUp SYBR Green Master Mix including primers was set on the StepOne Plus Real-Time PCR system. The final data was analyzed by software V2.3 (Thermo Fisher Scientific).The expression levels of target genes were measured using $2^{-\Delta\Delta CT}$ method.

2.5. LncRNA identification

We discarded transcripts below 200 nucleotides (nt) and then mapped short reads with genome and spliced to be a new longer transcript. We further verified long transcripts with refGenes from UCSC and determined as “refGene transcripts”, “intergenic transcripts”, “intronic transcripts” and “antisense transcripts”. The last three transcripts were used to go through non-labeled transcripts as indicated. Finally, we chose the expected transcripts from 27 individual samples referred to the level with > 1 count per million by TMM method.

2.6. Co-expression networks construction

All DE genes chosen in cells with EGCG treatment (100 μmol/L) for 48 h were determined. Fig. 2 illustrated that lots of key categories were composed of many GO terms. The Pearson correlation coefficient was calculated according to the expression value between lncRNA-mRNA pair. The co-expressed DEG-lncRNA pairs with the absolute value of Pearson correlation coefficient ≥ 0.75 were selected as cutoff. All pre-filtered refGenes and lncRNAs were combined to establish co-expression networks with Cytoscape V3.2.1. GO and KEGG analysis were performed with DAVID.

2.7. Transfection

siRNAs, si-LINC00511 along with negative shRNAs (sh-NC) were obtained from Genechem (Shanghai, China). The sequences of si-LINC00511, 5′-CCCAUGUCUGCUGUGCCUUUGUACU-3′; si-NC, 5′-UUCUCCGAACGUGUCACGUTT-3′.

AGS or SGC7901 cells were transfected with the LINC00511 or KDM2A by Lipofectamine2000 (Invitrogen). Cells transfected with empty pcDNA3.1 vector was negative control. Cells transfected with miR-29b mimics/NC mimics or miR-29b inhibitors/ NC inhibitors. All the transfected cells were detected after 48 h transfection.

2.8. CCK-8 assay

AGS and SGC7901 cells were transfected with expected plasmids at indicated time point and then subjected to determine cell proliferation. After 12 h of transfection, we seeded the cells (1×10^3 cells/well) into the 96-well plates. At a specific time, we added CCK-8 (10 μL per well) to the cells after transfection and incubated them for 2 h before measuring the absorbance at 450 nm. The OD value of 450 nm was collected using microplate reader (Bio-Tek Instruments, USA).

2.9. Transwell migration and invasion assays

Transwell chamber was used to conduct the assays of cell migration and invasion, which was coated with the matrigel mix for invasion assay or without it for migration assay. After 12 h of transfection, we seeded the cells (1×10^5 cells/well) into the upper Transwell chamber. After incubation for 48 h, we used cotton swabs to scrape the cells which were settled on the upper surfaces of the transwell chambers, and to fixed the cells settled on the lower surfaces for DAPI (Solarbio, Beijing, China) staining. Next, we observed the number of cells in the transwell chambers under a fluorescent inverted microscope and took a photo.

2.10. Cell cycle assays

Treatment of AGS and SGC7901 cells with 100 μM EGCG for 48 h. For cell cycle assay, G1, S and G2 peaks were detected from the propidium iodide-stained GC cells by flow cytometry (BD Bioscience, NJ, USA) and the Modfit software was used for analysis.

2.11. Subcellular fractionation

We followed the cell fractionation assay according to the previous report [31].

2.12. Luciferase reporter assay

pmirGLO dual-luciferase vector containing LINC00511-WT/MUT or KDM2A-WT/MUT was constructed, and separately named pmirGLO-LINC00511-WT/MUT or pmirGLO-KDM2A-WT/MUT. AGS or SGC7901 cells were transfected with pmirGLO-LINC00511-WT/MUT, pmirGLO-KDM2A-WT/MUT, miR-29b mimics or NC mimics by Lipofectamine 2000 (Invitrogen), followed by Dual-Luciferase Reporter Assay.

