Circular RNA CEP128 promotes bladder cancer progression by regulating Mir-145-5p/MYD88 via MAPK signaling pathway

Ming Sun1, Wenyao Zhao2, Zhaofu Chen1, Ming Li1, Shuqiang Li2, Bin Wu1 and Renge Bu1

1Department of Urology, Shengjing Hospital of China Medical University, Shenyang, Liaoning, China
2Department of General Surgery, Shengjing Hospital of China Medical University, Shenyang, Liaoning, China

The present experiment was designed for exploring the regulatory mechanism of circ-CEP128/miR-145-5p/MYD88 axis in bladder cancer. MiRNAs and circRNAs expression data were derived from Gene Expression Omnibus database with bladder tumor tissues and paracarcinoma tissue samples. Differentially expressed genes in tumor were analyzed via R software. Interaction network of differently expressed miRNAs and differently expressed mRNA was established by means of Cytoscape software. CircCEP128 and miR-145-5p expression levels were determined using qRT-PCR. The expression of MAPK signaling-related proteins MYD88, p38, ERK and JNK was examined by western blot. The relationship between circCEP128 and miR-145-5p was validated using RNA immunoprecipitation. The level of cell propagation and migration was determined by CCK-8 and wound healing assay, 5-bromo-2'-deoxyuridine assay and migration assay. Cell apoptosis rate and cell cycle were detected via flow cytometry. Tumor xenograft assay was implemented to investigate the function of circCEP128 in vivo. CircCEP128 and MYD88 were overexpressed in bladder cancer based on microarray analysis and miR-145-5p was a potential targeting factor in bladder cancer. CircCEP128 targeted miR-145-5p and miR-145-5p targeted MYD88. Expression of miR-145-5p was decreased in cancer samples. Knockdown of circCEP128 induced the inhibition of cell viability and mobility and cell cycle arrest. Overexpression of miR-145-5p or knockdown of circCEP128 promoted MAPK signaling pathway and related proteins expression. In addition, knockdown of circCEP128 suppressed the growth of bladder cancer tumor tissues in vivo. Overexpression of circCEP128 promoted bladder cancer progression through modulating miR-145-5p and MYD88 via MAPK signaling pathway.

Introduction
Bladder cancer represents the second most common neoplasm among urological malignancies and the fourth in general among all neoplastic pathologies for the male gender accounting for ~3% of all cancer-related deaths.1 Roughly 70% of bladder cancer patients are diagnosed with noninvasive bladder cancer that can be physically excised, while the others underwent the hazard of progression to muscle-invasive bladder cancer and metastasis to distant organs.2 Although combined therapeutic approaches such as transurethral electrosurgery of bladder tumors and radical cystectomy with adjuvant chemotherapy postoperation have made tremendous progress, the recurrence rate remains high. Furthermore, common chemotherapy is also accompanied by adverse reaction.3 Therefore, the search for new therapeutic agents is of great importance.

Circular RNAs (circRNAs) are another class of noncoding RNAs that are widely expressed in mammals.4 They have covalently linked ends of a single RNA molecular and appear highly stable comparing to their linear types.5 In the past two decades, they were thought to be functionless owing to errors in splicing.6 To date, plenty of circRNAs have been identified in different cell lines and species. A growing body of previous studies has suggested that circRNA mainly acts as a sponge of mRNA to modulate gene expression and is closely associated with carcinogenesis.7 For instance, cir-ITCH functions as a tumor inhibitor in esophageal squamous cell carcinoma8 and in hepatocellular...
carcinoma, circRNA Cdr1as acts as a tumor promoter through targeting mir-7 expression.\(^9\)

The discovery of noncoding RNA in the human genome changed approaches in cancer research.\(^10\) The dysregulated expression of noncoding, single-stranded RNAs termed microRNAs (miRNAs) have been closely associated with the pathogenesis of human cancers, as miRNAs have been shown to regulate cellular phenomena associated with tumorigenesis, including cellular differentiation, adhesion and apoptosis.\(^11\) MicroRNA (miRNA) is a class of small noncoding RNAs, and they are known to be implicated in the repression or degradation of target RNA transcripts in a sequence-dependent manner.\(^12\) MiRNAs regulated multiple critical biological functions and exerted an important function on cancer progression.\(^13\) Strategies to identify aberrant expression of miRNA-mediated cancer pathways are developing as a new direction in cancer research in the postgenome sequencing era. Such as there was a change in expression of miRNA-27a contributes to cisplatin resistance in bladder cancer through modulating the expression of the SLC7A1\(^14\) and miR-133b expressed low level in bladder cancer.\(^15\)

Herein, we delved into the role of miR-145-5p as it was significantly downregulated in bladder cancer cells,\(^16\) but the interaction mechanism of circCEP128 and miR-145-5p was still unclear.

