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Original Article

lncRNA TUG1 Promotes Cisplatin Resistance by Regulating CCND2 via Epigenetically Silencing miR-194-5p in Bladder Cancer

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Taurine-upregulated gene 1 (TUG1) has been involved in tumorigenesis of several human cancers, but its precise biological role in bladder cancer remains largely elusive. In this study, we found that TUG1 was upregulated in bladder cancer and the expression of TUG1 was positively and negatively correlated with CCND2 and miR-194-5p, respectively. MiR-194-5p expression was frequently decreased through promoter hypermethylation, while it was epigenetically increased following cisplatin and 5-aza-2′-deoxycytidine (5-Aza-DC) treatment. Furthermore, knockdown of TUG1 attenuated the expression of epigenetic regulator Enhancer of zeste homolog 2 (EZH2), and it alleviated the promoter hypermethylation of miR-194-5p and induced its expression. Increased miR-194-5p expression or decreased TUG1 expression significantly sensitized bladder cancer cells to cisplatin, inhibited the proliferation, and induced apoptosis. Besides, CCND2 was a direct target of miR-194-5p, while miR-194-5p was regulated by TUG1. CCND2 could partially restore the tumor-suppressive effects on cell proliferation and cisplatin resistance following TUG1 silencing. Additionally, TUG1 expression was correlated with clinical stage, lymphatic metastasis, and patient prognosis. In conclusion, TUG1 promotes bladder cancer cell growth and chemoresistance by regulating CCND2 via EZH2-associated silencing of miR-194-5p. Our study may be conducive to elucidating the molecular mechanism of and providing novel therapeutic target and biomarker for bladder cancer.

INTRODUCTION

Bladder cancer is one of the most common urinary malignancies in both China and worldwide, and its incidence has been increasing in recent years.[1] The predominant pathological type of bladder cancer is transitional epithelial carcinoma, while non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) account for approximately 70% and 30% of bladder cancer in clinical stage, respectively.[2] Generally, transurethral resection of bladder tumors was considered to be the standard therapy of NMIBC, while radical cystectomy and pelvic lymph node dissection was imperative to treat MIBC. Unfortunately, approximately 30% of MIBC patients have distant metastases or lymph node involvement when diagnosed, which lowers the five-year survival rate after radical cystectomy to only about 50%.[3]

The unsatisfactory prognosis of this population highlights the importance of early diagnosis and effective management of advanced diseases. Apart from the surgical procedures, chemotherapy also occupies a critical position for the comprehensive therapy for advanced bladder cancer. One of the agents for chemotherapy, cisplatin, was shown to significantly improve the 5-year survival rate of sensitive patients, although only half of the patients showed a positive response to cisplatin.[4] Although recent years have witnessed a series of etiological signaling, therapeutic targets, and promising biomarkers for facilitating the clinical management,[5] a panorama of the pathogenesis of bladder cancer and feasible treatment schemes for bladder cancer are still lacking. Therefore, further research to develop new therapeutic agents or biomarkers for bladder cancer is warranted, as well as efforts to improve the efficiency of existing therapies such as cisplatin chemotherapy.

Long noncoding RNAs (lncRNAs) are transcripts with a length greater than 200 nt and devoid of coding potential of functional proteins.[6] Next-generation sequencing has identified certain lncRNAs that are dysregulated in and may contribute to several pathological and physiological processes,[7] providing a robust driving force to illustrate the regulatory roles and underlying mechanisms of these novel transcriptional molecules in human diseases. Diverse lncRNAs may have distinct sequence length and super structure, exerting regulatory roles on downstream target genes at an epigenetic, transcription, or posttranscriptional level. Currently, lncRNAs have been demonstrated to interact with RNA-binding proteins to regulate protein function, participate in chromatin remodeling and histone modification, function as competing endogenous RNA (ceRNA), and so on.[8]