2.13. Statistical analysis

The experiment was repeated three times independently. We

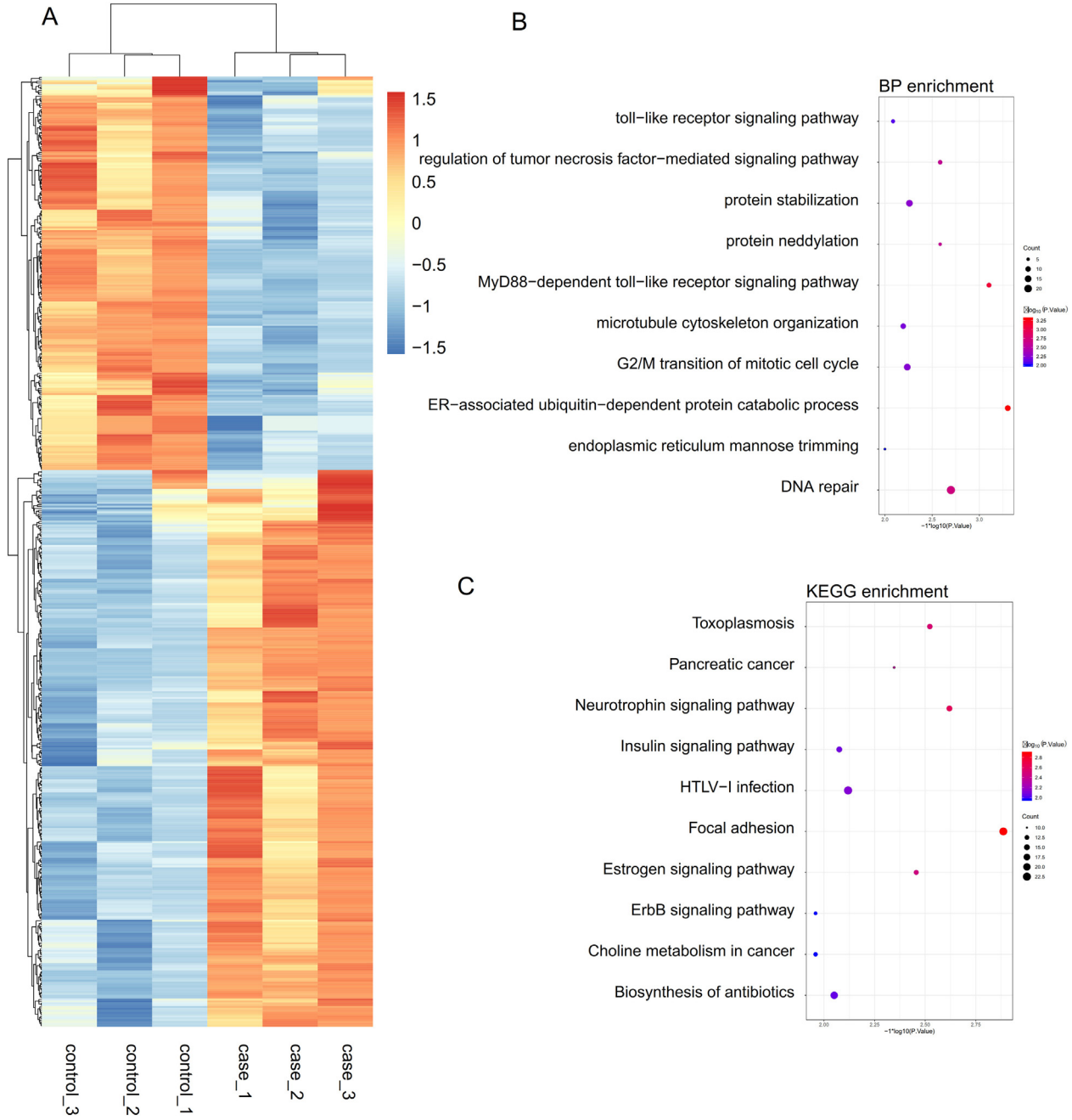

Fig. 2. Identification of EGCG regulating genes in GC. (A) Heatmap analysis of differently expressed genes in GC after EGCG treatment. (B) GO analysis of EGCG regulating genes in GC. (C) KEGG pathway analysis of EGCG regulating genes in GC.

applied the average value ± SD to express continuous variables and performed one-way ANOVA or Student's *t*-test for multiple comparisons. In this paper, a statistically significant difference was demonstrated by p values < 0.05.

3. Results

3.1. EGCG inhibits AGS and SGC7901 cell proliferation

To characterize the effect of EGCG on the proliferation of GC cells, we performed CCK8 detection to determine cell viability with treatment of EGCG. In order to further validate the biological functions of EGCG, we treated AGS and SGC7901 cells with EGCG in a dose-dependent manner. We used 0 μM, 20 μM, 40 μM, 60 μM, 80 μM, 100 μM EGCG to treat AGS and SGC7901 cells. After 48 h, the cell proliferation rate was measured. With the increase of EGCG treatment concentration, the survival rate of AGS and SGC7901 cells showed a whole dose-dependent decrease. Therefore, we chose to treat AGS and SGC7901 cells with 100 μM EGCG (Fig. 1A, B). The proliferation of the AGS and SGC7901 cells was dramatically inhibited after treatment with EGCG (100 μM). After 48 h, the cell proliferation rate was measured, which showed a dose- and time-dependent effect (Fig. 1C, D). The data suggested growth of GC cells could be suppressed by EGCG.

3.2. Overall change of gene expression in SGC7901 cells with treatment of EGCG

We deeply validated the function of EGCG in SGC7901 cells, we performed the next generation sequencing at high-depth using the Illumina HiSeq 2500 system. In summary, EGCG or DMSO-treated