The pathogenesis of bladder cancer appears closely linked to distinct molecular pathways.\(^17\) Mitogen-activated protein kinase (MAPK) family members are crucial intracellular signaling molecules in various cellular processes, including proliferation, migration, invasion and apoptosis.\(^18\) MAPK signal pathways play vital roles in the carcinogenesis of bladder cancer.\(^19\)

However, it remains undefined whether circCEP128 can regulate MAPK signal pathways in bladder cancer.

To detect microarray differential expression and to better interpret findings, gene-class testing or pathway analysis has become increasingly popular published a sophisticated method, called “Gene Network Enrichment Analysis”, which is similar to standard “GSEA” and applies hypothesis testing to evaluate pathways.\(^20\) We utilized Gene Expression Omnibus database (GEO), TargetScan, miRanda and GSEA to predict the related circRNAs, miRNAs and genes. What’s more, we analyzed with Gene Ontology and KEGG Pathway to rank significant pathways after enrichment. Our study was aimed at figuring out the relationship of circCEP128, miR-145-5p and MYD88 in bladder cancer and how they influenced cellular differentiation, cell viability and mobility and apoptosis via MAPK signaling pathway.

### Materials and Methods

#### Gene expression profiles

Gene expression data were derived from GEO (https://www.ncbi.nlm.nih.gov/geo/). CircRNAs were analyzed with GSE92675 on GPL19978 platform including four bladder tumor tissues and four paracarcinoma tissues. There were 3,423 circRNAs in GSE92675 and 433 circRNAs were displayed differential expression level among these circRNAs. The raw data was displayed in Supporting Information Table S1. MiRNAs were analyzed with GSE40355 on GPL8227 including eight paracarcinoma tissues and eight bladder cancer tissues (Bladder_HG1-8). There were 34,172 mRNAs in GSE40355 carried out the differential expression analysis and 3,430 mRNAs were displayed differential expression level. The raw data was displayed in Supporting Information Table S2. Heatmap was generated using heatmap package and p-value <0.05 (adjusted by BH), |log2 (foldchange)| >1 of expression level.

#### MiRNA predictions and correlation network analysis of miRNAs and mRNAs

StarBase (http://starbase.sysu.edu.cn/index.php) was used to predict the miRNAs targeted by circRNAs and Targetscan and miRanda were used to predict miRNAs targeted by miRNAs. Venn diagram manifested the intersection of predicted miRNAs and differently expressed miRNAs. Pearson correlation analysis was used with package “psych” R v.3.4.1 to investigate the correlations among differentially expressed (DEGs) of miRNAs and DEGs of mRNA in MAPK signaling pathway, relevance requirement >0.9 and p-value <0.05. The networks were constructed by means of Cytoscape software version 3.5.1.

#### Functional annotation and pathway enrichment analysis of DEGs

The functional annotation of DEGs was confirmed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v.6.8. In order to analyze enrichments of DEGs in KEGG, GO_Biological Process (BP), GO_Cellular Component (CC) and GO_Molecular Function (MF), we used DAVID (https://david.ncifcrf.gov/), while p-value cutoff <0.05 represented statistical significance.

#### Gene set enrichment analysis

KEGG database was utilized for gene set enrichment analysis (GSEA). The expression data of total normalized mRNAs were uploaded to GSEA version 3.0 software, followed by KEGG pathway...
enrichment analysis. GSEA for genes in KEGG_MAPK_signaling pathway were carried out using GSEABase package. The pathways of the KEGG and GO were obtained from the online website DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/) according to DEGs. The dotplot and joyplot were rendered using ggplot2, DOSE, clusterProfiler and ggjoy packages with R version 3.4.1 based on GSEA (p-value < 0.05).