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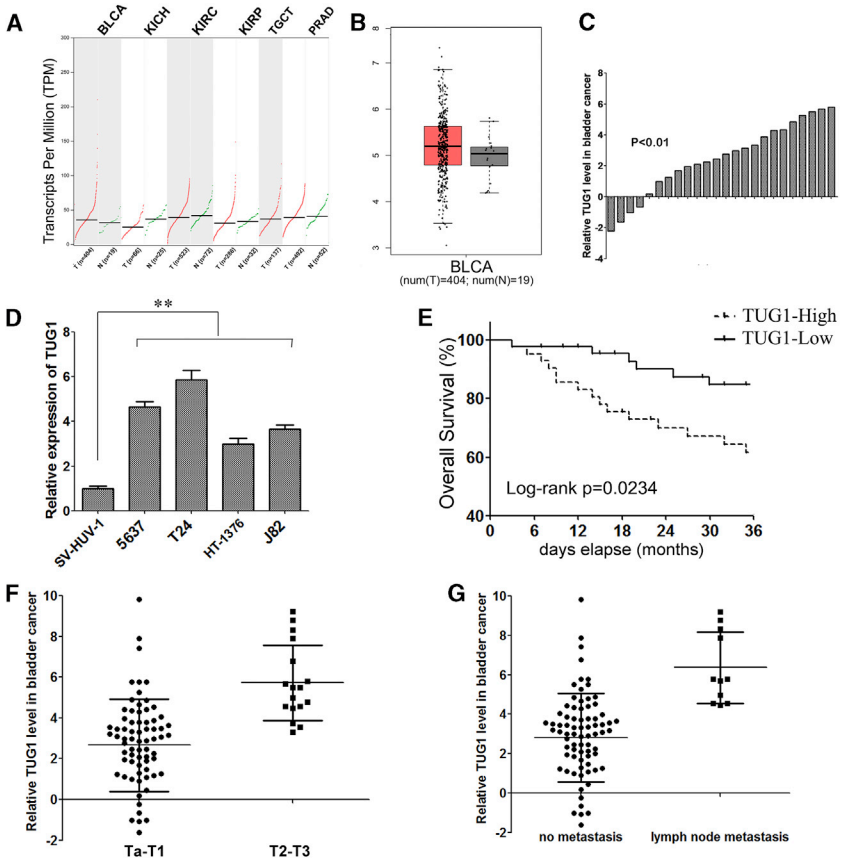

Figure 1. TUG1 Was Upregulated in Bladder Cancer, and Its Expression Was Correlated with Clinicopathological Characteristics

(A) The expression of TUG1 among urological cancers, including bladder urothelial carcinoma (BLCA), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), prostate adenocarcinoma (PRAD), and testicular germ cell tumors (TGCT) from The Cancer Genome Atlas (TCGA) Data Portal from GEPIA (http://gepia.cancer-pku.cn/). The expression of TUG1 in normal or cancer is shown with green or red dots. (B) The expression of TUG1 in normal and bladder cancer from TCGA Data Portal. (C) The expression of TUG1 was evaluated by real-time PCR in bladder cancer tissues; GAPDH was used for internal control. $p < 0.05$. (D) The expression of TUG1 was evaluated by real-time PCR in bladder cancer cell lines. *$p < 0.05$. (E) Kaplan-Meier survival curves for patients with bladder cancer according to the expression level of TUG1. (F) Bladder cancer samples with higher tumor stage (T2-T3) showed upregulated expression of TUG1 compared to that of lower tumor stage (Ta-T1). $p < 0.05$. (G) Bladder cancer samples with lymph node metastasis showed upregulated expression of TUG1. $p < 0.05$.

Taurine-upregulated gene 1 (TUG1) is a 7.6-kb lncRNA that is encoded by the ENSG00000253352 gene on chromosome 22q12; it was named for its upregulation after taurine treatment.[9] TUG1 expression is significantly dysregulated in diverse cancer types. In non-small-cell lung cancer, TUG1 is transcriptionally regulated by p53, and it functions as a tumor suppressor via recruiting a key component of the polycomb-repressive complex 2 (PRC2), Enhancer of zeste homolog 2 (EZH2), to the promoter region of HOXB7, causing H3K27 trimethylation and HOXB7 downregulation.[10] In contrast, TUG1 is significantly overexpressed in small-cell lung cancer, and it induces the expression of LIMK2b via binding with EZH2 to promote cell proliferation and chemotherapy resistance.[10] Subsequent studies indicate that TUG1 not only regulates downstream gene transcription in the nucleus by interacting with PRC2 but also affects downstream targets in the cytoplasm by functioning as a sponge to downregulate microRNAs (miRNAs).

