ORIGINAL ARTICLES

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Long noncoding RNA CCAL promotes gastric cancer cell proliferation and migration in a Myc dependent way

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Aims: Long non-coding RNAs (lncRNAs) play key roles in cancers, yet their potential molecular mechanisms are not well understood. The objective of this study is to examine the expression, biological functions and mechanism of lncRNA CCAL in gastric cancer (GC). *Methods*: MTT and Colony formation assay were used to detect cell proliferation and the colony formation ability of gastric cancer cells. Wound healing, Migration, and invasion assay were respectively used to explore the migration, and invasion in gastric cancer cell lines. Real-time polymerase chain reaction (RT-PCR) was performed to determine the expression level of CCAL. Western Blot was used to determine the expression of related proteins. *Results*: In the present study, we found that CCAL was upregulated in gastric cancer cell lines. Patients whose tumors had high CCAL expression had a shorter overall survival than patients whose tumors had low CCAL expression. Overexpression CCAL promoted the proliferation, migration and invasion of GC by regulating the expression of myc. *Conclusion*: The present study reveals that CCAL is an oncogenic lncRNA that promotes the tumorigenesis and progression of GC.

1. Introduction

Gastric cancer as one of the most common malignant tumors is the second leading cause of cancer-related mortality and with the third highest incidence in China. Gastric cancer has a high incidence, a low rate of early diagnosis and a low 5-year survival in China. Chemotherapy is an important means of treatment of advanced gastric cancer, but drug resistance has become a big bottleneck.

The human transcriptome comprises a large number of protein-coding messenger RNAs and a large set of nonprotein coding transcripts, including long non-coding RNAs and small non-coding RNAs (Krol et al. 2010), which account for a much greater proportion than that of protein-coding mRNAs in the transcriptome. Long non-coding RNAs (lncRNAs) do not encode proteins and by definition more than 200 nucleotides in length (Ørom et al. 2010). lncRNAs which are characterized by the complexity and diversity of their sequences and the mechanisms of action are different from microRNAs and are thought to function as rather primary or spliced transcripts (Spizzo et al. 2012). It has been shown that lncRNAs are involved in the regulation of gene expression and may interact with known cancer genes (Ørom UA et al. 2010; Geisler et al. 2012; Huarte et al. 2010). lncRNAs can interact with chromatin DNA, mRNA or protein, thus maintaining chromatin accessibility, mRNA stability and protein activity or stability (Guttman and Rinn 2011; Lau 2014). Previous studies have shown that lncRNAs may play important roles in the regulation of proliferation, metastasis, and drug resistance of a variety of human tumors (Kim et al. 2015; Tsai et al. 2010; Yuan et al. 2014), including colorectal cancer (Zheng et al. 2014), non-small-cell lung cancer (Yang et al. 2013), pancreatic cancer (Liu et al. 2014), and gastric cancer (Li et al. 2014). Overexpression of lncRNA CCAL is correlated with poor prognosis of patients with several tumorigenesis and progression, such as colorectal cancer (Ma et al. 2016), and osteosarcoma (Zhou et al. 2017). Although many studies have shown that CCAL plays an important role in cancer development, the biological functions of CCAL in GC tumorigenesis, progression remain unknown. Here, we sought to investigate the clinical significance and possible functional mechanisms of CCAL in GC.

2. Investigations and results

2.1. CCAL is upregulated in gastric cancer cells

RT-PCR was used to determine the expression level of CCAL. As shown in Fig. 1A, the expression of CCAL in gastric cancer cell lines, such as BGC-823, MKN-45, AGS, SGC-7901 and MGC-803, was significantly increased compared with that in GES-1 ($P<0.01$ and $P<0.001$). As shown in Fig. 1B, the expression level of CCAL protein was significantly higher in GC tumor tissue than that in the normal tissues by Northern Blot. Furthermore, increased levels of CCAL were detected in GC tissue relative to adjacent normal tissue levels in 50 GC patients ($P<0.001$, Fig. 1C). A few GC patient tissues showed lower CCAL expression in cancer tissues relative to paired adjacent noncancerous tissue (Fig. 1D).

2.2. Overexpression of CCAL promotes proliferation and invasion of GC

As shown in Fig. 2A, over-expression of CCAL could significantly increase the expression level of CCAL ($P<0.001$). MTT was used to detect the cell viability of GC (Fig. 2B). The result indicated that over-expression of CCAL could significantly promote the cell viability of GC after two days ($P<0.05$). Colony formation assay showed that over-expression of CCAL significantly increased the colony formation number ($P<0.001$, Fig. 2C). To analyze the role of CCAL in cell migration and invasion, wound healing and transwell assays were performed in MGC-803 cells. Cells treated with oeCCAL were distinctively more migratory and invasive than the control cells ($P<0.001$, Fig. 2D and 2E). Western Blot was used to determine the expression of the metastasis related proteins (Fig. 2F). The data showed that over-expression of CCAL could decrease the expression of E-cadherin and p21, and increase the expression of MMP2, MMP9, and cyclin D1.

