MBNL1-AS1 attenuates tumor cell proliferation by regulating the miR-29c-3p/BVES signal in colorectal cancer

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Abstract. Dysregulation of long non-coding RNAs (lncRNAs) is involved in the development of colorectal cancer (CRC). In the present study, the identification of muscle blind like splicing regulator 1 antisense RNA 1 (MBNL1-AS1) lncRNA was reported. Firstly, Cell Counting Kit-8, EdU and colony formation assays were uesed to explore the role of MBNL1-AS1 in regulating the proliferation of CRC cells. According to TCGA database, it was found that MBNL1-AS1 was correlated with microRNA (miR)-29c-3p and blood vessel epicardial substance (BVES) expression in CRC cells. Then, the regulation among MBNL1-AS1, miR-29C-3P and BVES was detected by dual luciferase reporter assay and the function of MBNL1-AS1/miR-29C-3P/BVES axis was explored by rescue assay. The results demonstrated that MBNL1-AS1 expression was decreased in CRC and was associated with the size of tumors derived from patients with CRC. Functionally, the upregulation of MBNL1-AS1 suppressed CRC cell proliferation *in vitro* and inhibited tumor growth *in vivo*, while knockdown of MBNL1-AS1 expression caused the opposite effects. MBNL1-AS1 expression correlated with BVES expression in CRC tissues and MBNL1-AS1 enhanced the stability of BVES mRNA by functioning as a competing endogenous RNA to sponge miR-29c-3p; the latter directly targeted MBNL1-AS1 and BVES mRNA 3'UTR. Collectively, the results indicated that MBNL1-AS1 suppressed CRC cell proliferation by regulating miR-29c-3p/BVES signaling, suggesting that the MBNL1-AS1/miR-29c-3p/BVES axis may be a potential therapeutic target for CRC.

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Introduction

Colorectal cancer (CRC) is the third most common cause of cancer-related death in the United States (1). In 2020, 147,950 individuals were predicted to be diagnosed with CRC, and 53,200 succumbed to the disease (1). The high mortality of patients with CRC is attributed to various factors, among which the late discovery leading to the loss of optimal chance of surgery may be the most important (2). However, although surgery accompanied with appropriate chemoradiotherapy are used in clinical practice, once patients with CRC have reached the advanced stage of the disease, a small part of them benefit from these therapeutic strategies; this outcome is notably noted for patients with CRC and distal metastasis (3). Therefore, the identification of novel biomarkers and therapeutic targets is required.

Long non-coding RNAs (lncRNAs) are a class of single-stranded RNAs >200 nucleotides (nt) in length, which lack the ability to encode proteins. In the past decade, increasing experimental evidence has indicated that lncRNAs play crucial roles in the development of cancer, including CRC (4,5). For example, lncRNA CRART16 promoted the resistance of CRC to 5-fluorouracil by sponging microRNA (miR)-193b-5p (6); lncRNA AGAP2-AS1 expression was upregulated by E2F transcription factor 4, which accelerated the progression of CRC by regulating the miR-182-5p/cofilin 1 axis (7); lncRNA small nucleolar RNA host gene 17 (SNHG17) promoted the tumorigenesis and metastasis of CRC by regulating the tripartite motif containing 23-pescadillo ribosomal biogenesis factor 1 axis and the miR-339-5p-FOS like 2-SNHG17 positive feedback loop (8). Currently, lncRNA muscle blind-like 1 antisense RNA 1 (MBNL1-AS1) was identified to be downregulated in several types of cancer and to be involved in cancer development (9,10). For example, MBNL1-AS1 suppressed the progression of retinoblastoma by regulating the miR-338-5p/Wnt/β-catenin signaling pathway (11). MBNL1-AS1 expression has been shown to be downregulated and to suppress the progression of colon cancer by regulating the miR-413-3p/myosin light chain 9 signaling (12). However, the underlying mechanisms by which MBNL1-AS1 suppresses the progression of CRC are unclear.

Blood vessel epicardial substance (BVES) is a tight junction-associated protein which is downregulated in the majority of cancer types, including CRC (13). BVES has been identified as a tumor suppressor in CRC, due to the fact that restoration of its expression can reduce the proliferation of CRC cells (14). With regard to the associated mechanism of action, BVES can induce β-catenin redistribution to inhibit WNT/β-catenin signaling, and the interaction of BVES with tight junction protein 1 and Rho/Rac guanine nucleotide exchange factor 2 can result in the decreased levels of activated ras homolog family member A in CRC (14). Several studies have demonstrated that the downregulation of BVES expression is involved in promoting hypermethylation in cancer cells (13,15,16). However, whether the stability of BVES mRNA is altered in CRC remains unknown.

In the present study, the expression levels of MBNL1-AS1 were decreased in CRC tissues compared with those noted in matched normal tissues. The co-expression analysis based on The Cancer Genome Atlas (TCGA) database indicated the strongest correlation between the expression of MBNL1-AS1 and BVES mRNA in CRC. Furthermore, MBNL1-AS1 enhanced the stability of BVES mRNA in CRC cells and suppressed their proliferation by acting as a competing endogenous RNA (ceRNA) to sponge miR-29b-3p, which in turn directly targeted BVES mRNA 3' untranslated region (UTR).

Materials and methods

Patients and samples. A total of 20 patients with CRC were enrolled in the present study. Inclusion criteria were as follows: i) CRC was diagnosed by pathological examination; ii) patients had no other severe diseases, including other cancers and high blood pressure with complications and iii) patients were willing to participate and signed the informed consents. Exclusion criteria were as follows: i) Incomplete information and ii) patients received chemo- or radiotherapy before collecting tissue samples. The fresh CRC samples were collected after the patients had been completed the surgery but within 10 min and stored in liquid nitrogen immediately. All fresh CRC samples were collected from November 2020 to December 2021 in the Affiliated Hospital of Southwest Medical University. The present study was approved (approval no. KY2023025) by the Ethics Committee of the Affiliated Hospital of Southwest Medical University (Luzhou, China), and informed consent was signed by all patients. Clinicopathological characteristics of patients are provided in Table I.

Animal model. A total of 24 4-week-old female BALB/c nude mice (weight, 16±3 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. and housed in laminar flow cabinets under a specific pathogen-free condition (37˚C; 12/12-h light/dark cycle; free access to food and water). Mice experiments were approved (approval no. swmu20230055) by the Ethics Committees of Southwest Medical University. The nude mice were divided into four groups randomly, and each group included six nude mice. A total of ~1x10^7 cells resuspended in 50 μl PBS were injected into the subcutaneous tissues of each nude mice. A total of seven, 10, 13, 16, 19 and 22 days after the cells were injected, the formed xenografts were measured, respectively. The nude mice were sacrificed before the longest diameter of xenografts exceeded 15 mm. They were euthanized by cervical dislocation after receiving satisfactory anesthesia by injection of 50 mg/kg pentobarbital sodium. Then, the xenografts were taken out and weighted. The volumes of xenografts were calculated based on the following formula: V=π/6 x L x W x H, which is used to calculate the volume of an ellipse.

Cell culture and transfection. Human colon cell lines (SW480, SW620, HCT116 and T84) and the normal epithelial cell line, CD 841 CoN were purchased from the National Collection of Authenticated Cell Cultures and cultured with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (FBS; cat. no. SV30087.03; Hyclone; Cytiva), in 5% CO_2 at 37˚C. Cell transfection was performed by using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Routinely, the transfection was carried out in six-well plates at room temperature. For each well, 2 μg overexpression/shRNA vector or 50 nM siRNA were used to incubate with cells for 24 h. After that, cells were harvested for subsequent analysis.