However, whether TUG1 can regulate the development, progression, and chemoresistance of bladder cancer via similar mechanisms is still elusive. Our previous study demonstrated that TUG1 was upregulated and affected cisplatin sensitivity in bladder cancer; we also detected a negative correlation between TUG1 and methylation-regulated miR-194-5p. Previous studies also supported that miR-194-5p could act as a tumor suppressor in several cancers by targeting downstream genes, including RAP2B in bladder cancer.[11–13] Taken together, these results formed the hypothesis that TUG1 might regulate the expression level of miR-194-5p, via EZH2-associated methylation or a ceRNA-based mechanism, to exert its oncogenic roles and regulate cisplatin resistance in bladder cancer.

In an attempt to elucidate the biological roles and mechanism of TUG1 in bladder cancer, we focused on its biological relevance in influencing cell proliferation and chemotherapy sensitivity, and we further investigated its regulatory roles on miR-194-5p and CCND2 in bladder cancer. Our study has the potential to identify potent biomarkers for prognostic prediction and effective therapeutic target, and it may also facilitate the mechanistic elucidation of the pathophysiological basis of bladder cancer.

RESULTS

TUG1 Is Upregulated in Human Bladder Cancer, and Its Expression Is Correlated with Clinicopathological Characteristics

To study the role of TUG1 in the carcinogenesis of bladder cancer, we first inspected the expression data of TUG1 in all urological cancers from Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html), which demonstrated that TUG1 exhibited significantly higher expression in bladder cancer (Figure 1A). Comparing with that of noncancerous counterparts, the expression of TUG1 was significantly upregulated in bladder cancer tissues (Figure 1B). Then we validated the expression of TUG1 in