2.3. Knock-out CCAL inhibits proliferation and invasion of GC

As shown in Fig. 3A, knock-out CCAL was significantly decrease the expression of CCAL ($P<0.001$). Furthermore, we found that

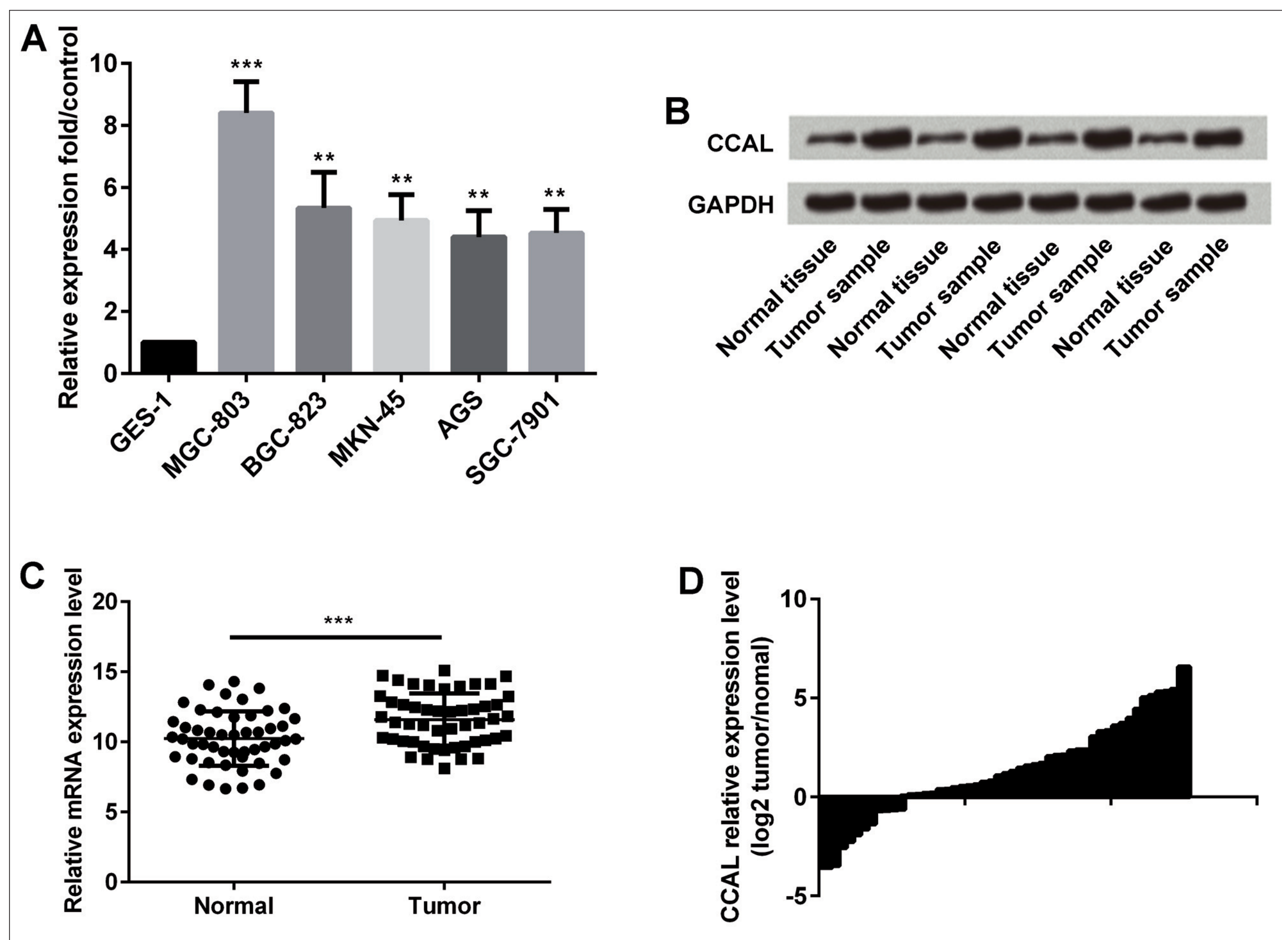

Fig. 1: Expression of CCAL in gastric cancer (GC) samples and cell lines. (A) The expression of CCAL in cell lines was determined by RT-PCR. (B) CCAL expression levels were measured in 4 paired (tumor and adjacent normal tissue) GC tissues by Northern blot. (C) CCAL mRNA expression levels were measured in 50 paired (tumor and adjacent normal tissue) GC tissues by RT-PCR. (D) CCAL mRNA expression level in 50 paired GC tissues (Log2 cancer vs. normal).
**, P<0.01; ***, P<0.001

the cell viability and colony number of knock-out CCAL showed the reverse trend. Knock-out CCAL inhibited the proliferation of MGC-803 cells (Fig. 3B and 3C). Wound healing and transwell assays showed that knock-out CCAL could significantly inhibit the migration and invasion of MGC-803 (P<0.001) (Fig. 3D and 3E). Western Blot showed that siCCAL could increase the expression of E-cadherin and p21, and decrease the expression of MMP2, MMP9, and cyclin D1 (Fig. 3F).