RNA extraction. Total RNAs of transfected SW480 or SW620 cells were extracted by using an RNA Extraction kit (Chongqing Van der Waals Biotechnology, Co., Ltd.) according to the manufacturer's protocol. Briefly, 10^6 cells were lysed using 100 μl Lysis Buffer I, and then 300 μl Lysis Buffer II was added. The solution was added into RNA Binding Columns and centrifuged at 13,000 x g for 30 sec at 4˚C. After washing, 50 μl Elution Buffer was added into the RNA Binding Columns to dissolve the RNAs. The stability of mRNA was performed by incubating 1 μg total RNA with 2 μg/ml Actinomycin D (Sigma Aldrich; Merck KGaA) at indicated time points, and analyzed by PCR.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNAs were reversely transcribed into cDNAs by using a PrimeScript™ RT reagent kit (Takara Bio, Inc.) and the cDNAs were amplified by using a SYBR® Premix Ex TaqTM II reagent (Takara Bio, Inc.). Initial denaturation was performed at 95˚C for 10 min, followed by 40 cycles of denaturation (95˚C for 10 sec), annealing (60˚C for 30 sec), and extension (72˚C for 30 sec). The expression of miR-29c-3p and BVES was calculated by using the $2^{-\Delta\Delta Cq}$ method (17). ACTB served as the internal reference. The primers of miR-29c-3p were purchased from (Shanghai, GenePharma Co., Ltd.), and the primers of MBNL1-AS1, BVES and ACTB were shown in Table SII.

Construction of vectors and small interfering RNA (siRNA) for BVES. The full-length sequence of MBNL1-AS1 was synthesized and inserted into pcDNA3.1 vectors (Addgene, Inc.) or pLV-Puro vectors (Addgene, Inc.,) to construct the MBNL1-AS1-overexpressing vectors or the cell lines that stably overexpress MBNL1-AS1. The short hairpin RNA (shRNA) sequence for MBNL1-AS1 was synthesized and inserted into pLV-Puro vectors (Addgene, Inc.) to construct the cell lines that stably low express MBNL1-AS1 expression. The wild-type or mutant sequences of 3'UTR of BVES mRNA

Table I. Clinical information of the patients with colorectal cancer.

Clinicopathological characteristics	Expression level of MBNL1-AS1		P-value
	Low	High	
Sex			0.7301
Male	15	13	
Female	5	7	
Age, years			1.0000
≤63	11	12	
>63	9	8	
Tumor size, cm^3			0.0500
≤28	16	9	
>28	4	11	
TNM stage			0.7403
I and II	6	8	
III and IV	14	12	
Lymph node metastasis			0.7150
Yes	16	14	
No	4	6	

MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1.

or MBNL1-AS1 containing the binding sites of miR-29c-3p were inserted into pGL3-Basic vectors (Addgene, Inc.) to construct the wild-type pGL3-BVES 3'UTR vector, the mutant pGL3-BVES 3'UTR vector, the wild-type pGL3-MBNL1-AS1 vector, or the mutant pGL3-MBNL1-AS1 vector. The recombinant vectors were selected by ampicillin after transforming in receptive bacteria. The shRNA sequences were shown in Table II. The siRNAs for BVES were purchased from Thermo Fisher Scientific, Inc. (cat. no. AM16708) and sequences were unavailable due to confidentiality issues. The transfection of these vectors or siRNAs were performed as aforementioned.

Dual-luciferase reporter assay. A total of ~1 μg of the wild-type pGL3-BVES 3'UTR vectors, the mutant pGL3-BVES 3'UTR vectors, the wild-type pGL3-MBNL1-AS1, or the mutant pGL3-MBNL1-AS1 vectors, and 0.05 μg of pRL-TK vectors (Promega Corporation) which served as control, were transfected into HEK293T cells, respectively, while the cells were also transfected with NC or miR-29c-3p mimics using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A total of 24 h after transfection at 37˚C, the cells were lysed, and the luciferase activity was measured using GloMax 20/20 Luminometer (Promega Corporation). The relative luciferase activity was calculated by normalizing Firefly luciferase to *Renilla* luciferase activity.

Cell proliferation. For Cell Counting Kit-8 (CCK-8) assay, ~2,000 transfected SW480 or SW620 cells were seeded into 96-well plates and cultured for 1, 2, 3, 4, and 5 days, respectively. For each well, 10 μl CCK-8 reagent (MedChemExpress) were diluted into 100 μl medium to incubate the cell and analyzed their proliferation based on the absorbance at 450 nm. For colony formation assay, ~1,600 transfected SW480 or SW620 cells were seeded into six-well plates and cultured for 2 weeks. Then the cell plates were fixed by 4% paraformaldehyde for 10 min at room temperature and stained by 0.1% crystal violet for 10 min at room temperature. For 5-ethynyl-2'-deoxyuridine (EdU) assay, the transfected cells were cultured in 15-mm slides and incubated with EdU solution for 2 h at 37˚C. The staining was performed by EdU-567 kit (Guangzhou RiboBio Co., Ltd.) for 30 min at 37˚C and the nucleus was stained by 5 mg/ml DAPI.

Cell apoptosis by flow cytometry. A total of ~20,000 transfected SW480 or SW620 cells were cultured in six-well plate and were harvested by trypsin without EDTA. Cells were resuspended by binding buffer, containing 2.5 μl AnnexinV-FITC and 2.5 μl PI for 30 min in the dark (cat. no. BD 556570; BD Biosciences). The percentage of apoptotic cells was recorded using a Beckman Flow Cytometer (Beckman Coulter, Inc.) using count model and analyzed using FlowJo software (Version 10.0; FlowJo LLC).

Western blot analysis. Total proteins of transfected SW480 or SW620 cells extracted by RIPA lysis buffer (Beyotime Institute of Biotechnology) and concentration were measured by BCA kit (Beyotime Institute of Biotechnology). For electrophoresis, 30 μg protein samples were loaded each lane and were separated using 10% SDS-PAGE gels. Then, the proteins in the gels were transferred onto PVDF membranes, which were then incubated in a blocking buffer (3% bovine serum albumin; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h at room temperature. Then, the PVDF membranes were incubated with anti-BVES antibody (1:500; cat. no. 12920-1-AP; Proteintech Group, Inc.) or anti-GAPDH antibody (1:10,000; cat. no. 60004-1-Ig; Proteintech Group, Inc.) at 4˚C overnight. After washing by 0.1% TBST solution, the PVDF membranes were incubated with HRP-conjugated goat-anti-rabbit secondary antibody (1:1,000; cat. no. 7074;) or goat-anti-mouse secondary antibody (1:1,000; cat. no. 7076; both from Cell Signaling Technology, Inc.) at room temperature for 1 h. After washing, the PVDF membranes were exposed using SuperSignal West Dura Extended Duration Substrate Kit (Thermo Fisher Scientific, Inc.).

Bioinformatic analysis. The enrichment of signaling pathways were analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/). The correlation of the expression between two genes were acquired from TCGA database (https://www.cancer.gov/ccg/research/genome-sequencing/tcga) and analyzed using Spearman's correlation (two-tailed). The binding sites of miR-29b-3p and miR-29c-3p were predicted by TargetScanHuman 7.1 (https://www.targetscan.org/vert_71/).