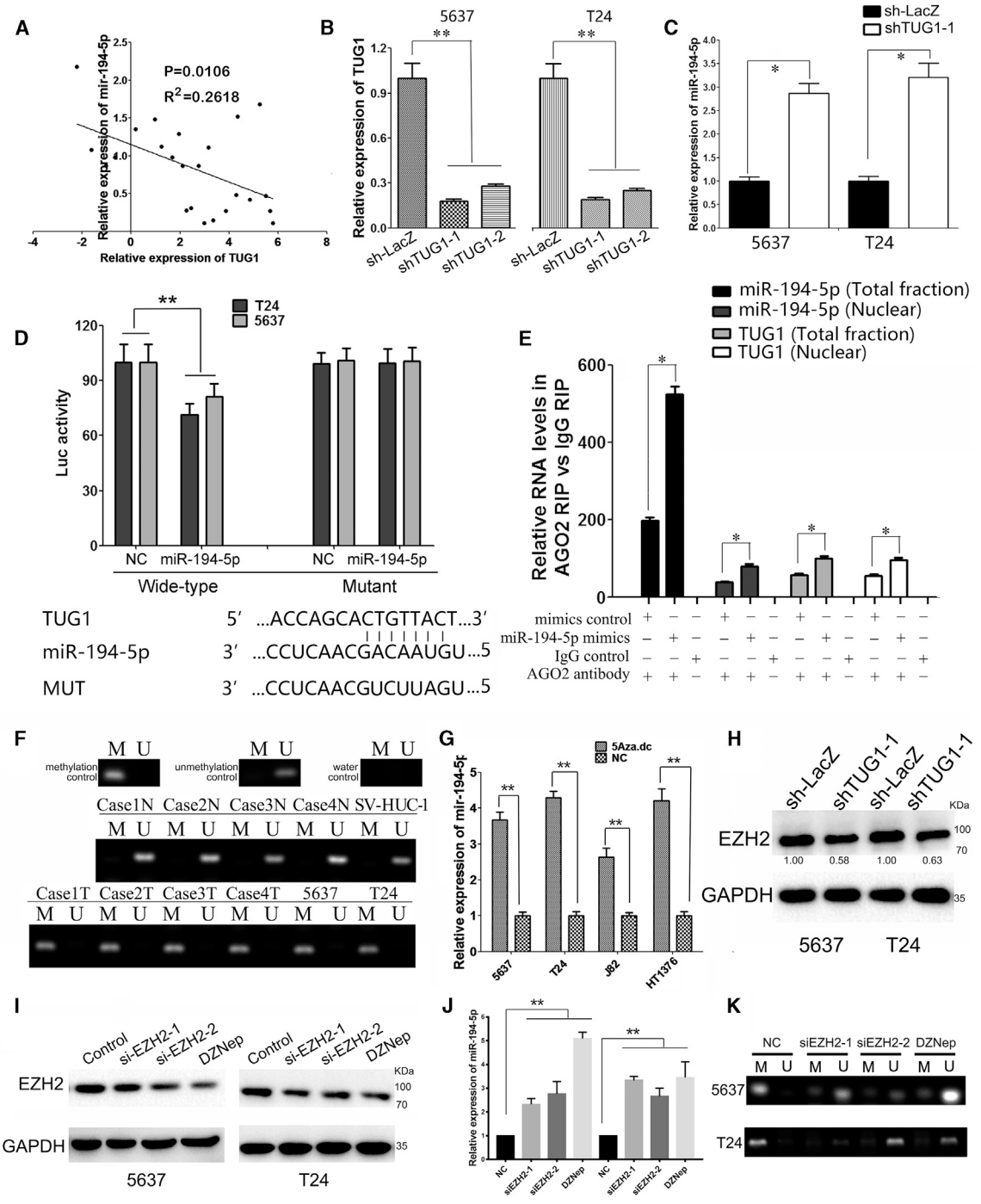

Figure 2. miR-194-5p Could Be Negatively Regulated by TUG1

(A) The expression of miR-194-5p was negatively correlated with that of TUG1. (B) The efficiency of TUG1 silencing in short hairpin RNA stably transduced bladder cancer cell lines 5637 and T24. (C) The expression of miR-194-5p in bladder cancer cell lines T24 and 5637 after transfecting with sh-TUG1 or sh-LacZ by real-time PCR. $p < 0.01$. (D) Luciferase reporters harboring putative target sites in the 3′ UTR of TUG1 were co-transfected with 100 nM of the indicated small RNA molecules (wild-type or mutant miR-94-5p) in T24 and 5637 cells. *$p < 0.01$. Relative luciferase activity was plotted as the mean ± SEM of three independent experiments. (E) The amount of miR-194-5p and TUG1 bound to AGO2 was measured by qPCR in nuclear and total fractions in the presence of miR-194-5p mimics or mimic NC. (F) Methylation-specific PCR was performed using primers specific for the unmethylated (U) or methylated (M) miR-194-5p promoter region in the genomic DNA deriving from bladder cell lines and tissues.

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24 pairs of bladder cancer fresh tissues. Comparing with their adjacent normal bladder tissue specimens, we observed 20 of the 24 bladder cancer tissues had a significantly higher expression of TUG1, while the expression of TUG1 decreased in the remaining four pairs (Figure 1C). Besides, we detected the expression of TUG1 in bladder cancer cell lines (5637, HT1376, J82, and T24) and a noncancerous human uroepithelial cell SV-HUC-1 by qPCR. We found that all four of the bladder cancer cell lines exhibited a significantly higher expression of TUG1 over SV-HUC-1, and the strongest overexpression of TUG1 was detected in bladder cancer cells T24 and 5637 (Figure 1D).