2.4. *CCAL regulated the expression of Myc by recruiting NURF*

As shown in Fig. 4A, RNA pulldown assay showed that CCAL interacted with UNRF components, including BPTF, RNF21, and RBBP4. We also verified this result using RNA immuno-precipitation (RIP) and found CCAL could be enriched in BPTF precipitates, further proving that CCAL interacts with BPTF (P<0.001, Fig. 4B). Domain mapping analysis showed that the length of CCAL that interacted with BPTF was 500-1000nt (Fig. 4C). Western blot analysis showed that siCCAL inhibited the expression of Myc, and over-expression of CCAL increased the expression of Myc (Fig. 4D). Moreover, siBPTF inhibited the expression of Myc, and over-expression of BPTF increased the expression of Myc (Fig. 4E). ChIP assay showed that a specific region of the Myc promoter was enriched upon BPTF precipitation (P<0.001, Fig. 4F). Furthermore, we found that siCCAl could significantly inhibit the enrichment of BPTF, while over-expression CCAL could significantly promote the enrichment of BPTF n Myc promoter (P<0.05, Fig. 4G). ChIP assay showed that either siCCAL or siBPTF could significantly decrease the enrichment of H3K4me3 in Myc promoter (P<0.001, Fig. 4H). In 2 samples, we found that siCCAL decreased the expression of Myc, while over-expression of CCAL increased the expression of Myc (Fig. 4I). The expression of proliferation and migration related proteins were inhibited in oeCCAL+simyc group (Fig. 4J). In contrast, the expression of proliferation and migration related proteins were inhibited in siCCAL+oemyc group (Fig. 4K). These data indicated that CCAL played its role through Myc. Finally, we found that the expression of Myc was upregulated in CCAL^{high} samples, while the expression of Myc was down-regulated in CCAL^{low} samples (Fig. 4L).

2.5. *High levels of CCAL reveal bad prognosis and malignancy of the carcinoma*

The result showed that over-expression of CCAL was associated with the grading of the tumor (Fig. 5A). That is, the higher expression of CCAL, the higher tumor grade was found. The expression of CCAL in grade was significantly higher than that in grade II and I (P<0.01). To examine the relationship between CCAL expression levels and GC prognosis, the GC samples were divided into two groups according to CCAL expression levels and Kaplan-Meier survival analysis was performed. Both in overall survival or in disease-free survival, CCAL^{high} patients had a poor prognosis, and CCAL^{low} patients could survive longer, indicating the critical role of CCAL in GC prognosis (Fig. 5B and 5C). Then we observed