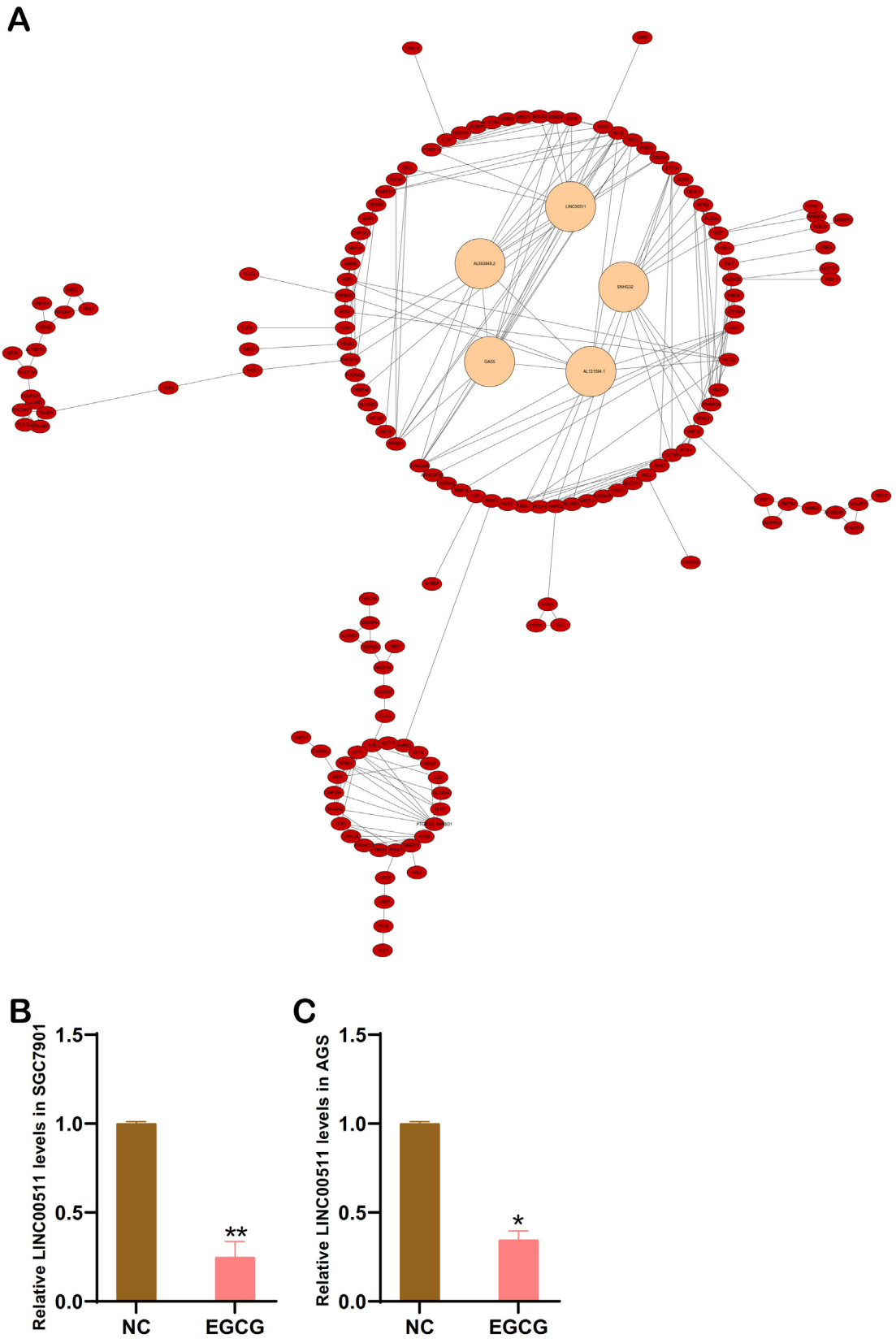

Fig. 3. EGCG can regulate the expression of lncRNAs at the core of the network. (A) Construction of lncRNA co-expression network revealed the key lncRNAs after treatment of EGCG in GC. (B, C) RT-PCR was used to detect the level of LINC00511 in SGC7901 (B) and AGS (C) after EGCG treatment.