**Human bladder tissue samples**

Human bladder tissue specimens and blood samples were collected from 40 bladder cancer patients undergoing surgery between March 2016 and October 2016 at Shengjing Hospital of China Medical University. All tissues were histologically confirmed by pathologists, and this experiment was authorized by Shengjing Hospital of China Medical University and obtained informed consent from all the patients.

**Cell culture**

Normal human uroepithelium cell line SV-HUC-1 and human urinary bladder cancer cell lines 293T, J82 and T24 were bought from BeNa Culture Collection (Beijing, China). T24 cell line was cultured in RPMI 1640 (Gibco BRL, Paisley, UK) with 10% fetal bovine serum (FBS). SV-HUC-1 and 293T cells were cultivated in Dulbecco’s Modified Eagle Medium containing 10% FBS (Gibco BRL). J82 cell was fostered in Eagle’s minimum essential medium (MEM) supplemented with 10% FBS. All cells were cultured in a humidified atmosphere with 5% CO2 at 37°C.

**Cell transfection**

Small interfering RNA of circCEP128 (si-circCEP128: ATGAGA TTTGGATTTGTGTAC), miR-145-5p mimics, miR-145-5p inhibitor, small interfering RNA of MYD88 (si-MYD88), NC were purchased from Sangon Biotech (Shanghai) Co., Ltd. Si-circCEP128, miR-145-5p mimics, miR-145-5p inhibitor and si-MYD88 were respectively transfected into T24 cells using the Lipofectamine 3000 reagent (Life Technologies Inc., Carlsbad, CA) following the instructions. Transfection efficiency of cells was observed after 1–2 days.

**QRT-PCR**

Total RNA was isolated from the cells using TRIzol reagent (Gibco BRL). RNase-R treatment was carried out at 37°C using RNase-R (Epicient) 2 U/mg for 15 min. Treated RNA was directly reverse transcribed on the RETROscript System (Thermo Fisher Scientific, Waltham, MA) with random primers. Reverse transcription of RNA into cDNA was implemented using using TaqMan™ Advanced miRNA cDNA Synthesis Kit (#A28007, ThermoFisher Scientific (China) Inc.). Then 2 μl cDNA was subjected to PCR using an UltraSYBR Mixture (#CW0957, CWBiotech, China). The primers were produced by Shanghai Sangon Biotech (China). GAPDH or U6 was used as an internal control. The relative expression was calculated using 2^−ΔΔCt method. All experiments were set with three parallel experiments. Primers used in qRT-PCR showed in Supporting Information Table S3.

**Western blot assay**

We extracted total proteins from lysed cells using RIPA buffer (R0010, Beyotime, Shanghai, China) with 1% phenylmethylsulfonyl fluoride. About 600 μg proteins were electrophoretically segregated and transferred onto a polyvinylidene fluoride membrane (#FP36, Beyotime). The primary antibodies (anti-MYD88: 1:1000, #ab2064; anti-p-MYD88: 1:1000, #ab31828; anti-p-p-MYD88: 1:1000, #ab47363; anti-MYD88: 1 μg/ml, #ab54230; anti-p-MYD88: 1:1000, #ab65142; anti-JNK: 1:1000, #ab179461; anti-p-JNK: 1:1000, #ab124956; anti-GAPDH: 1:2500, #ab9485; Abcam, Cambridge, UK) were attenuated using TBST and blocked by pathologists, and this experiment was authorized by Shengjing Hospital of China Medical University and obtained informed consent from all the patients.

**Flow cytometry analysis of apoptosis and cell cycle**

Cell suspension was supplemented with 5 μl Annexin V-FITC and 5 μl PI, followed by incubation for 20 min. Then cell apoptosis was observed by flow cytometry (FCM) after 48 hr. We detected with Annexin V-FITC Apoptosis Detection Kit (#C1062, Beyotime Biotech, Shanghai, China). Cell cycle assay was performed with Cell Cycle and Apoptosis Analysis Kit (#C1062, Beyotime Biotech, Shanghai, China). 0.5 ml per tube cell sample iodide organism dye was added and avoid light warm bath for 30 min at 37°C, and then deposited ice bath away from light. The flow test should be completed within 24 hr after finishing dyeing. All experiments were set with three parallel experiments.