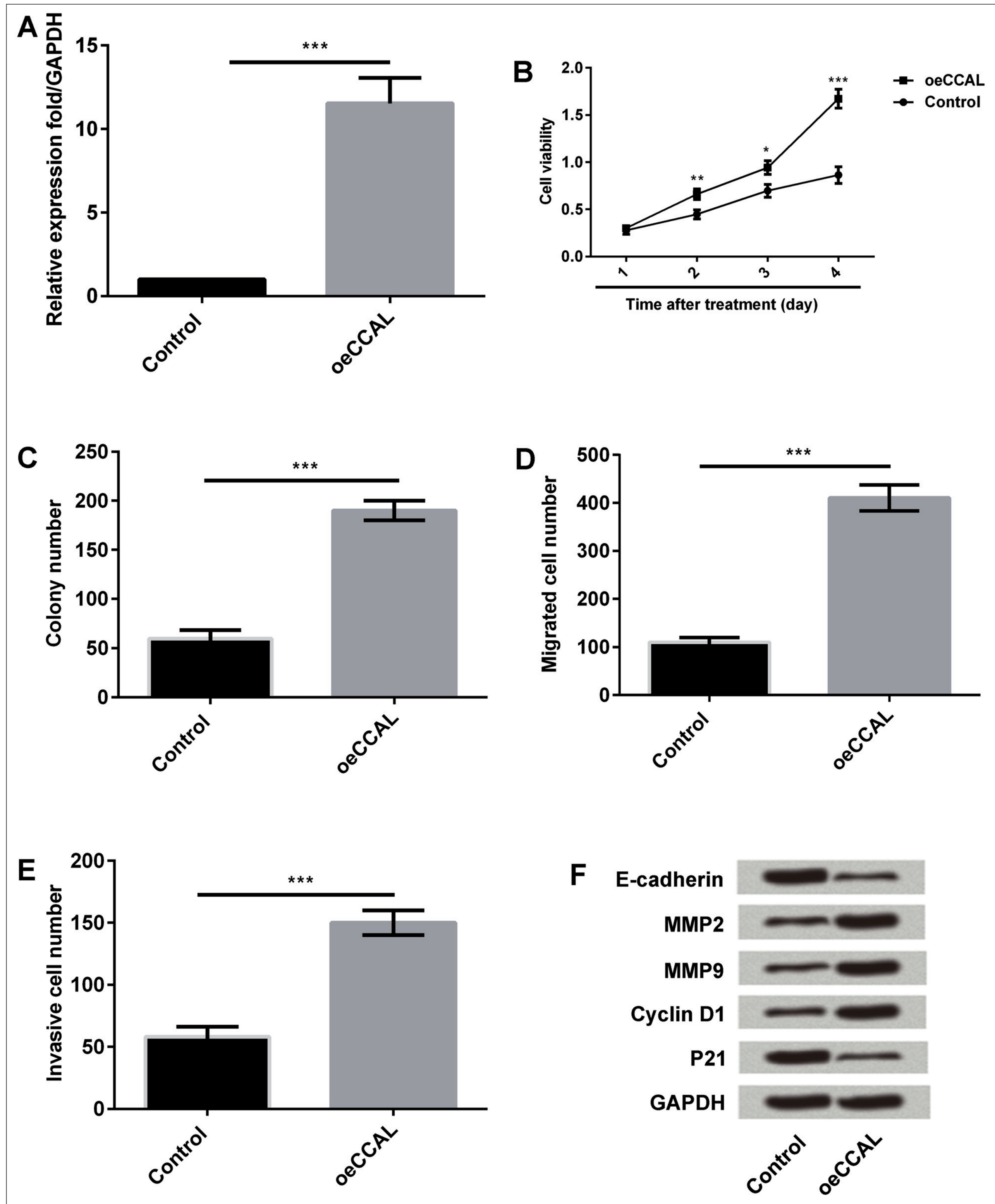

Fig. 2: Increased CCAL expression promotes GC cell line proliferation, clone formation, migration and invasion. (A) The expression of CCAL was increased in MGC-803 by stabling over CCAL express. (B) MTT assay. (C) Colony formation assays. (D) Cell migration assays. (E) Invasion assays. (F) The expression of the metastasis related proteins were determined by Western blot.
*, P<0.05; **, P<0.01; ***, P<0.001

the expression of the metastasis related proteins by Western blot in CCAL^{low} and CCAL^{high} group, respectively (Fig. 5D). The results showed that the CCAL^{low} group has a higher expression of E-cadherin and p21 than CCAL^{high} group. The expression of MMP2, MMP9, and cylin D1 in CCAL^{high} group was higher than that in CCAL^{low} group.

3. Discussion

During recent years, studies focused on the role of lncRNAs in tumorigenesis. lncRNAs such as HOTAIR, CUDR, MVIH, PCGEM1, and MALAT1 have been considered as the biological hallmarks of cancer, including evading growth suppressors, sustaining proliferative signaling, enabling replicative immortality,