Furthermore, we expanded our sample sizes of bladder cancer patients to evaluate its association with clinicopathological characteristics and patients' prognosis. A further cohort of 87 bladder cancer patients included 48 males and 39 females, with a median age of 53 years. Lymph node metastasis was found in 11 patients and muscle invasion (stage T2 or T3) was found in 17 patients, and radical cystectomy was adopted for them. According to the Kaplan-Meier curves and the log-rank test, increased expression of TUG1 was associated with shorter overall survival ($p = 0.0234$) (Figure 1E). Besides, bladder cancer patients with higher tumor stage (muscle invasive, T2 or T3) showed significantly higher expression of TUG1 than patients with non-muscle-invasive disease (Ta or T1) (Figure 1F). In addition, patients with positive lymph node metastasis showed upregulated expression of TUG1 (Figure 1G). Therefore, these results indicated that increased TUG1 expression might play a critical role in bladder cancer development and progression and might have the potential to function as a valuable biomarker for prognostic prediction of this cancer.

miR-194-5p Is Frequently Decreased in Bladder Cancer through the ceRNA Capacity of TUG1

First, the StarBase database (http://starbase.sysu.edu.cn/) was utilized to identify potential ceRNA networks for specific lncRNA, and hsa-miR-194-5p was selected for multiple binding sites of it on TUG1 transcript and its downregulation pattern in bladder cancer via previous evidence.[14] We first validated the miR-194-5p expression in bladder cancer cell lines and paired clinical tissues, which indicated that miR-194-5p expression was significantly downregulated in primary bladder cancer samples as well as in bladder cancer cell lines (5637, HT1376, J82, and T24) in comparison with noncancerous controls (Figures S1A and S1B). Furthermore, by conducting Pearson correlation analysis using the expression of TUG1 and miR-194-5p in bladder cancer, we found that the expression of TUG1 and miR-194-5p was negatively correlated ($p = 0.0106$, $R^2 = 0.2618$) (Figure 2A). These results indicated that TUG1 might regulate the expression of miR-194-5p to exert its biological functions in bladder cancer. LncRNAs can function as miRNA sponges to regulate the expression of specific miRNAs through the miRNA response elements (MREs) on lncRNAs; we then evaluated the possibility of TUG1 as a ceRNA to regulate miR-194-5p.

To evaluate the effects of TUG1 on miR-194-5p in bladder cancer, we stably silenced TUG1 expression in two bladder cancer cell lines (5637 and T24) with lentiviral plasmid carrying short hairpin RNA (shRNA) for TUG1, while control cells were also established with lentiviral vehicle bearing nonspecific shRNA (LacZ). As demonstrated in Figure 2B, two shRNAs specific for TUG1 (shTUG1-1 and shTUG1-2) achieved satisfactory interference efficiency in bladder cancer cells, while the silencing effect was more pronounced in cells transfected with shTUG1-1. Then we detected the expression of miR-194-5p in these cells with qPCR, and we found that the expression of miR-194-5p was significantly induced after TUG1 knockdown in two cell lines (Figure 2C). Besides, we constructed the luciferase reporter bearing the putative binding fragment of TUG1 with miR-194-5p, which showed that the activity of luciferase containing TUG1 was suppressed by wild-type miR-194-5p mimics, while the luciferase activity was not affected by mutant miR-194-5p in T24 and 5637 cells, compared with that in corresponding control cells (Figure 2D). Considering that miRNAs are able to regulate coding and noncoding RNA by recruiting nuclear localized AGO2 and form the RNA-induced silencing complex (RISC), we further employed an AGO2-RNA immunoprecipitation assay. As desired, we found that miR-194-5p mimic transfection caused an enrichment of TUG1 in AGO2 immunoprecipitates in both total and nuclear extracts (Figure 2E), which also proved that miR-194-5p could bind to TUG1. Taken together, TUG1 may contribute to the downregulation of miR-194-5p via acting as a miRNA sponge.

miR-194-5p Is Also Decreased in Bladder Cancer through TUG1-Associated EZH2-Mediated Promoter Hypermethylation

Previous studies indicated that miR-194-5p was downregulated in bladder cancer as a result of the upregulation of TUG1. However, whether the ceRNA theory was the only underlying mechanism of its downregulation was still elusive. MiRNAs are also transcribed by the genome, and the expression of miRNAs can also be affected by promoter methylation, one common epigenetic regulation causing gene silencing. First, we carried out bisulfite modification of the genomic DNA from 4 pairs of bladder tissues and bladder cell lines 5637 and T24, and we conducted the methylation-specific PCR. Our data indicated that the miR-194-5p promoter was hypermethylated in bladder cancer tissues and cell lines, while its promoter was hypomethylated in noncancerous tissues and SV-HUC-1 cells (Figure 2F).