**Dual luciferase reporter gene assay**

The 293T cells were cultivated in 6-well plates. PGL3-MYD88-3’UTR-wt or pGL3-MYD88-3’UTR-mut were constructed by inserting wild-type and mutated MYD88-3’UTR sequences into pGL3 vectors. PGL3-circCEP128-wt or pGL3-circCEP128-mut was constructed by inserting wild-type and mutated circCEP128 sequences into pGL3 vectors. The constructs were cotransfected with miR-145-5p mimics or NC. The fluorescence activities of cells were detected 2 days later.

**RNA immunoprecipitation**

Biotin-labeled circCEP128 probe: 5’-GAAGGTCACCAACCCTT TTATTTC-3’ was synthesized by Sangon Biotech and the RIP assay which was performed as previously described by minor modification. T24 cells were fixed for 10 min with 1% formaldehyde, and then they were lysed and ultrasonicated. Approximately 50 μl supernatant was retained after centrifugation, and we incubated the remaining part with circCEP128 specific probes-streptavidin dynabeads (Invitrogen, Carlsbad, CA), mixing overnight at 30°C. Next day, washing the dynabeads-probes-circRNAs mixture and incubating with 200 μl lysis buffer were accomplished, and the formaldehyde crosslinking was reversed by proteinase K. In the last, the mixture was added with TRIzol in order to extract and detect RNA.
5-Bromo-2’-deoxyuridine cell proliferation assay
Cells were labeled with 5-bromo-2’-deoxyuridine (BrdU; #B5002-100MG, Sigma) which was a nucleotide analog of thymidine. The culture was pulse-labeled for 30 min by administering BrdU (100 μM/ml) and incubating at 37°C. The culture was then washed thoroughly with 1× PBS before fixing it with 4% paraformaldehyde, followed by addition of anti-BrdU antibody (#ab6326, Abcam) and DAPI dye solution. Then cells were rinsed in PBS thrice. BrdU positive cells were observed through fluorescence microscopy.

Cell count kit-8 assay
Cell counting kit-8 (#40203ES60, Yeasen, Shanghai, China) was used for cell viability measurement. About 100 μl cell suspensions (2 × 10^4 cells/ml) with 10% FBS medium was seeded to a 96-well plate and incubated for 5 days at 37°C. Then, 10 μl of CCK-8 was supplemented into each well at the indicated time and incubated for 3 hr at 37°C. Absorbance was evaluated at 450 nm through a Rayto-6000 system (Rayto, China).

Wound healing assay
After cultured in a six-well plate for 1 day, the confluent monolayer of cells was formed and scratched using a 200-μl sterile pipette tip. Having been rinsed in PBS, cells were incubated in serum-free RPMI1640 medium, and then the wound healing area was photographed at 0 and 48 hr using a microscope (200×, Olympus).

Nude mouse tumorigenicity assay
Twelve male nude mice (4-weeks-old) were bought from Shanghai SLAC Experimental Animal Center (China). These male nude mice divided into two groups equally (NC, si-circCEP128). Six mice were injected with T24 cells (2 × 10^6 cells) transfected with si-circCEP128 at right limb. The rest were injected with T24 cells through at right limb. After 35 days, then all the mice were sacrificed, and the tumors were excised and weighted. The tumor volumes were measured every 4 days from the seventh day. The tumor volume was measured following the formula of (length × width^2)/2.

Immunohistochemical staining
Approximately 4% paraformaldehyde dehydrated in graded ethanol was utilized to immobilize the mice tumor tissues, which was then embedded in paraffin. Having been sectioned and stained using a 200-ml sterile pipette tip. Having been rinsed in PBS, cells were incubated in serum-free RPMI1640 medium, and then the wound healing area was photographed at 0 and 48 hr using a microscope (200×, Olympus).