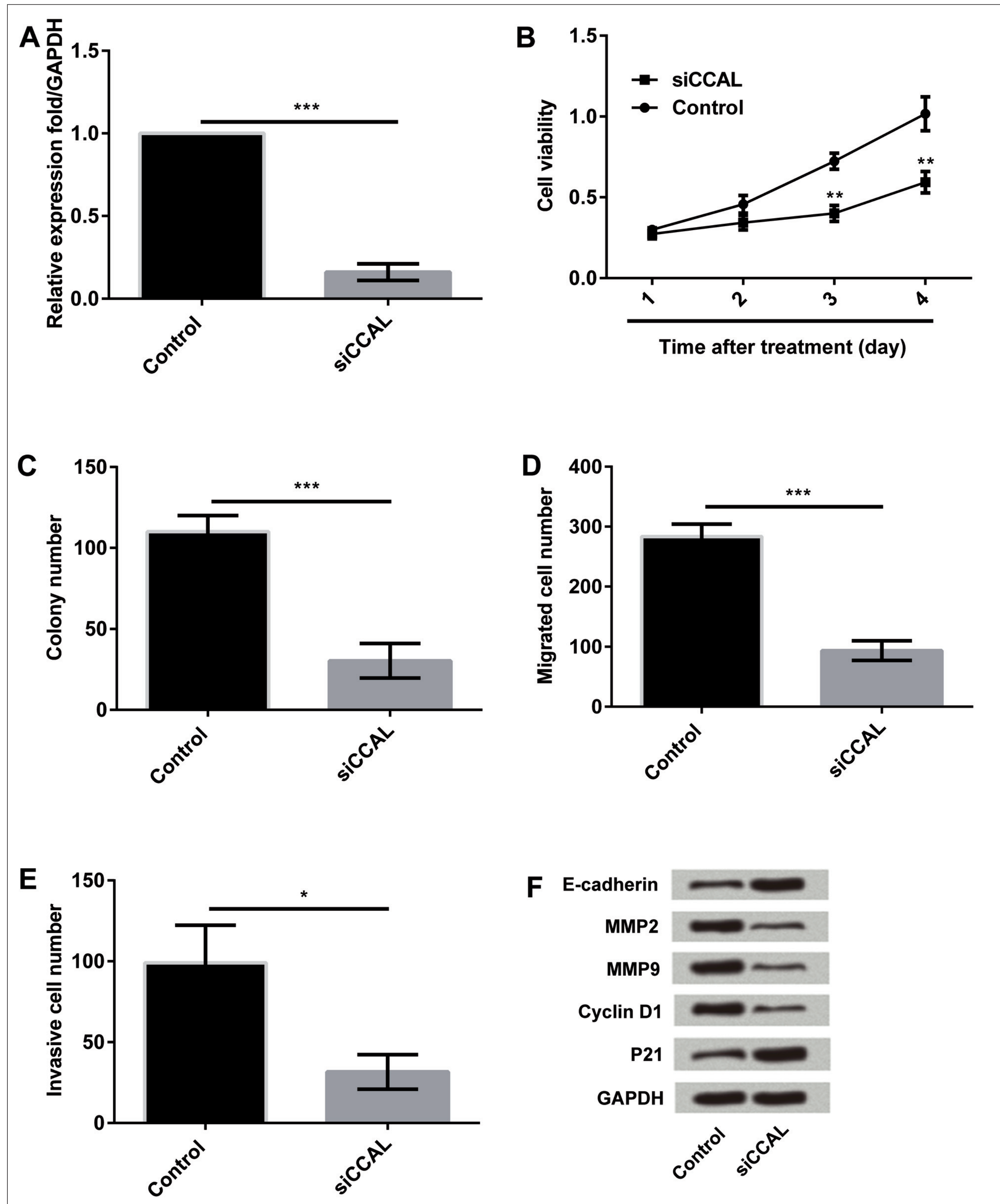

Fig. 3: Decreased CCAL expression inhibits GC cell line proliferation, clone formation, migration and invasion. (A) The expression of CCAL was decreased in MGC-803 by known-down CCAL express. (B) MTT assay. (C) Colony formation assays. (D) Cell migration assays. (E) Invasion assays. (F) The expression of the metastasis related proteins were determined by Western blot.
*, P<0.05; **, P<0.01; ***, P<0.001

inducing angiogenesis, activating invasion and metastasis, and resisting cell death (Dinger et al. 2009; Gutschner and Diederichs, 2012; Ji et al. 2003; Wilusz et al. 2009). CCAL was initially found to be associated with the progression of colorectal cancer (CRC), which could promote the deterioration of CRC (Ma et al. 2016). In addition, previous studies have shown that over-expression CCAL promoted tumor metastasis in osteosarcoma (Zhou et al. 2017). In this study, we found that upregulated CCAL could promote the proliferation, colony formation and migration ability of GC cells. In contrast, these promotion effects were reversed by decreased