Statistical analysis. The data are presented as the mean ± standard deviation (SD). The differences between two groups were analyzed using unpaired Student's t-test or Mann-Whitney U test and among more than two groups were analyzed using

Table II. The DNA sequences used in the present study.

Name	Sequence (5'→3')
MBNL1-AS1	Sense: CAGGAGAGTGGCAGGAGATGAC Antisense: GTGGTTCGCAGGCATTCTAAGC
BVES	Sense: GGCGTCGTCCTCTTCACAGA Antisense: GCACAGCATCCTACCATTCCAA
ACTB	Sense: TGTCCACCTTCCAGCAGATGT Antisense: TGTCACCTTCACCGTTCCAGTT
MBNL1-AS1-shRNA1	Sense: GATCCGAACGAAAGGAGCAGGGTATTTCAAGAG AATACCCTGCTCCTTTCGTTTTTTA Antisense: AGCTTAAAAAAACGAAAGGAGCAGGGTATT CTCTTGAAATACCCTGCTCCTTTCGTTCG
MBNL1-AS1-shRNA2	Sense: GATCCGCCAGAACCTAGTCTCATGTTTCAAGAGA ACATGAGACTAGGTTCTGGTTTTTA Antisense: AGCTTAAAAACCAGAACCTAGTCTCATGTTC TCTTGAAACATGAGACTAGGTTCTGGCG
NC-shRNA	Sense: GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAG AACGTGACACGTTCGGAGAATTTTT Antisense: AGCTAAAAATTCTCCGAACGTGTCACGTTCT CTTGAAACGTGACACGTTCGGAGAAGGG

MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; shRNA, short hairpin RNA; NC, negative control.

one-way ANOVA followed by Tukey's HSD test. $P<0.05$ was considered to indicate statistically significant difference. Graphs were generated by GraphPad Prism 8.0.1 (Dotmatics).

Results

MBNL1-AS1 is involved in the PI3K-AKT signaling pathway and is downregulated in CRC tissues and cell lines. To investigate the potential lncRNAs that regulate the development of CRC, TCGA database was used to explore the altered signaling pathways. Enrichment analysis of KEGG revealed that the PI3K-AKT signaling pathway had the highest number of dysregulated genes among other pathways, with 46 downregulated genes and 26 upregulated genes. Additionally, the Calcium signaling pathway showed 46 downregulated genes in CRC tissues compared with their corresponding expression levels in normal tissues (Table SI, Fig. 1A). Subsequently, the dysregulated lncRNAs implicated in these pathways were examined. Notably, the present analysis revealed that the lncRNA MBNL1-AS1 exhibited significantly high enrichScores in the Calcium, PI3K-AKT and cAMP signaling pathways (Fig. 1B, Table SII), indicating its potential involvement in CRC progression.

Subsequently, the expression levels of MBNL1-AS1 were analyzed in CRC using the TCGA database; the analysis included 620 human CRC tissue samples and 830 normal colorectal tissue samples. The results indicated that MBNL1-AS1 expression was significantly decreased in CRC tissues compared with that noted in normal tissues (Fig. 1C). However, MBNL1-AS1 expression did not correlate with the TNM stage, lymph node metastasis, or distal metastasis of CRC (Fig. 1D-F). Moreover, MBNL1-AS1 expression was also significantly decreased in four CRC cell lines, including SW480, SW620, HCT116, and T84 compared with that noted in the normal epithelial cell line, CD 841 CoN (Fig. S1). Taken together, these results suggested that MBNL1-AS1 expression is decreased in CRC and that it is associated with the PI3K-AKT signaling pathway.

MBNL1-AS1 suppresses CRC cell proliferation in vitro and in vivo. Given that MBNL1-AS1 expression was not altered in the metastatic CRC tissues compared with that noted in the non-metastatic CRC tissues, the effect of MBNL1-AS1 was investigated on the proliferation of CRC cells. Given that SW620 and SW480 cells exhibited the lowest and highest, respectively, expression of MBNL1-AS1 than the other CRC cell lines (Fig. S1), forced expression of MBNL1-AS1 was established in SW620 cells (Fig. 2A); the stable knockdown variant of MBNL1-AS1 was also established in SW480 cells (Fig. 2B). By using CCK-8, colony formation and EdU assays, it was found that the ectopic expression of MBNL1-AS1 significantly inhibited the proliferation of SW620 cells, while knockdown of its expression significantly promoted the ability of SW480 cells to proliferate (Fig. 2C-L). Moreover, both overexpression or knockdown of MBNL1-AS1 expression displayed no apparent impact on cell apoptosis (Fig. 2M-P).

To further evaluate the roles of MBNL1-AS1 on the proliferation of CRC, CRC cells were subcutaneously injected into nude mice. One week after the injection, the xenografts were formed, and their sizes were measured and recorded. A total of four weeks after the injection, the nude mice were sacrificed, and the xenografts were removed (Fig. 3A and D). Ectopic expression of MBNL1-AS1 significantly decreased