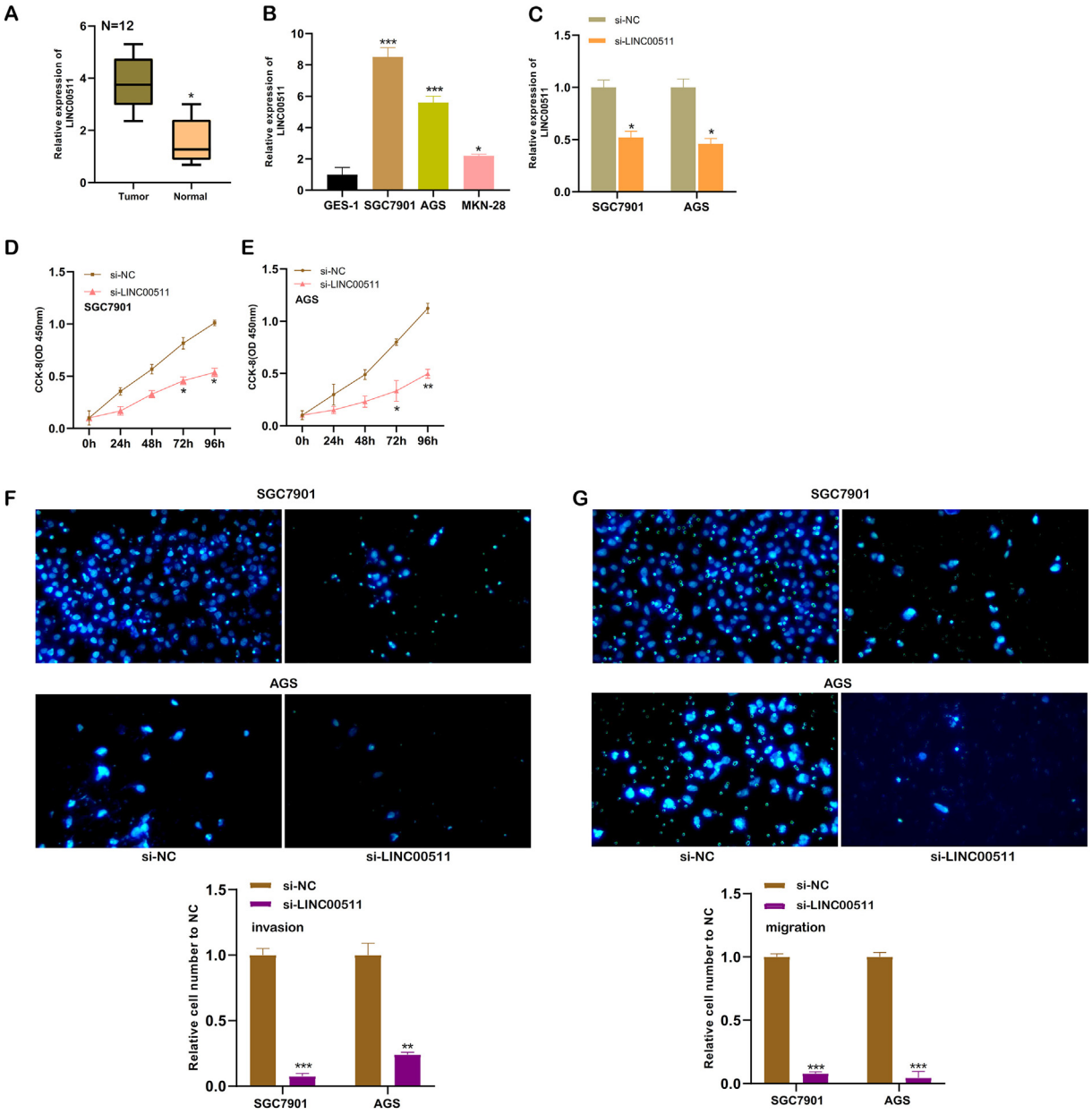

Fig. 4. Knockdown of LINC00511 inhibited the migration, invasion and proliferation capacity of SGC7901 and AGS cell lines, and LINC00511 high expression in tumor tissues. (A) LINC00511 expression in GC tissues was greatly higher than that in adjacent tissues by qRT-PCR. (B) LINC00511 was obviously raised in SGC7901 and AGS cells in comparison with that in GES-1 and MKN-28 cells. (C) Si-LINC00511 reduced LINC00511 expression level in GC cells. (D, E) GC Cells with si-LINC00511 had lower rate of proliferation. (F) Knockdown of LINC00511 inhibits invasion ability of SGC7901 and AGS. (G) Knockdown of LINC00511 inhibits migration ability of SGC7901 and AGS.

SGC7901 cells for 48 h with > 732 million 100 bp paired-end reads were sequenced. Edger was used to determine obviously differentially expressed (DE) genes. The DE genes are shown in the heatmap (Fig. 2A).

3.3. Analysis of pathways in EGCG-treated SGC7901 cells

All DE genes chosen in cells with EGCG treatment (100 μM) for 48 h were determined. Fig. 2 illustrated that lots of key categories was composed of many GO terms.

Next, we further explored the implicated pathways induced by EGCG. The selected DE genes in cells with EGCG treatment (100 μM) were analyzed. Cells with 24 h- EGCG treatment expressed as “steroid hormone biosynthesis” were overrepresented (Fig. 2A). Most of DE genes attributing to GO terms were increased among the selected ones (Fig. 2B). We used the KEGG pathway to analyze genes regulated by EGCG in gastric cancer (Fig. 2C).

Cell cycle assays using flow cytometry were used to validate the bioinformatics analysis results. Our data suggested the ratio of S phases was obviously low in EGCG-treated AGS cells, but dramatically promoted in G0/G1 phase, (Fig. 1F).

3.4. EGCG can regulate the expression of lncRNAs at the core of the network

We constructed co-expression networks to validate possible association between lncRNA and EGCG in AGS cells. As presented in Fig. 3A, 5 lncRNAs (AL593848.2, GAS5, SNHG32, AL121594.1, LINC00511) and 87 mRNAs were included in this network.