Results
Differentially expressed circRNAs and miRNAs in bladder cancer tumor tissues and paracarcinoma tissues
Differential expressed circRNAs was obtained based on GSE92675, Figure 1a showed the top 10 upregulated and downregulated circRNAs in bladder cancer tumor tissues and paracarcinoma tissues, including circCEP128 overexpressed in bladder cancer. While Figure 1b showed the top 10 upregulated and downregulated miRNAs in bladder cancer tumor tissues and paracarcinoma rely on GSE40355, miR-145-5p was suppressed in bladder cancer. Through starbase, a bioinformatics prediction website, we entered circCEP128 into starbase and search the potential targeting miRNAs, Figure 1c presented a pattern diagram of miRNAs, which owned a targeted relationship with circCEP128. In addition, the intersection of miRNA predictions and differently expressed miRNA was revealed via the Venn diagram, through the intersecting 242 possible miRNAs (obtained from the local computing server by integrated code) and 29 differential miRNAs (confirmed from the differential expressed miRNAs analysis), two common miRNAs (miR-145-5p and miR-494) were identified (Fig. 1d). Furthermore, we found that there existed some binding sites of miR-145-5p on circCEP128, and the binding sites of miR-145-5p on 3’ UTR of circCEP128 were showed in Figures 1e and 1f. And then, in terms of GO_Biological Process (GO_BP), GO_Cellular Component (GO_CC), GO_Molecular Function (GO_MF) and KEGG, we ranked top 10 pathways after enrichment analysis for further study on bladder cancer (Supporting Information Fig. S1).

MAPK pathway was upregulated in bladder cancer
The activated and suppressed KEGG pathways were exhibited in dotplot and joynplot. The dotplot displayed that the MAPK signaling pathway was one of the significantly activated pathways in bladder cancer (Fig. 2a). The joynplot also indicated the remarkable activation of MAPK signaling pathway in bladder cancer (Fig. 2b). Having crosschecked the consequences, we narrowed down our interesting pathways into a mutual option. GSEA enrichment plot displayed that many genes of MAPK signaling pathway were identified in the region, where most were highly expressed in bladder cancer (Fig. 2c). Heat map showed the top 10 upregulated and downregulated mRNAs in bladder tumor tissues and paracarcinoma tissues, including MYD88 overexpressed in bladder cancer (Fig. 2d). In addition, the network of interactions between miRNAs and mRNAs showed that miR-145-5p was negatively
associated with MYD88 compared to miRNAs associated with differently expressed gene (DEGs) in bladder cancer (Supporting Information Fig. S2a). Meanwhile, we also discovered that there existed some binding sites of miR-145-5p on 3’UTR of MYD88 (Supporting Information Fig. S2b).

Circ-CEP128 was significantly upregulated in bladder cancer tissues and blood

QRT-PCR revealed that circCEP128 was remarkably upregulated in bladder cancer (p < 0.01, Supporting Information Figs. S3a and S3b). The expression of circCEP128 was overexpressed in high tumor differentiation stage of bladder cancer (p < 0.05, Supporting Information Figs. S3b and S3f). What’s more, circCEP128 was overexpressed in III–IV T stage of bladder cancer (p < 0.01, Supporting Information Figs. S3c and S3g). Likewise, circCEP128 also presented considerably high expression in N2–N3 lymph node metastasis stage of bladder cancer (p < 0.01, Supporting Information Figs. S3d and S3h). As a result, circCEP128 was observably upregulated train bladder cancer. Data of correlation between clinicopathologic characteristics and circCEP128 expression in cancer tissues and blood showed in Supporting Information Table S4.

Circ-CEP128 and MYD88 were overexpressed while miR-145-5p expression was suppressed in bladder cancer

MiR-145-5p was significantly down-regulated while MYD88 expression was remarkably increased in bladder cancer as confirmed
by qRT-PCR \((p < 0.01, \text{Figs. 3a and 3b})\). Additionally, we also found that circCEP128 and MYD88 were highly expressed while miR-145-5p was low expressed in bladder cancer \((p < 0.01, \text{Fig. 3c})\). Meanwhile, circCEP128 was the most expressed and miR-145-5p was the least expressed in T24 compared to other bladder cancer cell lines, so we chose T24 for next experiments. In addition, to further confirm the characteristics of circCEP128, we used a highly processive 3’ to 5’ exoribonuclease (RNase R enzyme) that does not act on circRNAs but linear RNAs. As expected, circCEP128 was resistant to RNase treatment in contrast to GAPDH as shown in Figure 3d. Expression of p-p38, p-ERK and p-JNK were upregulated in T24 compared to SV-HUC-1 detected by western blot \((p < 0.01, \text{Fig. 3e, Supporting Information Fig. S5a})\). MYD88 was overexpressed and inhibited miR-145-5p in bladder cancer. By RIP experiments, we found a specific enrichment of circCEP128 and miR-145-5p as compared to the controls and it meant that circCEP128 binds to miR-145-5p \(\text{(Fig. 3f)}\). Dual-luciferase reporter assay also validated the
targeted relationship between miR-145-5p mimics to the MYD88 since the luciferase activity of MYD88 WT was notably repressed after transfection with miR-145-5p mimics ($p < 0.05$, Fig. 3g).