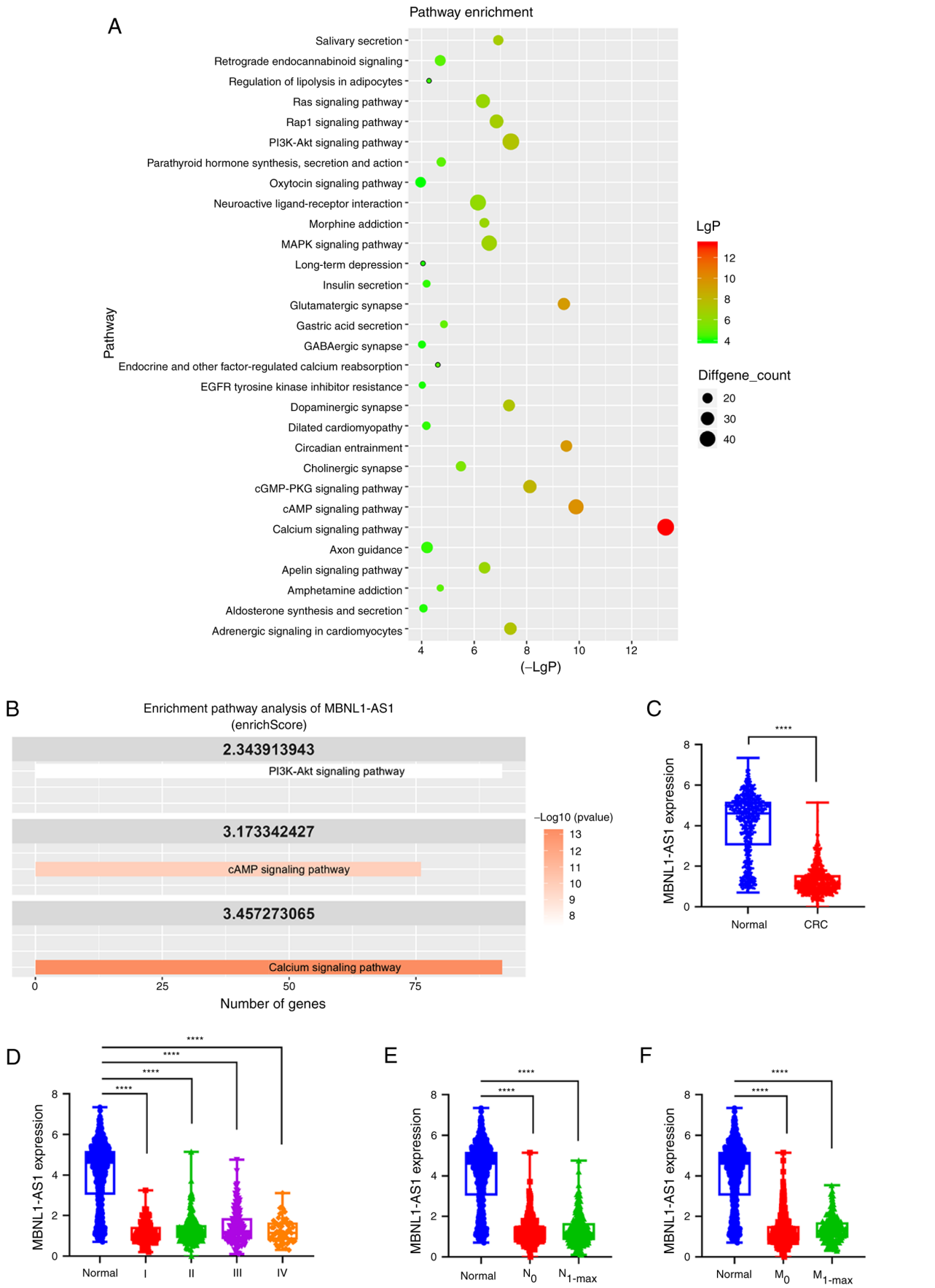

Figure 1. MBNL1-AS1 is involved in the PI3K-AKT signaling pathway and its expression is downregulated in CRC tissues and cell lines. (A) Pathway enrichment analysis in CRC and normal tissues based on the TCGA database. (B) Enrichment Pathway Analysis revealed that MBNL1-AS1 was associated with the Calcium, cAMP and PI3K-AKT signaling pathways. (C) MBNL1-AS1 expression in 620 human CRC tissues and 830 normal tissues based on the TCGA database. (D-F) MBNL1-AS1 expression in CRC tissues with TNM staging, lymph-node metastasis, or distal metastasis. ****P<0.0001. MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; CRC, colorectal cancer; TCGA, The Cancer Genome Atlas.

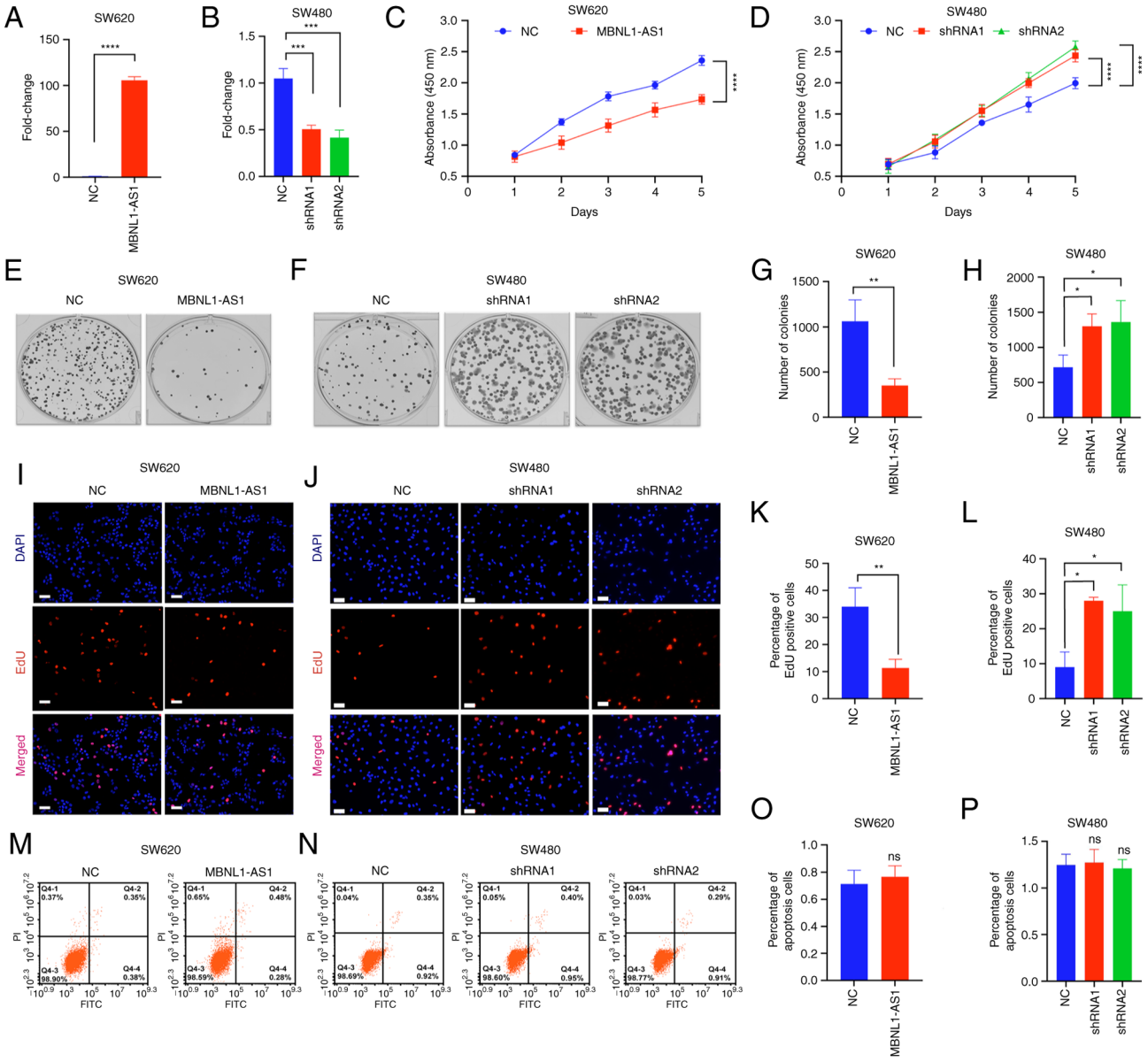

Figure 2. MBNL1-AS1 suppresses CRC cell proliferation *in vitro*. (A and B) MBNL1-AS1 expression in CRC cells which were transfected with the MBNL1-AS1-overexpressing vectors or the shRNA 1 or shRNA 2 sequences of MBNL1-AS1. (C and D) The absorbance of SW620 and SW480 cells as detected by the Cell Counting Kit-8. (E-H) The number of colonies of SW620 and SW480 cells, as detected by the colony formation assay. (I-L) The percentage of EdU positive cells of SW620 and SW480 cells, as detected by the EdU kit. (M-P) The percentage of apoptotic SW620 and SW480 cells, as detected by the Annexin-FITC/PI kit. For A-P, three individual replicates were performed for each experiment. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; CRC, colorectal cancer; EdU, 5-ethynyl-2'-deoxyuridine; shRNA, short hairpin RNA; NC, negative control.

the sizes and weights of the xenografts (Fig. 3B and C), while knockdown of MBNL1-AS1 expression revealed the opposite effects (Fig. 3E and F). Collectively, the results indicated that MBNL1-AS1 acted as a tumor suppressor that inhibited CRC cell proliferation.