To further validate that the lower expression of miR-194-5p was affected by promoter methylation, four bladder cancer cell lines (5637, T24, J82, and HT-1376) were treated with nonspecific

(G) miR-194-5p expression in 4 bladder cancer cell lines after 5-Aza-DC or mock treatment. *$p < 0.05$. (H) Western blot showed that EZH2 protein level was attenuated following the knockdown of TUG1 in bladder cancer cells. (I) Western blot indicated that EZH2 protein was effectively abrogated following specific siRNA or DZNep treatment. (J) qRT-PCR results indicated that miR-194-5p was significantly induced following the knockdown of EZH2. (K) Methylation-specific PCR showed that knockdown of EZH2 by siRNAs or DZNep relieved the promoter hypermethylation of miR-194-5p.

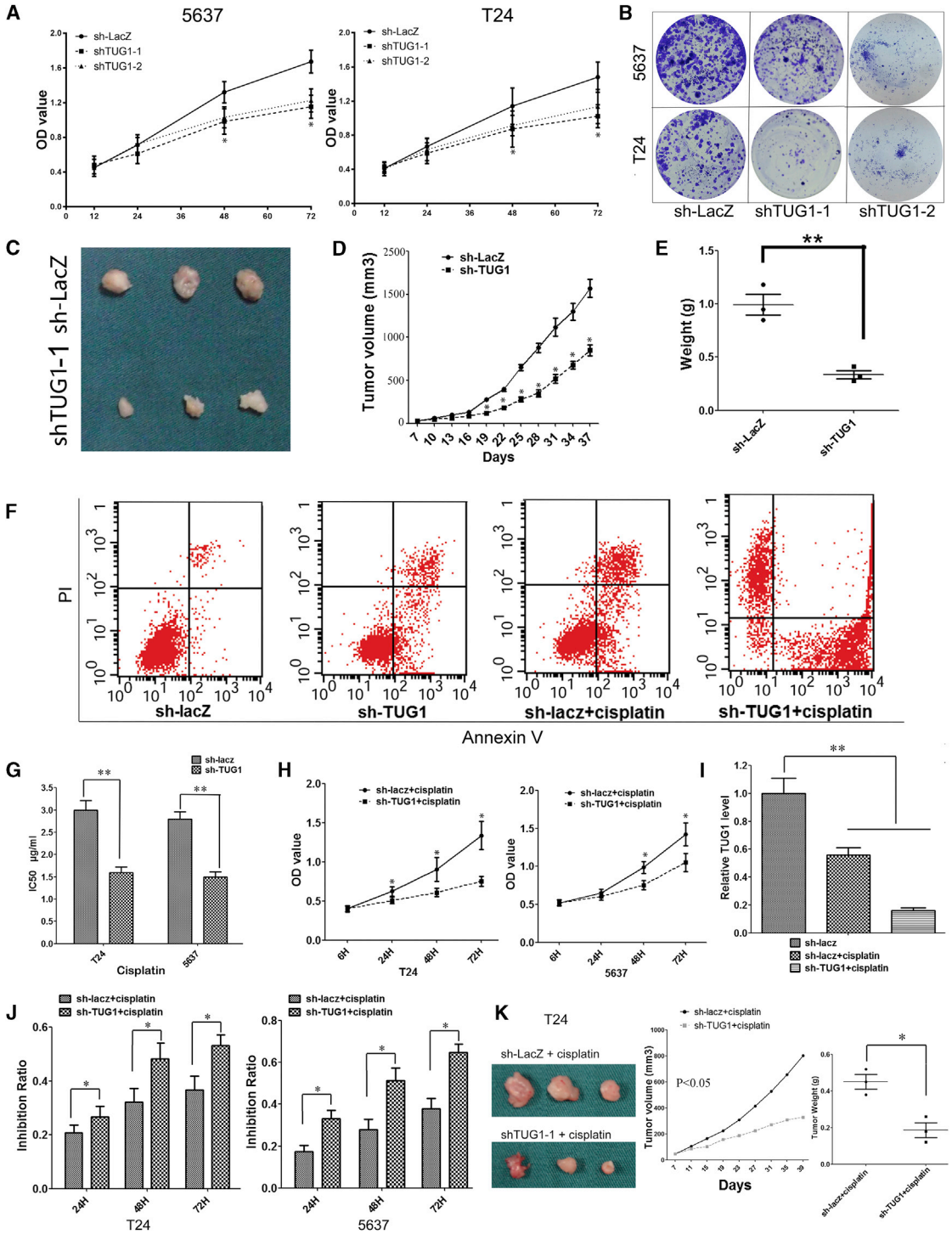

Figure 3. Knockdown of TUG1 Inhibited Cell Proliferation, Induced Apoptosis, and Sensitized Bladder Cancer Cells to Cisplatin

(A) MTS assays revealed cell growth curves of the indicated cells. (B) Representative micrographs of crystal violet-stained cell colonies analyzed by colony formation. (C–E) Photographs of tumors (C) excised 37 days after inoculation of stably transfected T24 cells into nude mice. Mean tumor volume (D) measured by caliper on the indicated days and tumor weight (E) of each nude mice at the end of 37 days are shown. $p < 0.01$. (F) Apoptosis detection after treating with sh-TUG1, cisplatin, and sh-TUG1 + cisplatin in bladder cancer cell lines T24 and 5637. $p < 0.01$. (G) The change of IC_{50} after transfecting sh-TUG1 into bladder cancer cell lines T24 and 5637. $p < 0.01$. (H) MTS assays