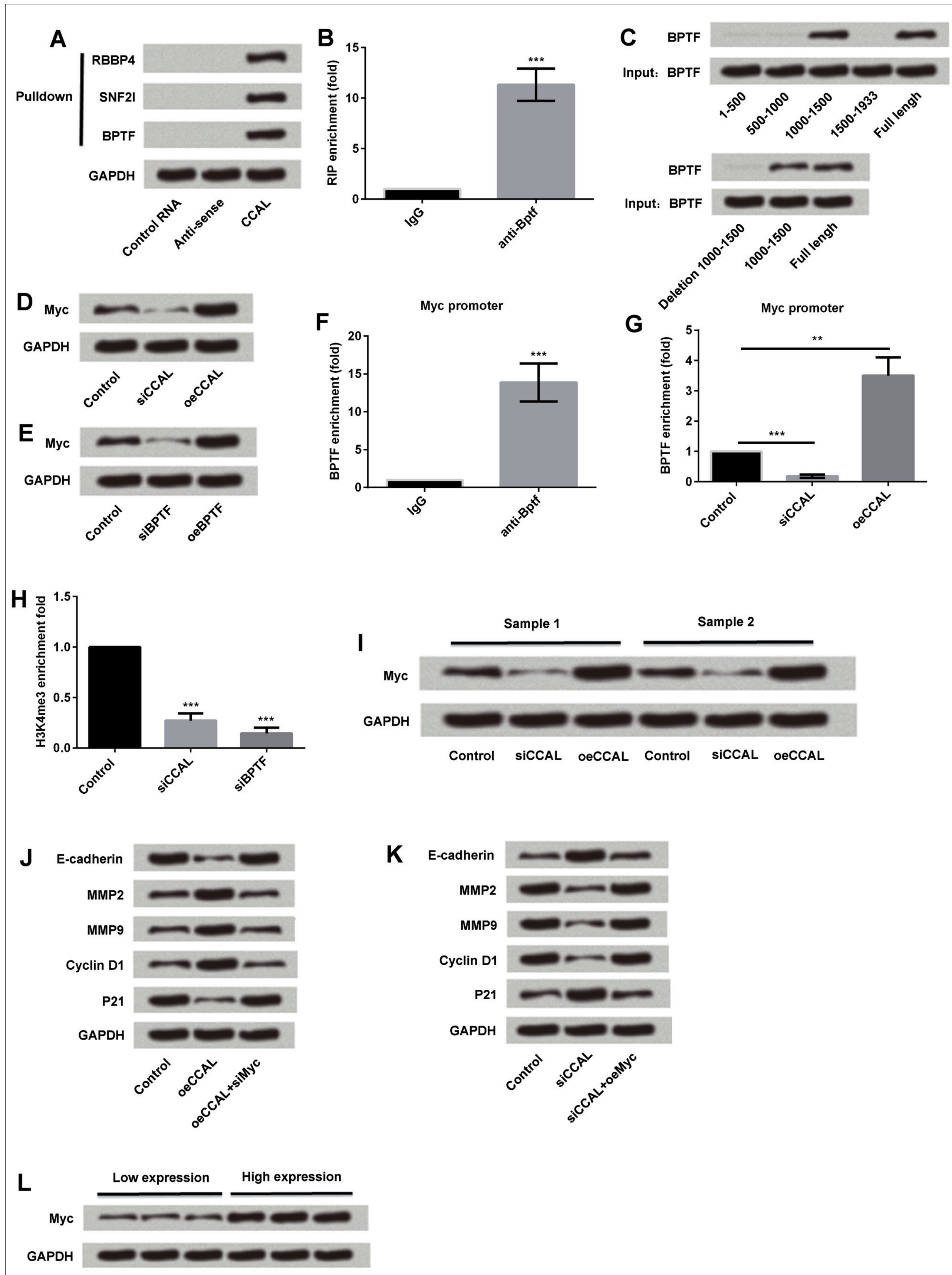

Fig. 4: CCAL regulated the expression of Myc by recruiting NURF. (A) The interaction between CCAL and Myc was confirmed by RNA pull-down and western blot. (B) The interaction between CCAL and BPTF was confirmed by RNAIP. (C) Domain mapping analysis was used to detect the length of CCAL that interacted with BPTF. (D and E) Western blot analysis was used to detect the expression of Myc by regulating the expression of CCAL and BPTF, respectively. (F) ChIP was performed using anti-BPTF and control lgG antibodies. (G) ChIP was used to determine the enrichment of BPTF in Myc promoter by regulating the expression of CCAL. (H) ChIP was used to determine the enrichment of H3K4me3 in Myc promoter by regulating the expression of CCAL or BPTF. (I and L) Western blot analysis was used to detect the expression of Myc in GC samples. (J and K) Western blot analysis was used to detect the expression of proliferation and migration related proteins by regulating the expression of CCAL and Myc.
**, P<0.01; ***, P<0.001