Besides that, silencing circCEP128 significantly decreased the expression of circCEP128, while increased miR-145-5p expression ($p < 0.01$, Figs. 3h and 3i).

Figure 3. Legend on next page.
Silencing circCEP128 repressed MYD88 and related proteins in MAPK pathway by regulating miR-145-5p

Expression of MYD88 was downregulated after knockdown of circCEP128 or transfection of miR-145-5p mimics while enhanced by miR-145-5p inhibitor. What’s more, there was no difference after cotransfection of si-circCEP128 and miR-145-5p inhibitor compared to NC group (p < 0.01, Supporting Information Fig. S4a). Meanwhile, western blot assay revealed that knockdown of circCEP128 or overexpression of miR-145-5p repressed the expressions of MYD88 and related proteins including p-p38, p-ERK and p-JNK (p < 0.01, Supporting Information Figs. S4b and S5b). MYD88 was markedly downregulated after transfection with Si-MYD88 (p < 0.01, Supporting Information Fig. S4c).

Knockdown of MYD88 was also found to significantly suppress the levels of p-p38, p-ERK and p-JNK (p < 0.01, Supporting Information Figs. S4d and S6a). Taken together, silence of circCEP128 repressed MYD88 and related proteins in MAPK pathway via regulation of miR-145-5p.

Silencing circCEP128 restrained bladder cancer cell viability and mobility while accelerated cell apoptosis and induced cell cycle arrest

BrdU staining, wound healing assay and migration experiments showed that downregulation of miR-145-5p significantly promoted migration ability of T24 cells, while knockdown of circCEP128 notably inhibited the cell migration ability (p < 0.01, Figs. 4a–4c, 4e and 4f). CCK8 assay indicated knockdown of circCEP128 significantly inhibited the growth of T24 cells and the inhibition of miR-145-5p promoted the growth of T24 cells compared to NC group (p < 0.05, Fig. 4d).

Additionally, FCM assay displayed that knockdown of circCEP128 and overexpression of miR-145-5p arrested more cells at G0/G1 phase, while miR-145-5p inhibitor reduced the number of cells on G0/G1 stage and allowed more cells to enter the S stage (p < 0.01, Figs. 5a and 5c). Moreover, the cell apoptosis rate was remarkably enhanced after transfection with si-circCEP128 or miR-145-5p mimics, whereas the apoptotic ratio of cells transfected with miR-145-5p inhibitor notably decreased. After transfection of si-circCEP128 into miR-145-5p inhibitor group, the apoptosis rate of cells observably increased (p < 0.01, Figs. 5b and 5d). Overall, knockdown of circCEP128 accelerated cell apoptosis and induced cell cycle arrest.

Silencing circCEP128 restrained tumor growth in bladder cancer through modulating miR-145-5p and MYD88

We randomly divided the mice into two groups, which were respectively injected with T24 cells transfected with si-circCEP128 and those transfected with NC. We found that after several weeks, tumor volume and tumor weight of mice in the si-circCEP128 group were notably reduced compared to the NC group (p < 0.01, Figs. 6a–6c). Immunohistochemical analysis demonstrated that when circCEP128 was downregulated, Ki-67 in si-circCEP128 group were significantly suppressed (p < 0.01, Figs. 6d and 6e). Knockdown of circCEP128 decreased the expression of MYD88 while increased the expression of miR-145-5p in tumor tissues compared to NC group (p < 0.01, Fig. 6f). Taken together, knockdown of circCEP128 exerted suppressive effects on tumor growth via regulation of miR-145-5p/MYD88.