MBNL1-AS1 expression is positively correlated with BVES expression in CRC tissues and MBNL1-AS1 promotes the stability of BVES mRNA in CRC cells. To further explore the underlying mechanisms by which MBNL1-AS1 suppresses CRC cell proliferation, co-expression analysis of lncRNAs was performed with mRNAs in CRC tissues based on the TCGA database (Table SIII). The data indicated that BVES and MBNL1-AS1 exhibited the highest correlation coefficient followed by cholinergic receptor muscarinic 2 (CHRM2), a crucial member in the PI3K-AKT signaling pathway (18) (Table SIV). However, the present study focused on BVES instead of CHRM2, since the former has been reported to be involved in the development of CRC. Correlation analysis revealed that BVES mRNA expression positively correlated with MBNL1-AS1 expression in CRC tissues based on the TCGA database (Fig. 4A).

Subsequent studies determined whether MBNL1-AS1 could regulate BVES expression and the data indicated that the ectopic expression of MBNL1-AS1 significantly increased the expression levels of BVES mRNA and protein (Fig. 4B and C), while knockdown of MBNL1-AS1 expression caused a significant decrease in these levels (Fig. 4D and E).

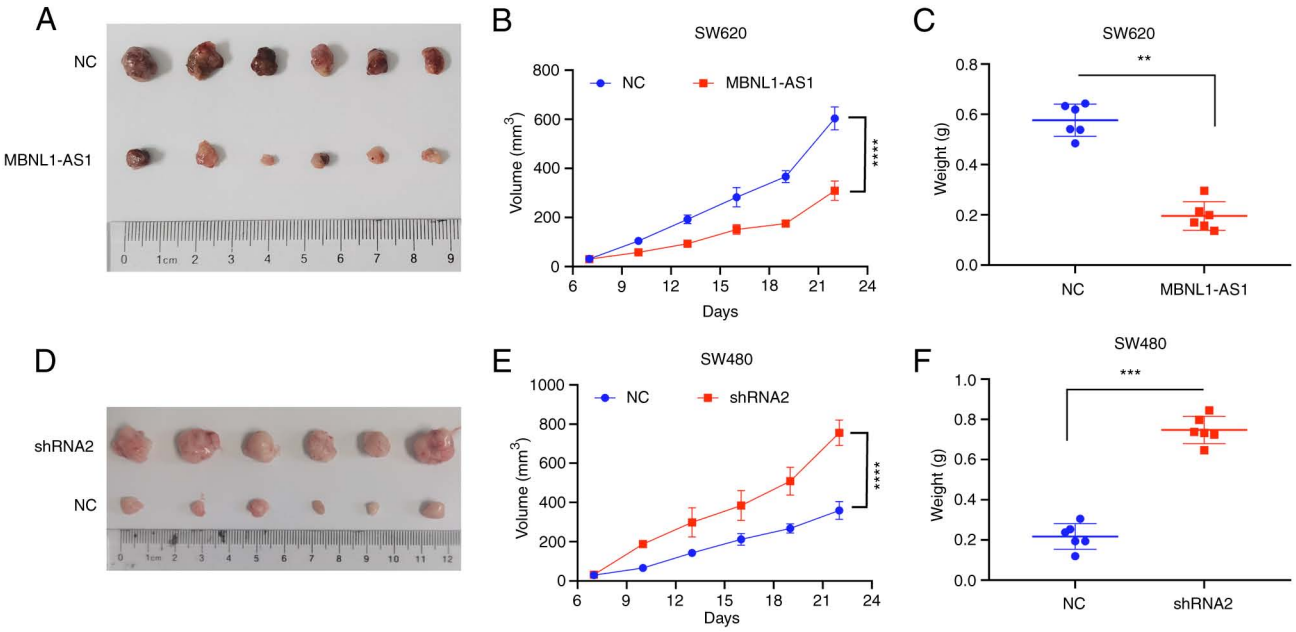

Figure 3. MBNL1-AS1 suppresses colorectal cancer cell proliferation *in vivo*. (A and D, each group included six mice) Representative images indicate the tumor xenografts. (B and E) The volumes of the xenografts are indicated. (C and F) The weights of the xenografts are indicated. **P<0.01, ***P<0.001 and ****P<0.0001. MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; shRNA, short hairpin RNA; NC, negative control.

Then, the forced expression of MBNL1-AS1 was established in SW620 cells (Fig. 4F); the stable knockdown variant of MBNL1-AS1 was also established in SW480 cells (Fig. 4G). However, BVES did not alter MBNL1-AS1 expression in CRC cells (Fig. 4H and I). These data suggested that MBNL1-AS1 was an upstream regulatory factor of BVES. Subsequent studies were performed to assess the mechanism by which MBNL1-AS1 increased BVES mRNA expression. Actinomycin D was used to inhibit RNA transcription in CRC cells and the data indicated that knockdown of MBNL1-AS1 significantly decreased BVES mRNA expression while the ectopic expression of MBNL1-AS1 increased BVES mRNA expression (Fig. 4J and K, respectively), suggesting that it regulated BVES mRNA expression at the post-transcriptional instead of the transcriptional level. Collectively, these results suggested that MBNL1-AS1 increased the stability of BVES mRNA. Moreover, their expression levels indicated a positive correlation in CRC tissues.

MBNL1-AS1 acts as a ceRNA to sponge miR-29b-3p, which directly targets BVES mRNA. A large number of studies have shown that lncRNAs serve as ceRNAs to sponge miRs, which target and regulate the mRNA molecules (19). To explore whether MBNL1-AS1 serves as a ceRNA to sponge miRs, co-expression analysis was performed for MBNL1-AS1, miRs and BVES in CRC tissues based on the TCGA database. The results indicated that miR-29b-3p and miR-29c-3p were candidate miRs (Table SV), and their expression levels were both upregulated in CRC tissues compared with those observed in normal tissues (Fig. 5A and B). TargetScanHuman 7.1 was used to predict the binding sites of miR-29b-3p and miR-29c-3p with BVES mRNA and MBNL1-AS1. The data indicated that the seed sequences of the two miRs were both complementary to the 3,217-3,223 nt of BVES mRNA and to the 1,214-1,222 nt of MBNL1-AS1 (Fig. 5C). Then, the forced expression of miR-29b-3p and miR-29c-3p were established in SW620 cells (Fig. 5D and E). Subsequent experiments demonstrated that miR-29c-3p mimics significantly decreased the mRNA and protein expression levels of BVES, whereas miR-29b-3p mimics exhibited a weaker effect compared with that of miR-29c-3p mimics (Fig. 5F-I). Therefore, the present study focused on miR-29c-3p. Furthermore, luciferase reporter vectors were constructed that contained the binding sites of miR-29c-3p to BVES mRNA and MBNL1-AS1, as well as the corresponding mutant vectors. The results revealed that miR-29c-3p mimics significantly decreased luciferase activity levels in 293 cells transfected with the wild-type vectors compared with those of the mutant vectors (Fig. 5J). Furthermore, a significantly negative correlation was observed between the expression levels of MBNL1-AS1 and miR-29c-3p, and between those of BVES and miR-29c-3p (Fig. 5K and L). Collectively, these results suggested that MBNL1-AS1 increases BVES expression by serving as a ceRNA to sponge miR-29c-3p in CRC cells.