Analysis of DE in refGenes displayed the level of LINC00511 was

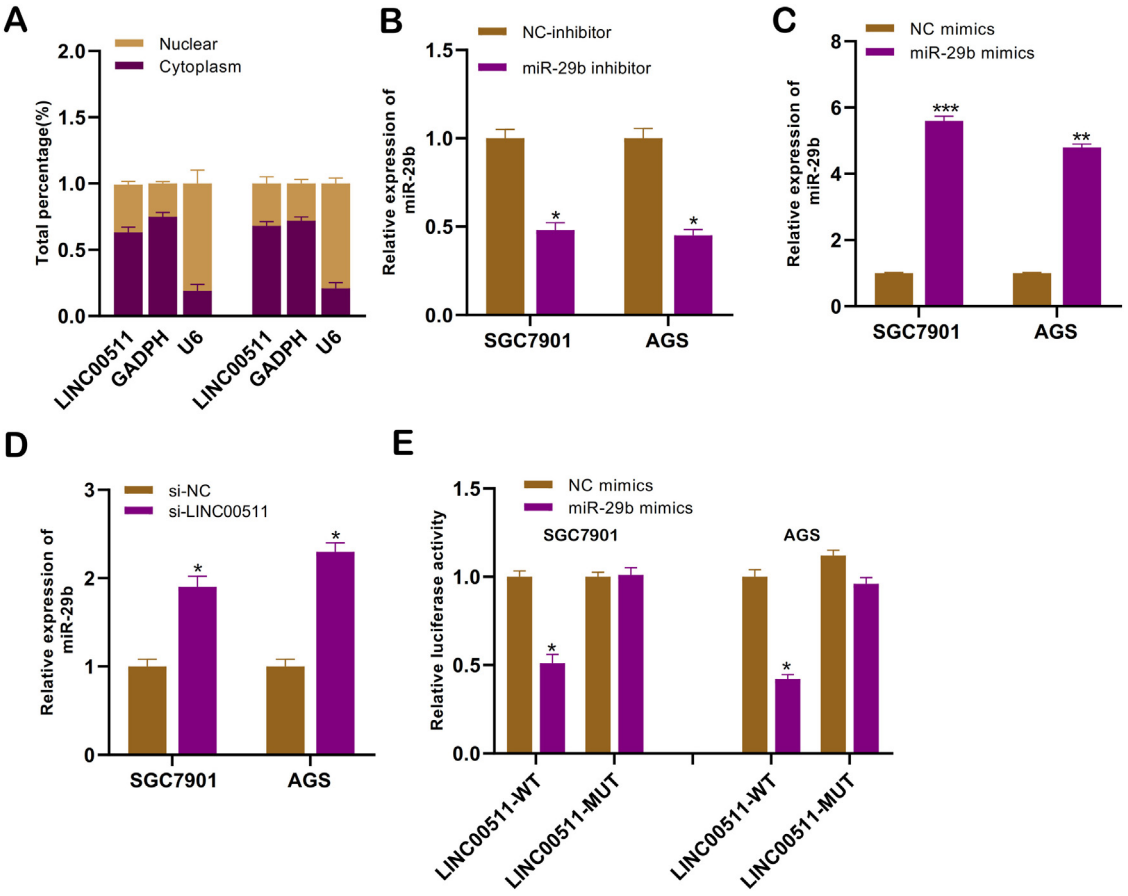

Fig. 5. LINC00511 was mainly located in cytoplasm and LINC00511 is target genes of miR-29b. (A) LINC00511 was mainly located in the cytoplasm of the GC cells. (B) The miR-29b inhibitor remarkably suppressed miR-29b expression in GC cells. (C) Upregulation of miR-29b remarkably increased miR-29b level in GC cells. (D) Knockdown of LINC00511 upregulated miR-29b expression in GC cells. (E) Luciferase reporter activity assay in SGC7901 and AGS cells after transfection with pLINC00511-MUT or LINC00511-WT and control miR-NC or miR-29b mimics.

reduced after the AGS and SGC7901 cells treated with EGCG (Fig. 3B, C). Notably, the expression of LINC00511 showed the most significant change after treatment with EGCG. The expression level of LINC00511 decreased by 61% in the AGS cells after treatment with EGCG and decreased by 73.3% in the SGC7901 cells after treatment with EGCG.

3.5. LINC00511 was found to be highly expressed and promoted cell proliferation and metastasis of GC cells

We found that the expression level of LINC00511 in gastric cancer tissues was significantly higher than that in normal paracancerous tissues (Fig. 4A). We evaluated LINC00511 expression in AGS, SGC7901, GES-1 and MKN-28 cells using qRT-PCR analysis. The results indicated that the expression of LINC00511 in AGS and SGC7901 were observably higher than that of GES-1 and MKN-28 cells (Fig. 4B). Then, we transfected si-LINC00511 and si-NC into AGS and SGC7901 cells and evaluated LINC00511 level. This result showed that the expression of LINC00511 was decreased observably (Fig. 4C). Down-regulation of LINC00511 inhibited cell growth. (Fig. 4D, E). Reduced LINC00511 apparently retarded cell migration and invasion (Fig. 4F, G). In conclusion, LINC00511 is more highly expressed in GC tissues than in adjacent paracancer tissues., and down-regulation of LINC00511 gene inhibited the proliferation and metastasis of GC cells.

3.6. LINC00511 targets miR-29b

Cell fractionation assay showed LINC00511 was accumulated in the cytoplasm (Fig. 5A). We further validated the probable binding sites of LINC00511 to miRNAs using starBase database. qRT-PCR results showed miR-29b expression was raised after treatment of miR-29b mimics not miR-29b inhibitor (Fig. 5B, C). Subsequently, qRT-PCR results further revealed miR-29b expression was increased by si-LINC00511 (Fig. 5D). Furthermore, luciferase reporter assays showed that miR-29b specifically bond to LINC00511. Luciferase activity was prominently reduced in cells transfected with LINC00511-WT and miR-29b mimics, but not in cells treated with LINC00511-Mut and miR-29b mimics (Fig. 5E), indicating LINC00511 could bind to miR-29b, negatively regulating this molecule.

3.7. LINC00511 promoted GC development after knocking down miR-29b expression

Fig. 6A–C individually illustrated cell proliferation, migration and invasion was suppressed after treatment of miR-29b mimics. Fig. 6D–F indicated reduced miR-29b could rescue influence of si-LINC00511 on GC cells growth, pointing miR-29b mimics curbed cell growth, but miR-29b inhibitors counteracted the effects of LINC00511 knockdown.

3.8. KDM2A was identified as a target of miR-29b

We performed luciferase reporter assays, which showed that LINC00511 competitively bond to miR-29b with KDM2A, thereby restoring plasmid activity (Fig. 7A). mRNA analysis demonstrated KDM2A level was enhanced after treatment of mir-29b inhibitor other than miR-29b mimics (Fig. 7B, C). In brief, KDM2A was a target of miR-29b.