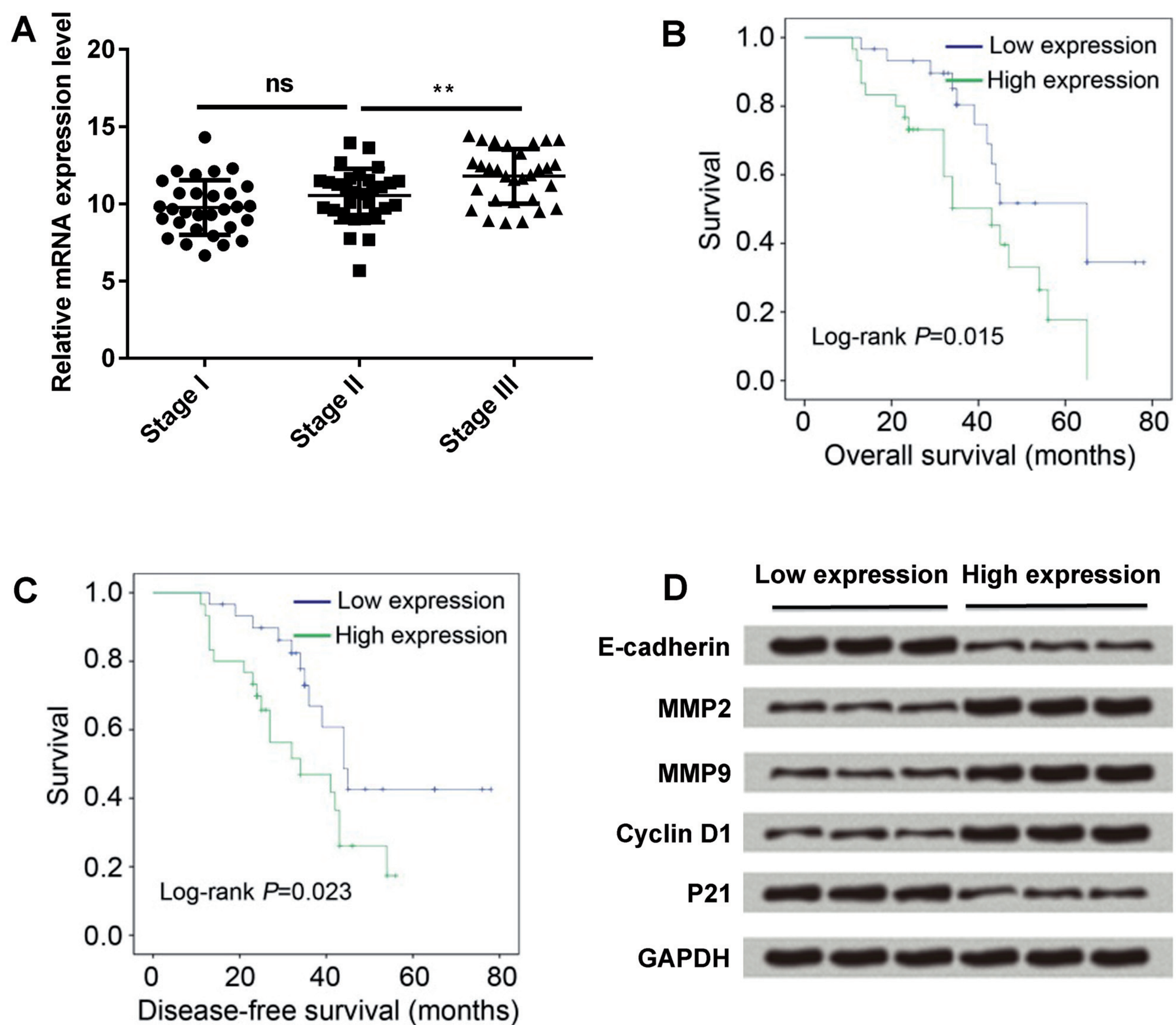

Fig. 5: High levels of CCAL reveal bad prognosis and malignancy of the carcinoma. (A) The expression of CCAL mRNA was detected by RT-PCR in the stage of GC. (B and C) The relationship between CCAL and survival was detected by Kaplan-Meier survival analyses in overall survival and disease-free survival. (D) Western blot analysis was used to detect the expression of proliferation and migration related proteins in GC samples.
**, P<0.01

expression of CCAL expression in GC cells. Moreover, we determined the expression of the metastasis related proteins, such as E-cadherin, MMP2, MMP9, Cyclin D1, and P21. The result showed that over-expression of CCAL promoted the expression of these proteins, suggesting that CCAL play an important role in the metastasis of gastric cancer.

In the current study, we demonstrated CCAL interacts with NURF complex, which played a critical role in Myc promoter activation. C-myc is a kind of cellular proto-oncogene, and its product is the nuclear transcriptional factor c-Myc protein. C-Myc protein can increase the transcriptional expression of numerous target genes by binding to the gene sequence and recruiting some related factors, thus promoting cell proliferation and tumorigenesis (Amati et al. 1993; Blackwood and Eisenman 1991). Previous studies have shown that activation or over-expression of c-myc is involved in the occurrence and development of many cancers (He et al. 1998; Kawate et al. 1999; Levens 2010; Shiraha et al. 2013). Our present results indicate that CCAL promote cell proliferation and migration by regulating the expression of myc. Finally, we investigated the prognostic significance of CCAL expression in a large number of GC patient samples. In GC patients, increased expression of CCAL was observed in cancer tissue compared with adjacent normal tissues. Moreover, the patients with positive CCAL staining had shorter survival time across all tumor stages. These results showed that the high levels of CCAL reveal bad prognosis and malignancy of the carcinoma.

In conclusion, the present study reveals for the first time that CCAL is an oncogenic lncRNA that promotes the tumorigenesis and progression of GC. Our results indicate that CCAL may serve as a potential prognostic marker and therapeutic target for the treatment of GC.

4. Experimental

4.1. *Cell culture*

Human GC cell lines BGC-823, MKN-45, AGS, SGC-7901, MGC-803 and human gastric mucosal epithelial cell line GES-1 were purchased from the Cell Bank of the Shanghai Institute of Cell Biology (Shanghai, China). They were cultured in RPMI-1640 culture media (HyClone, USA) supplemented with 10% Fetal Bovine Serum (FBS) and maintained in 5% CO_2 at 37 °C and passaged at 80-90 % confluency every three or four days.