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demethylation agent 5-aza-2′-deoxycytidine (5-Aza-DC), which showed that the expression of miR-194-5p could be strongly upregulated in all of the four bladder cancer cell lines (Figure 2G). These results suggested that miR-194-5p is frequently silenced through promoter hypermethylation in bladder cancer.

Besides, TUG1 was reported to directly interact with epigenetic regulator EZH2; we thus tried to figure out if TUG1 could regulate the methylation pattern via interacting with EZH2 in bladder cancer. Using the data from The Cancer Genome Atlas (TCGA), we found that the expression of EZH2 was positively correlated with that of TUG1 (Figure S2). Then we evaluated the protein expression of EZH2 after the knockdown of TUG1 in bladder cancer cell lines, and western blot indicated that EZH2 protein was significantly attenuated following TUG1 silencing (Figure 2H). In addition, we evaluated the expression of miR-194-5p following effective EZH2 knockdown (Figure 2I), which indicated that miR-194-5p could be induced via abrogating EZH2 (Figure 2J). Furthermore, EZH2 small interfering RNAs (siRNAs) and EZH2 inhibitor 3-deazaneplanocin A (DZNeP) also suppressed the hypermethylation of miR-194-5p promoter, as demonstrated by methylation-specific PCR (Figure 3K). These results joined together to show that the TUG1-EZH2 pathway also contributed to the attenuation of miR-194-5p via promoter hypermethylation.

TUG1 Knockdown Suppresses Bladder Cancer Cell Growth, Induces Apoptosis, and Sensitizes Bladder Cancer Cells to Cisplatin