MBNL1-AS1 inhibits CRC cell proliferation by regulating miR-29c-3p/BVES signaling. The present study further determined whether MBNL1-AS1 inhibited CRC cell proliferation by regulating miR-29c-3p/BVES signaling. BVES siRNAs were used to suppress the MBNL1-AS1-mediated increase in BVES expression (Fig. 6A and B). Furthermore, miR-29c-3p mimics also suppressed the MBNL1-AS1-mediated increase in BVES expression (Fig. 6C and D). CCK-8, colony formation and EdU assays indicated that significantly reversed the MBNL1-AS1-mediated decrease in the proliferation of SW620 cells, while miR-29c-3p mimics significantly suppressed the MBNL1-AS1-mediated decrease identified in the proliferation of SW620 cells (Fig. 6E-J) without affecting cell apoptosis (Fig. 6K and L). Collectively, these results suggested that MBNL1-AS1 inhibited CRC cell proliferation by regulating miR-29c-3p/BVES signaling.

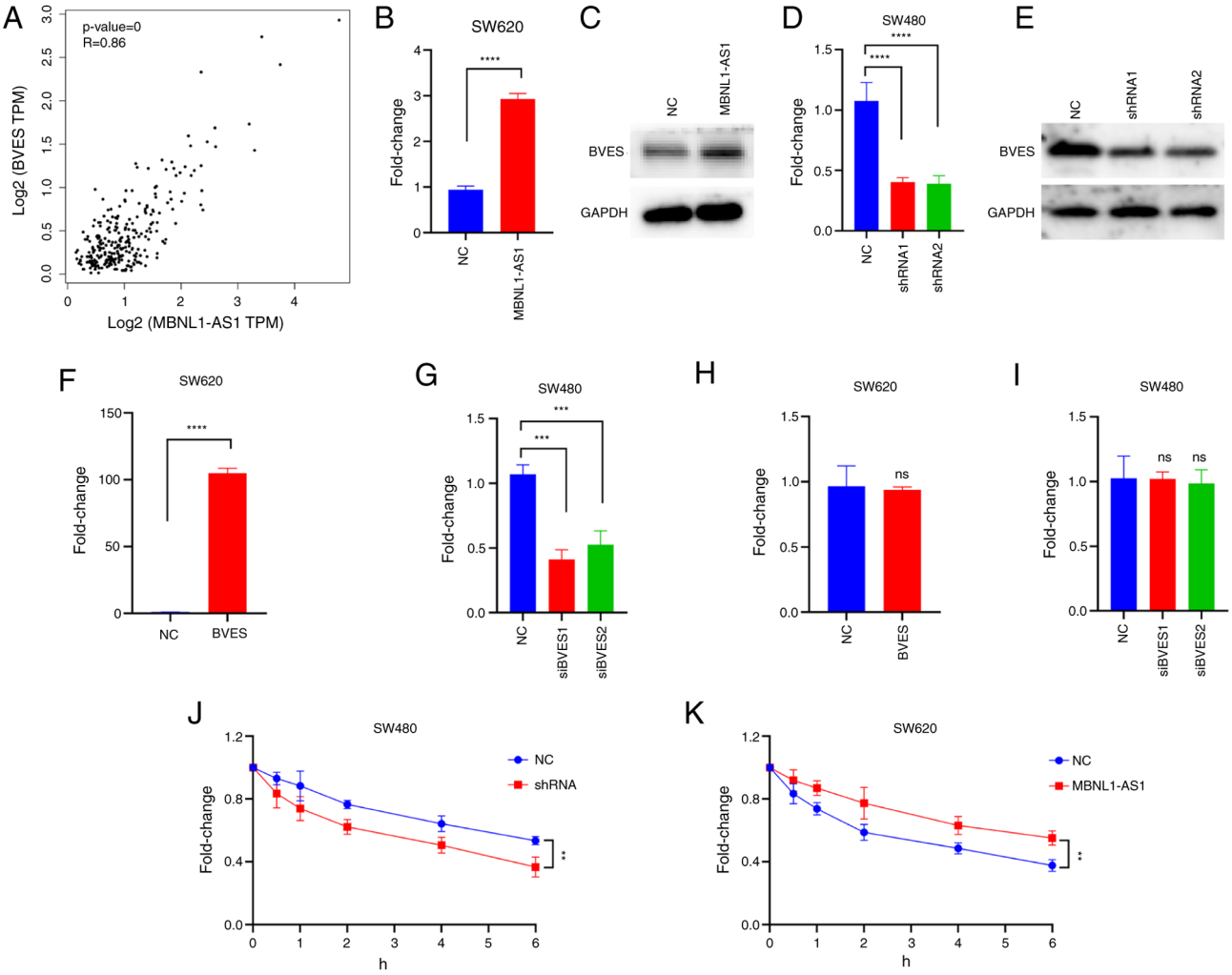

Figure 4. MBNL1-AS1 expression is positively correlated with BVES expression in CRC tissues and MBNL1-AS1 promotes the stability of BVES mRNA in CRC cells. (A) Pearson analysis indicated a significant correlation between the expression levels of BVES mRNA and MBNL1-AS1 in CRC tissues. (B and C) The mRNA and protein expression of BVES in SW620 cells, which were transfected with NC or MBNL1-AS1-overexpressing vectors are demonstrated. (D and E) The mRNA and protein expression levels of BVES in SW480 cells, which were transfected with NC or MBNL1-AS1-inhibiting vectors are indicated. (F and G) The mRNA expression levels of BVES in SW620 cells transfected with NC or BVES-overexpressing vectors or in SW480 cells transfected with NC or the siRNA sequences specific to BVES are revealed. (H and I) The mRNA expression levels of MBNL1-AS1 in SW620 cells transfected with NC or BVES-overexpressing vectors or in SW480 cells transfected with NC or the siRNA sequences specific to BVES are indicated. (J and K) The mRNA expression levels of BVES in SW480 cells transfected with NC or MBNL1-AS1-inhibiting vectors or in SW620 cells transfected with NC or MBNL1-AS1-overexpressing vectors in the presence of actinomycin D are shown. For B-K, three individual replicates were performed for each experiment. **P<0.01, ***P<0.001 and ****P<0.0001. MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; BVES, blood vessel epicardial substance; CRC, colorectal cancer; NC, negative control; siRNA, small interfering RNA; ns, non-significant.

Discussion

MBNL1-AS1 is involved in the development of several types of cancer (11,12,20). However, its roles and the underlying mechanisms are largely unknown in CRC. The present study indicated that MBNL1-AS1 expression was significantly decreased in CRC tissues compared with that observed in normal tissues. Moreover, MBNL1-AS1 suppressed the ability of CRC cells to proliferate. The expression levels of MBNL1-AS1 and BVES mRNA exhibited the strongest correlation in CRC tissues based on the TCGA database analysis and demonstrated that MBNL1-AS1 was an upstream regulatory factor which increased BVES expression in CRC cells. With regard to its mechanism of action, it was observed that MBNL1-AS1 served as a ceRNA to sponge miR-29c-3p, which directly targeted BVES mRNA 3' UTR. Finally, it was demonstrated that MBNL1-AS1 inhibited CRC cell proliferation by regulating miR-29c-3p/BVES signaling. Therefore, these findings suggested that the expression levels of MBNL1-AS1, miR-29c-3p and BVES may be used as potential biomarkers for the diagnosis of CRC. Moreover, the MBNL1-AS1/miR-29c-3p/BVES axis may be a potential therapeutic target for CRC. Also, for further studies, the signaling pathway involved in MBNL1-AS1/miR-29c-3p/BVES axis will be elucidated.