4.2. *Transfection and stable cell line construction*

The CCAL construct was generated by subcloning PCR-amplified full-length human CCAL cDNA into pcDNA3.1 for overexpression. siRNA was employed for downregulation. The CCAL-shRNA hairpin DNA sequence was annealed and synthesized. It was cloned into pYr-1.1 vector (Yinrun Biotechnology, Changsha, China) and linear-

ized by restriction enzymes BsaI. Lipofectamine 2000 (Invitrogen, USA) was used for transfection according to the manufacturer's protocol. Stable cell lines expressing CCAL or shCCAL were selected after 3-4 weeks by treating 200 mg/mL G418. Cells transfected with pcDNA3.1 or the pYr-1.1 vector (containing non-targeting control shRNA sequence) were considered as negative controls.

4.3. MTT assay

The cell proliferative and invasive capacities were determined using a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay and a Matrigel invasion chamber assay, respectively, according to standard methods described before. Each experiment was performed three times.

4.4 qRT-PCR

Total RNA was isolated from transfected cells by using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega). Reverse transcription was performed by using the MultiscribeRTkit (Applied Biosystems) and random hexamers or oligo (dT). The reverse transcription conditions were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C.

4.5 Western blot

The protein used for western blotting was extracted using RIA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane at 4 °C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the polyvinylidene difluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

4.6. Colony formation assays

For colony formation assays, cells (stable over-expression and downregulation of CCAL cells and their corresponding negative control cells) were seeded in 6-well plates with 100 cells and a final volume of 2 mL culture medium per well. The cells were maintained in 5% CO_2 at 37 °C and the culture medium was changed every four days for the following three weeks. Finally the colony formation cells of each group were stained with H&E staining.

4.7. Migration and invasion assays

The cell migration and invasion assays were done in 24-well Transwell plates with or without pre-coated Matrigel. For migration assay, the stable cells and the corresponding negative control cells were seeded in the upper chamber of the Transwell system (8.0 mm, pore size; 3422, Millipore, USA) with 1×10^4cells/well in 100 µL of serum-free RPMI-1640 medium, and the lower chamber was filled with 600 µL 30 % FBS RPMI-1640 culture medium. For invasion assay, 24 h after transfection, 2×105 cells/well of the stable cells and the corresponding negative control cells were seeded in the upper chamber with pre-coated Matrigel (8.0 mm, pore size; ECM554, Millipore, USA), and the lower chamber was filled with 600 µL 30% FBS RPMI-1640 culture medium. After 24 h or 48 h of incubation, cells remaining on the top layers of the inserts were removed by cotton swab scrubbing, and cells on the lower surface of the membrane were fixed and stained with H&E staining. The cell numbers in five random fields (200×) were counted for each chamber, and the average value was calculated.

4.8. Northern blot

Total RNA was extracted from GC cells according to TRIZOL methods (Invitrogen) for northern blot. LncRNA CCAL and GAPDH fragments were cloned into PCDNA4 plasmid and northern probes were produced using Biotin RNA Labeling Mix (catalogue 11685597910, Roche). T7 RNA polymerase (catalogue 10881767001, Roche) was used for in vitro transcription. For northern blot, the samples were separated by electrophoresis using formaldehyde gel, followed by membrane transferring. The membranes were incubated with hydration buffer supplemented with proper amount of probes, and then the nucleic acid signal was detected using Chemiluminescent Nucleic Acid Detection Module (catalogue 89880, Thermo Scientific).

4.9. RIP assay

For RIP, the cells were treated with 1% formaldehyde and the crashedwith RIPA buffer (150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1% NP40, 1 mM EDTA and 50 mM Tris pH 8.0) supplemented with RNase inhibitors and proteinase inhibitors for 30 min, followed by centrifugation. The supernatants were incubated with the indicated antibodies for 4 h, and then supplemented with protein A/G beads. The precipitates were washed with RIPA buffer followed by de-crosslinking. Finally RNA was extracted and LncCCAL enrichment was examined using RT-PCR.

4.10. ChIP

ChIP assays were performed following standard protocol (Upstate Biotechnology, Inc.). Cells were treated with 1% formaldehyde for 10 min, cracked with SDS lysis buffer followed by ultrasonication, then incubated with proper antibodies. After washing by low salt, high salt and LiCl buffer, the elution buffer was used to harvest the chromatin fragments. Finally the de-crosslinking was performed and enrichment was examined using RT-PCR.

4.11. Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean±SD. Statistical analyses were performed using SPSS 19.0 statistical software. The P-values were calculated using a one-way analysis of variance (ANOVA). A P-value of <0.05 was considered to indicate a statistically significant result.

Conflict of interest: None declared.

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