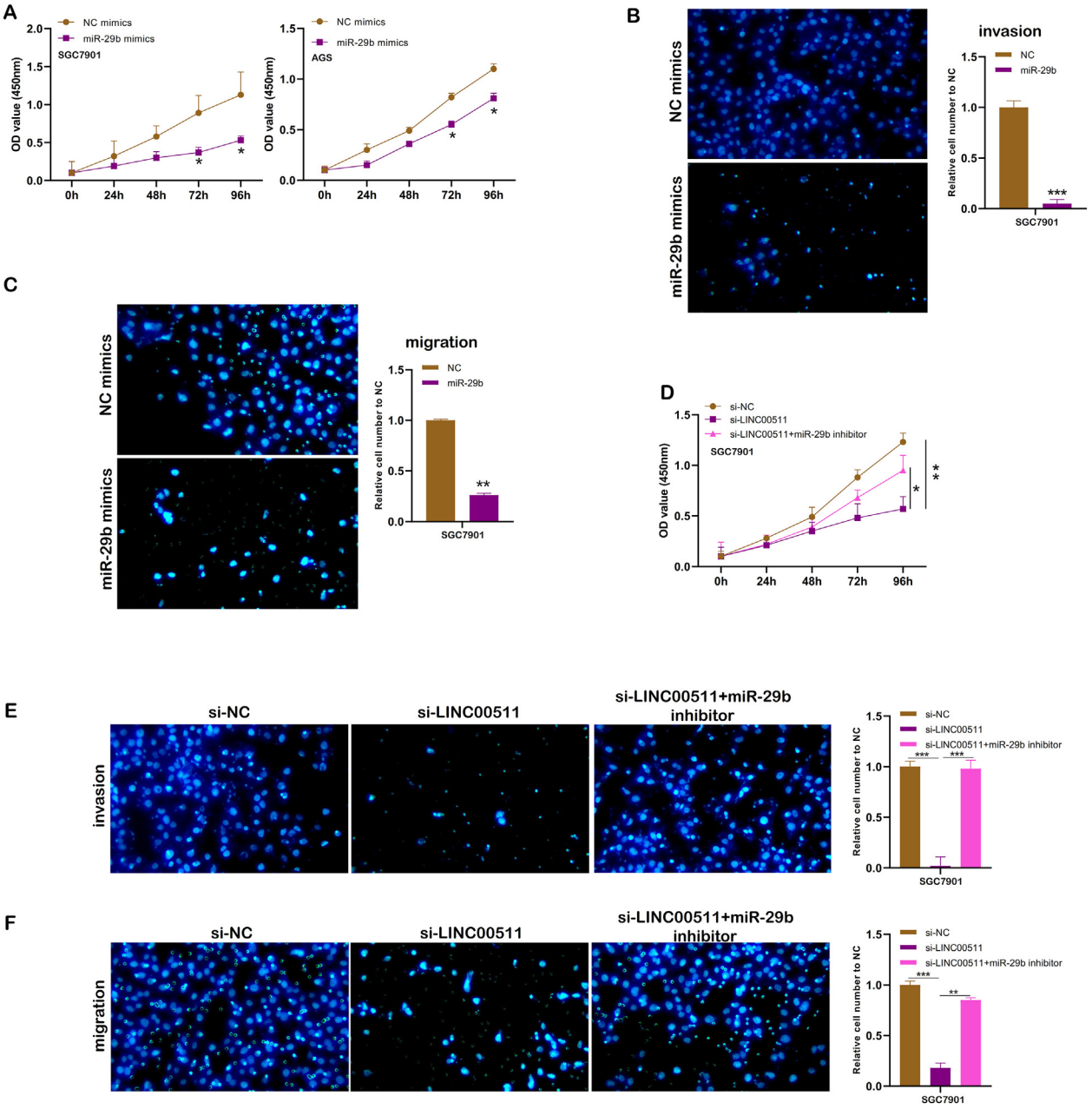

Fig. 6. The over-expression of miR-29b inhibited the migration, invasion and proliferation capacity of SGC7901 and AGS cell lines. (A) Increased miR-29b inhibited SGC7901 and AGS cells growth. (B) Upregulated miR-29b repressed SGC7901 cells invasion ability. (C) Enhanced miR-29b curbed SGC7901 cells migration ability. (D–F) GC cells proliferation (D), migration (E) and invasion (F) in the rescue experiment.

3.9. LINC00511 promoted GC by targeting the miR-29b/KDM2A axis

To demonstrate the effectiveness of the LINC00511/miR-29b/KDM2A axis in GC cells, we first detected KDM2A expression in cells treated with pcDNA3.1/KDM2A. qRT-PCR data showed that KDM2A expression was increased by pcDNA3.1/KDM2A (Fig. 8A). In the CCK8 experiment, the effect of si-LINC00511 on cell proliferation were rescued by pcDNA3.1/KDM2A (Fig. 8B). The rate of invasion and migration was decreased after downregulation of LINC00511, whereas that could be reversed after transfection of KDM2A (Fig. 8C, D). In conclusion, the effect of LINC00511 knockdown was reversed by KDM2A overexpression.