Currently, the TCGA database has accumulated numerous data from tens of thousands of clinical samples. The present study analyzed the aberrant expression of genes associated with the key signaling pathways in 620 CRC tissues and 830 normal colorectal tissues based on the TCGA database, which have been widely used to analyze gene expression in cancer (21,22). KEGG enrichment analysis indicated that 46

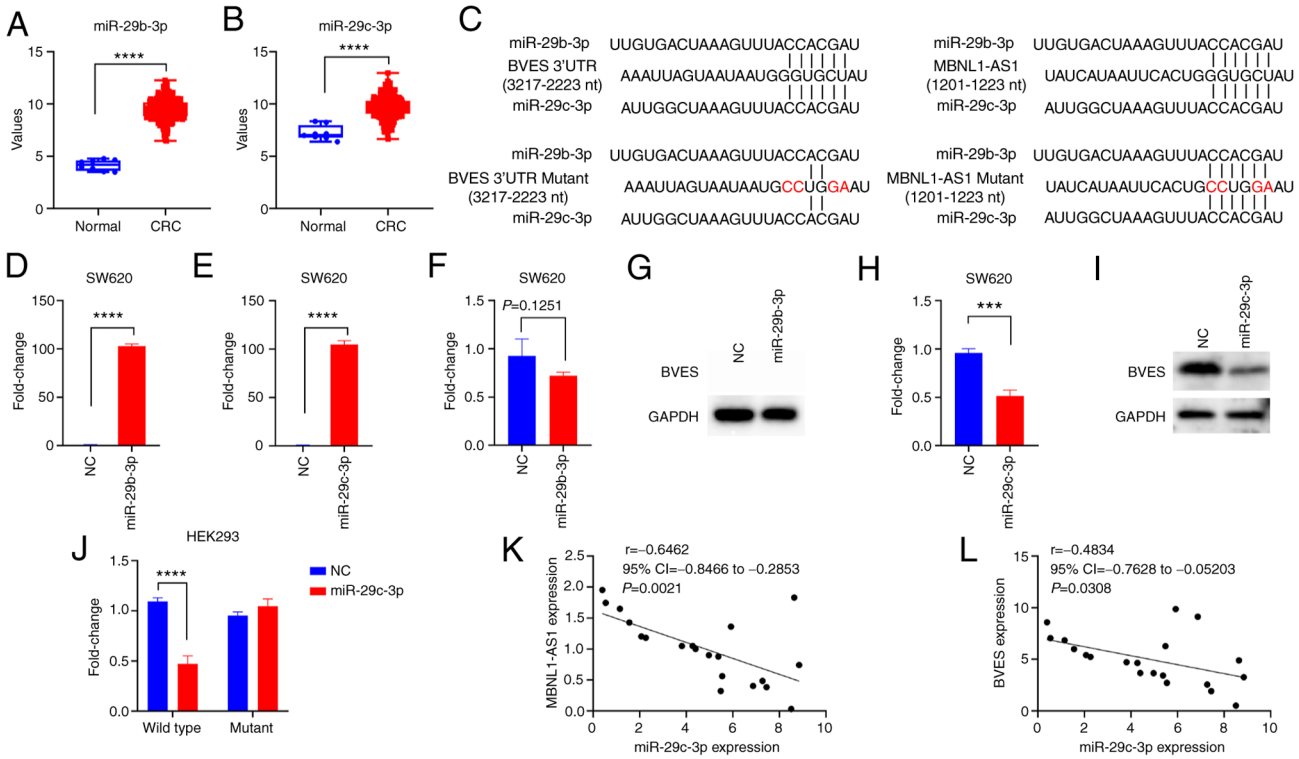

Figure 5. MBNL1-AS1 acts as a ceRNA to sponge miR-29b-3p, which directly targets BVES mRNA. (A and B) The expression levels of miR-29b-3p and miR-29c-3p in normal and CRC tissues derived from The Cancer Genome Atlas database are indicated. (C) The binding sites of BVES 3'UTR or MBNL1-AS1 to miR-29b-3p or miR-29c-3p, and their corresponding mutant binding sites. (D and E) The expression levels of miR-29b-3p or miR-29c-3p in SW620 cells transfected with NC, miR-29b-3p mimics, or miR-29c-3p mimics, are shown. (F-I) The mRNA and protein expression levels of BVES in SW620 cells transfected with NC, miR-29b-3p mimics or miR-29c-3p mimics are revealed. (J) The luciferase activity levels in 293 cells transfected with NC or the luciferase reporter vector containing the binding site of the wild-type or mutant BVES with miR-29c-3p are demonstrated. (K and L) Pearson's correlation analysis indicating negative correlations between the expression levels of miR-29c-3p and MBNL1-AS1 or BVES mRNA in CRC tissues (n=20). For D-J, three individual replicates were performed for each experiment. ***P<0.001 and ****P<0.0001. MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; ceRNA, competing endogenous RNA; miR, microRNA; BVES, blood vessel epicardial substance; CRC, colorectal cancer; siRNA, small interfering RNA; UTR, untranslated region; NC, negative control.

dysregulated genes were involved in the PI3K-AKT signaling pathway and a large number of studies have confirmed that this pathway plays crucial roles in the development of CRC, suggesting that it may be one of the most important pathways involved in the development of this disease (23). Subsequently, the dysregulated lncRNAs involved in this signaling pathway were analyzed and MBNL1-AS1 was identified as the lncRNA with the highest association since it had the highest enrich-Score in the PI3K-AKT signaling pathway. MBNL1-AS1 expression has been shown to be decreased in the majority of cancer types, such as in retinoblastoma (11), prostate (9), lung (24), bladder (25), gastric (20), colon (12) and breast cancers (26). With the exception of the clinical samples in the TCGA database, MBNL1-AS1 expression was detected in human clinical samples and the results demonstrated that it was significantly decreased in CRC tissues compared with that identified in matched normal tissues. Therefore, it was deduced that MBNL1-AS1 expression was downregulated in CRC.

The present findings revealed the highest correlation coefficient between the expression levels of MBNL1-AS1 and BVES mRNA in 620 CRC tissues. BVES has been shown to play important roles in cancer development (13-16,27), including CRC (14). Whether MBNL1-AS1 can regulate BVES expression remains unclear. The findings of the present study demonstrated that MBNL1-AS1 promoted the stability of BVES mRNA, since MBNL1-AS1 upregulation caused an increase in BVES mRNA expression in the presence of actinomycin D. By using the TargetScanHuman 7.1 and the co-expression analysis of the miRs with MBNL1-AS1 or BVES in CRC tissues, it was found that miR-29c-3p may be a potential candidate in the connection between MBNL1-AS1 and BVES. Subsequently, miR-29c-3p was shown to directly target both MBNL1-AS1 and BVES mRNA 3'UTR, suggesting that it increased BVES expression by serving as a ceRNA to sponge miR-29c-3p. Increasing experimental evidence has indicated that miR-29c-3p is upregulated in CRC tissues compared with the corresponding expression noted in normal tissues and that it promotes CRC cell proliferation (28,29). The findings of the present study indicated that MBNL1-AS1 inhibited CRC cell proliferation by regulating miR-29c-3p/BVES signaling. MBNL1-AS1 has been shown to suppress cell proliferation and enhance cell apoptosis by regulating the miR-135a-5p/PHLPP2/FOXO1 axis (25). It has also been revealed to suppress breast cancer progression by modulating the miR-423-5p/CREBZF axis (26). These data suggested that MBNL1-AS1 acts as a tumor suppressor. Additionally, the five-year survival rate of breast cancer patients with high expression of lncRNA MBNL1-AS1 was significantly improved than