Discussion

In this work, circCEP128 was overexpressed in bladder cancer while miR-145-3p was suppressed in bladder cancer. Furthermore, circCEP128 could sponged and downregulated miR-145-5p, thereby upregulating MYD88 and downstream proteins in MAPK signaling pathway. In addition, knockdown of circCEP128 inhibited the proliferation, apoptosis and cell cycle of bladder cancer cells and the growth of tumor in vivo. As a result, circCEP128 facilitated bladder cancer progression by regulating miR-145-5p/MYD88 via MAPK signaling pathway.

Numerous studies have confirmed the abnormal expression levels of some circRNAs and miRNAs in diverse types of cancers. For instance, hsa_circ_0013958 was confirmed to be upregulated in all of the LAC tissues, cells and plasma. Circ-FBXW7 was abundantly expressed in the normal human brain, and circ-FBXW7 expression positively associated with glioblastoma patient overall survival. In our study, circCEP128 was abundantly expressed in bladder cancer. What’s more, they found that the miR-143/-145 cluster was lowly expressed in all stages of bladder tumor. In another study, analysis of miRNA expression signature of bladder cancer by deep-sequencing revealed that miR-145-5p and miR-145-3p were significantly downregulated in bladder cancer tissues. Elevated levels of miR-143 and miR-145 were observed in tumors of higher stage and grade. These studies coincided with the result of our study in which miR-145-5p was low expression in bladder cancer.
Many studies had confirmed that circRNAs were proposed to harbor miRNAs and were found to be enriched with functional miRNA binding sites and ectopic expression of circRNAs and miRNAs could make effects on cancer cell functions. Li et al. found that circHIPK3 exerted inhibitory influence on human invasive bladder cancer T24T and UMUC3 cells by regulating miR-558.27
Additionally, previous researches revealed that circRNA-MYLK functioned as an endogenous sponge for miR-29a in bladder cancer. Whereas circRNA-MYLK knockdown decreased cell proliferation, motility and induced apoptosis. Herein, circCEP128 served as a sponge for miR-145-5p, further increased cell proliferation, motility and inhibited apoptosis.

Activation of MAPK signaling pathway was a frequent event in tumor progression and metastasis. In our study, MAPK signaling pathway and downstream proteins were upregulated in bladder cancer. With the development of science, more and more studies found that miRNAs and signaling pathways interacted in many kinds of cancers. In the present study, they confirmed that the MAPK signaling pathway in T24 cells were synergistically repressed by the cotreatment with miR-143/-145. Similarly,
through targeting NRAS, miR-145-5p could suppress cell functions by inhibiting MAPK pathways. These were the same exploration as us that miR-145-5p inhibited MYD88 and related proteins in MAPK signaling pathway.

In summary, circCEP128 and MYD88 were up-regulated, while miR-145-5p was lowly expressed in bladder cancer. Silence of circCEP128 and overexpression of miR-145-5p could inhibit bladder cancer cell viability and mobility and stimulated cell apoptosis. CircCEP128 acted as a sponge of miR-145-5p to modulate the expression level. In general, our findings further unraveled the mechanism of circCEP128/miR-145-5p/MYD88 axis in bladder cancer and provided novel therapeutic targets for the treatment of bladder cancer.

Author contributions
MS, WZ, SL and RB contributing to the conception and design; ZC, ML and BW analyzing and interpreting data; MS drafting the article; WZ, ML and BW revising it critically for important intellectual content; all authors approving the final version to be published.

Ethics approval and consent to participate
All tissues were histologically confirmed by pathologists, and this experiment was authorized by Shengjing Hospital of China Medical University and obtained informed consent from all the patients.

Availability of data and material
Human bladder tissue specimens and blood samples were collected from 40 bladder cancer patients undergoing surgery between March 2016 and October 2016 at Shengjing Hospital of China Medical University.

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Figure 6. Influence of circCEP128 and miR-145-5p on tumor growth. (a) Tumor tissues of mice in the NC group and si-circCEP128 group. (b, c) Silencing circCEP128 resulted in reduction of tumor volume and weight. (d, e) Ki-67 positive cells were detected by immunohistochemistry and analyzed with Image-ProPlus 4.5 software. (f) The expression level of circCEP128, miR-145-5p and MYD88 in bladder cancer cell line transfected with si-circCEP128. **p < 0.01, compared to NC group. [Color figure can be viewed at wileyonlinelibrary.com]
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