4. Discussion

Tea was originally used for therapeutical purposes and now has been the most popular beverage worldwide owing to its pleasant flavor and potential benefits for human health, including anticancer [32]. Among the multiple bioactive ingredients in green tea, EGCG has been considered to be mainly responsible for the anticancer potential. Despite many researches have been carried out on the anticancer mechanism of EGCG, its mechanisms still remained elusive [33–36]. We used whole transcriptome analysis to discover the probable role of EGCG in GC cells. Not only did we confirmed targets of EGCG in view of DE expression, but also, we characterized pathways related to EGCG.

We identified genes with significantly altered expression in EGCG-treated AGS cells. Our data revealed overall gene expression have been changed after treatment of EGCG, especially in high-dose condition,

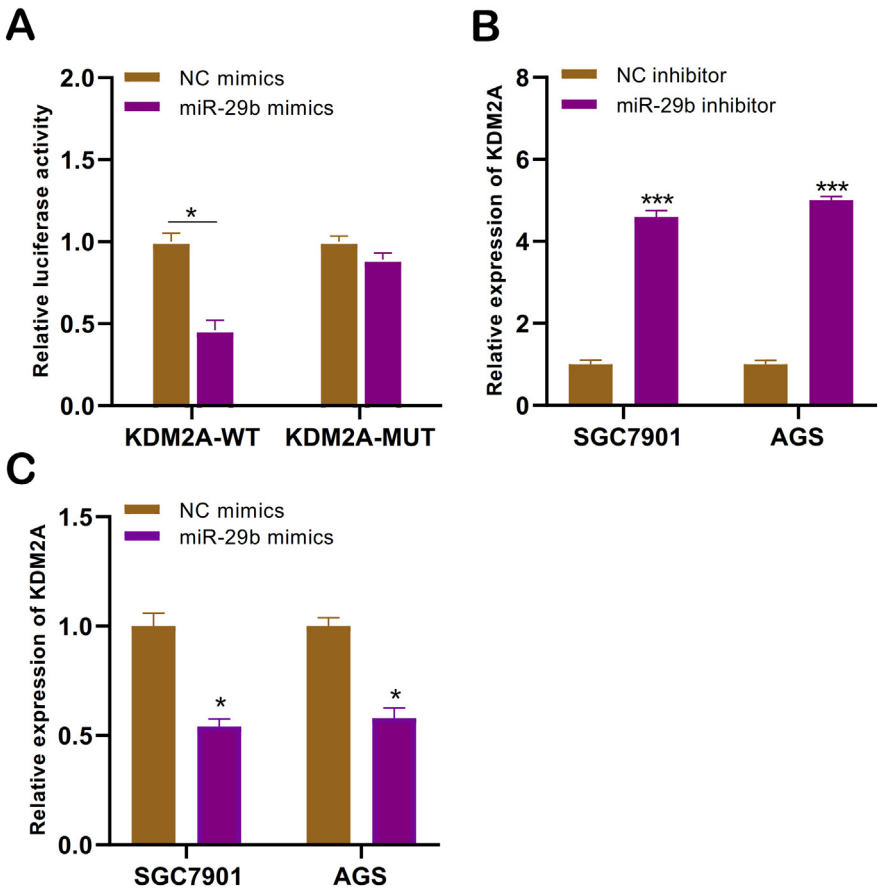

Fig. 7. KDM2A was a target of miR-29b. (A) miR-29b reduced the luciferase activity of KDM2A wt, not mutant KDM2A plasmids. (B) KDM2A expression was enhanced by the miR-29b inhibitor in AGS and SGC7901 cells. (C) KDM2A expression was reduced after treated with miR-29b mimics in indicated cells.

consistent with previous studies [37–40]. Besides, our data also revealed genes like *CYP1A1*, *AKR1C2* and *AKR1C3* participated in metabolism of xenobiotic compound and also upregulated. Previous studies revealed LINC00511 was overexpressed in multiple cancer [41–43], In our study, LINC00511 was apparently decreased in EGCG-treated cells. Currently, a lot of lncRNAs were reported to play principle role in progression of carcinogenesis stages [44,45]. We hypothesize that lncRNAs participated in EGCG-mediated gene regulation networks in GC cells and deeply investigated its role linked to corresponding pathways.

Previous researchers have identified a large number of lncRNAs functioned importantly in cancer [46,47]. Our data indicated part of lncRNAs would generate regulation networks and probably exclusively or distinguishably expressed in EGCG-treated GC cells. Moreover, this strategy can help us strongly understand overall transcription level change. We further held that EGCG treatment could affect cell cycle and other pathways in GC cells. In addition, we showed that LINC00511 was suppressed by EGCG in GC cells.

Here, we found that LINC00511 was increased in GC tissues and cells, similar to that in osteosarcoma [48]. Inhibited cell proliferation and promoted cell death was observed in GC cells after knocking down LINC00511, suggesting LINC00511 functioned importantly in GC progression. However, the present data was not enough to support LINC00511 exerted important effects on GC. In addition, nuclear–cytoplasmic fractionation data showed LINC00511 accumulated in cytoplasm, consistent with the study revealed LINC00511 concentrated in cytoplasm in pancreatic adenocarcinoma [49]. Importantly, we have found interaction between LINC00511 and miR-29b. Our results indicated enhanced miR-29b could curb cell proliferation and raise cell death in GC cells. Further results showed that KDM2A could interact with miR-29b. Previously, KDM2A has been confirmed to have oncogenic function [50]. Our present data exhibited increased KDM2A could reverse LINC00511-mediated cell growth and death, indicating LINC00511 regulated some pathways in GC metastasis.