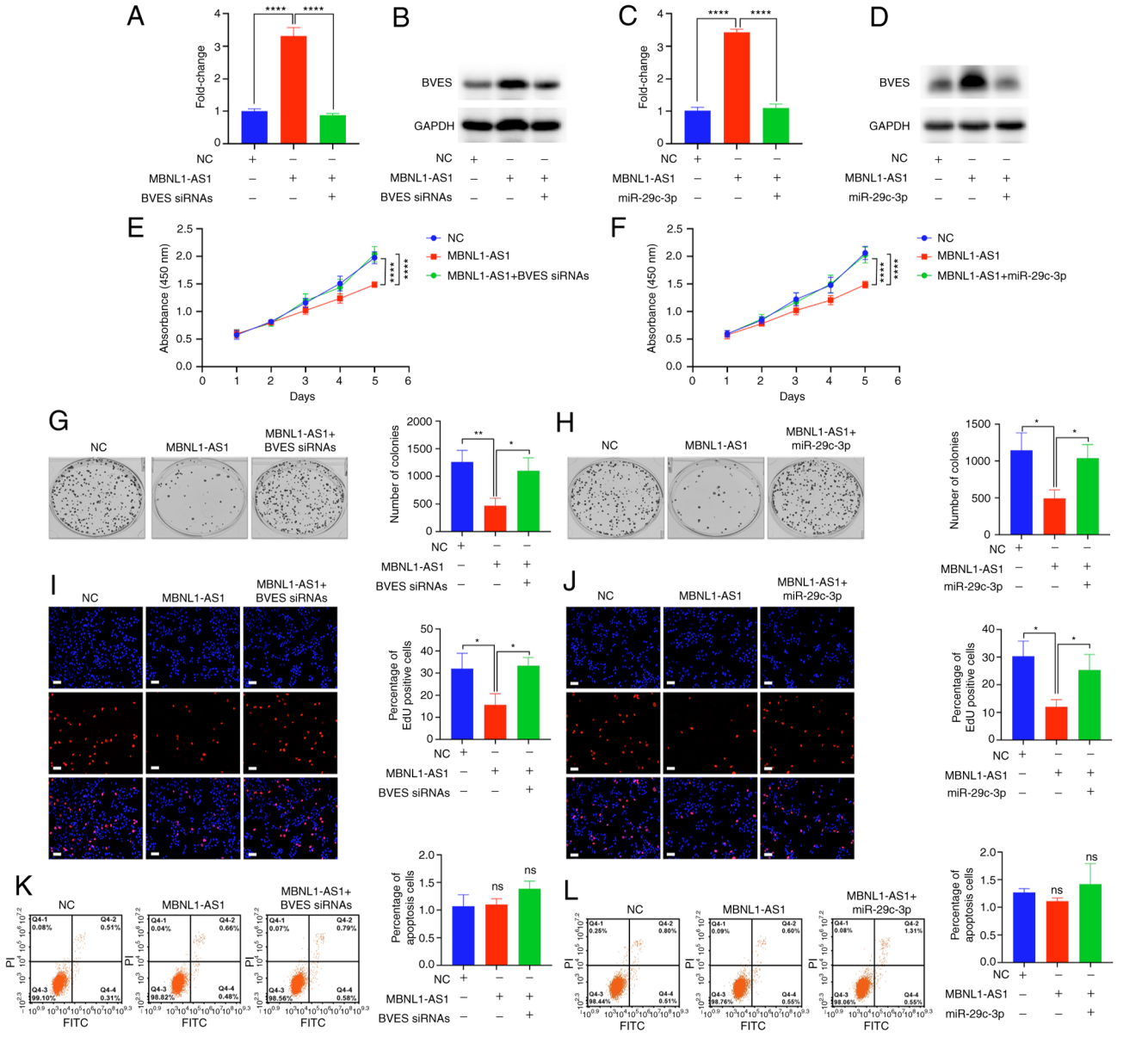

Figure 6. MBNL1-AS1 inhibits colorectal cancer cell proliferation via regulating the miR-29c-3p/BVES signal. (A and B) The mRNA and protein expression levels of BVES in SW620 cells transfected with NC, MBNL1-AS1-overexpressing vectors or co-transfected with MBNL1-AS1-overexpressing vectors and BVES siRNAs are indicated. (C and D) The mRNA and protein expression levels of BVES in SW620 cells transfected with NC, MBNL1-AS1-overexpressing vectors or co-transfected with MBNL1-AS1-overexpressing vectors and miR-29c-3p mimics are shown. (E and F) The absorbance (450 nm) of SW620 cells transfected with NC, MBNL1-AS1-overexpressing vector or co-transfected with MBNL1-AS1-overexpressing vectors and BVES siRNAs, or co-transfected with MBNL1-AS1-overexpressing vectors and miR-29c-3p mimics is demonstrated. (G and H) The number of colonies of SW620 cells transfected with NC, MBNL1-AS1-overexpressing vector or co-transfected with MBNL1-AS1-overexpressing vectors and BVES siRNAs, or co-transfected with MBNL1-AS1-overexpressing vectors and miR-29c-3p mimics is indicated. (I and J) The percentage of 5-ethynyl-2'-deoxyuridine positive cells of SW620 cells transfected with NC, MBNL1-AS1-overexpressing vector or co-transfected with MBNL1-AS1-overexpressing vectors and BVES siRNAs, or co-transfected with MBNL1-AS1-overexpressing vectors and miR-29c-3p mimics is shown. (K and L) The percentage of apoptotic cells of SW620 cells transfected with NC, MBNL1-AS1-overexpressing vector or co-transfected with MBNL1-AS1-overexpressing vectors and BVES siRNAs, or co-transfected with MBNL1-AS1-overexpressing vectors and miR-29c-3p mimics is indicated. For A-L, three individual replicates were performed for each experiment. *P<0.05, **P<0.01 and ****P<0.0001. MBNL1-AS1, muscle blind like splicing regulator 1 antisense RNA 1; miR, microRNA; BVES, blood vessel epicardial substance; siRNA, small interfering RNA; NC, negative control; ns, non-significant.

that of those with low expression, as observed in a cohort of 80 patients (30). However, whether the expression levels of MBNL1-AS1, miR-29c and BVES were associated with the survival rates of patients with CRC requires further investigation.

In conclusion, the data of the current study indicated that the expression levels of MBNL1-AS1 and BVES were downregulated and those of miR-29c-3p were upregulated in CRC tissues compared with those noted in normal tissues, demonstrating that MBNL1-AS1 increased BVES expression by serving as a ceRNA to sponge miR-29c-3p. The latter directly targeted BVES mRNA 3'UTR and MBNL1-AS1. Collectively, the findings revealed that MBNL1-AS1 inhibited CRC cell proliferation by regulating miR-29c-3p/BVES signaling and suggest its application as a potential therapeutic target for CRC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WSC performed all experiments and drafted the manuscript. XZ and ZFZ participated to perform the cell culture and RT-qPCR experiments. XMC designed this study and drafted the manuscript. WSC and XMC confirm the authenticity of all raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. KY2023025) by the Ethics Committee of the Affiliated Hospital of Southwest Medical University (Luzhou, China), and informed consent was obtained from all participants for use of their tissue. Mice experiments were approved (approval no. swmu20230055) by the Ethics Committee of Southwest Medical University (Luzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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