Several limitations should be noted in this study. First, the expression level of LINC00511, miR-29b and KDM2A were not validated using clinical samples. Thus, we should collect more clinical GC samples and validate the correlation among them in the near future. Second, the in vivo assay should be further conducted to demonstrate functional importance of these genes in GC.

In summary, our findings suggested that EGCG can greatly hold up GC cell growth and metastasis. Bioinformatics studies have shown that EGCG is widely participated in cell metastasis regulation. Furthermore, co-expression network analysis showed that the long noncoding RNA LINC00511 is widely involved in the regulation of EGCG downstream networks. Knockdown of LINC00511 can significantly inhibit the proliferation, metastasis and invasion of GC. Experimental verification showed that LINC00511 could competitively bind miR-29b to induce KDM2A expression. In this study, EGCG can inhibit the development of GC. At the same time, these results indicate that LINC00511 and miR-29b/KDM2A can be used as diagnostic and therapeutic targets for GC.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

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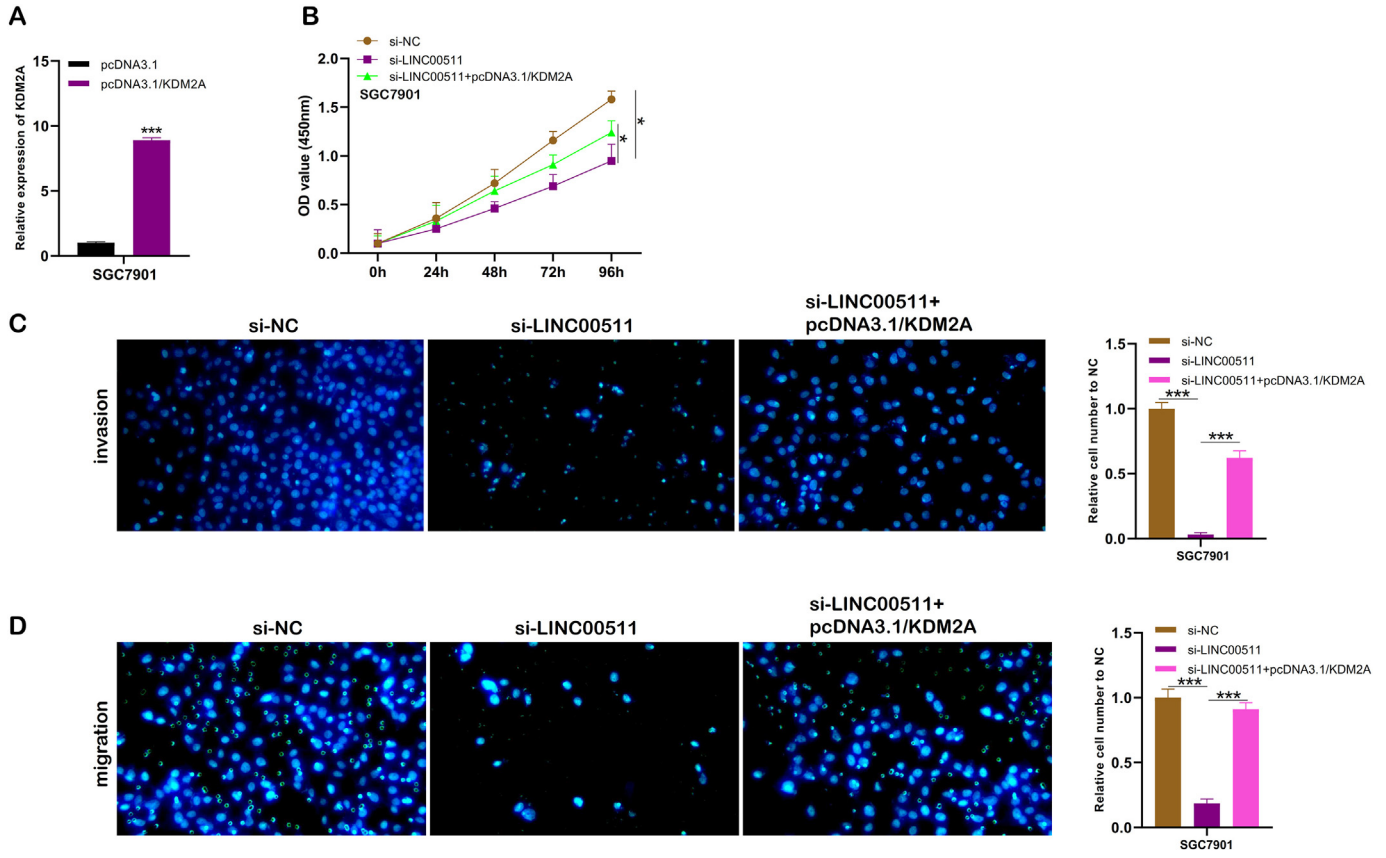

Fig. 8. LINC00511 promoted GC by targeting the miR-29b/KDM2A axis. (A) KDM2A level was increased after transfection of pcDNA3.1/KDM2A. (B) CCK8 assay to determine si-LINC00511–mediated cell viability in cells transfected with pcDNA3.1/KDM2A. (C) Assessment of si-LINC00511–mediated cell invasion in cells transfected with pcDNA3.1/KDM2A. (D) Assessment of si-LINC00511–mediated cell migration in cells transfected with pcDNA3.1/KDM2A.

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