Aberrant expression of lncRNAs may also play indispensable roles in multiple cellular processes of cancer biology. To clarify the biological functions of TUG1 in the carcinogenesis of bladder cancer, we stably silenced TUG1 expression in 5637 and T24 (Figure 2B), and we conducted a series of *in vitro* and *in vivo* functional studies. MTS assay showed that downregulating TUG1 could inhibit the proliferation of bladder cancer cell lines 5637 and T24 (Figure 3A). Colony formation assay also indicated that knockdown of TUG1 could inhibit the colony formation capacity of bladder cancer cell lines (Figure 3B). To confirm the pro-proliferative roles of TUG1 *in vivo*, we utilized T24 cells to generate bladder cancer xenograft on BALB/c nude mice, and we observed a dramatic decrease in tumor volume and weight in the sh-TUG1-1 group compared with the sh-LacZ group (Figures 3C–3E). To investigate the effect of TUG1 on the apoptosis induction of bladder cancer cell line T24, we used flow cytometry for measuring the apoptosis rate. Compared to the negative control group, deprivation of TUG1 significantly induced the apoptotic rate of cancer cells (Figure 3F).

Apart from the functions on proliferation and apoptosis, we also focused on the impacts of TUG1 on cisplatin chemoresistance in bladder cancer. To determine whether TUG1 plays a role in chemosensitivity, we determined the cell growth inhibition in cells with decreased TUG1 after cisplatin treatment. We detected that the half maximal inhibitory concentration (IC_{50}) of cisplatin was decreased in the two bladder cancer cell lines when TUG1 was attenuated (Figure 3G; $p < 0.05$). In accordance, the sh-LacZ + cisplatin (2 μg/mL, 1-day treatment) group had stronger proliferative capacity compared with the sh-TUG1 + cisplatin (2 μg/mL, 1-day treatment) group (Figure 3H; $p < 0.05$). In addition, we treated the T24 cells with sh-LacZ, sh-LacZ + cisplatin (2 μg/mL), or sh-TUG1 + cisplatin (2 μg/mL), and we proved that the expression of TUG1 decreased successively in the three experimental groups, implying that TUG1 expression decreased significantly following cisplatin treatment (Figure 3I).

We then examined the effect of TUG1 on cell inhibition rates by treating cells with sh-LacZ + cisplatin (2 μg/mL) or sh-TUG1 + cisplatin (2 μg/mL); the inhibition rate was gradually increased in both groups as time went by, while the inhibition rate of bladder cancer cells in the sh-LacZ + cisplatin group was significantly lower than that in the sh-TUG1 + cisplatin group at different time points (Figure 3J). Furthermore, we examined the cell apoptosis rate in the same experimental group via flow cytometry; the apoptosis rate of bladder cancer cells in the sh-LacZ + cisplatin group was significantly lower than that in the sh-TUG1 + cisplatin group (Figure 3F). This observation was further confirmed in a T24 cell-based xenograft model, which demonstrated a dramatic decrease in tumor volume and weight in the sh-TUG1 + cisplatin group (Figure 3K). All these results indicated that TUG1 acts as an oncogene in bladder cancer and reduces the sensitivity of bladder cancer cells to cisplatin. Knockdown of TUG1 might exert a synergistic effect in cisplatin-based chemotherapy for treating bladder cancer.

miR-194-5p Can Be Upregulated by Cisplatin and Sensitize Bladder Cancer Cells to Cisplatin

The preceding data proved that TUG1 exerted regulatory roles on miR-194-5p; we were thus curious whether the aberrant expression of miR-194-5p might be involved in the proliferation, apoptosis, and chemoresistance of human bladder cancer. To investigate the effect of miR-194-5p on the growth and apoptosis induction of bladder cancer cells, overexpression of miR-194-5p was achieved by miR-194-5p mimic transfection, followed by the MTS assay and flow cytometry to determine the proliferation and apoptosis rates, respectively. Compared to the negative control group, overexpression of miR-194-5p significantly inhibited bladder cancer cell proliferation and induced apoptosis (Figures 4B and 4C; $p < 0.05$) in both cell lines.

We next determined the cell growth inhibition after cisplatin treatment to measure the precise role of miR-194-5p in chemosensitivity. As shown in Figure 4A, the IC_{50} of bladder cancers decreased after miR-194-5p induction ($p < 0.05$). Then we examined the inhibitory

revealed cell growth curves of the indicated cells. (I) The expression of TUG1 was evaluated by real-time PCR in bladder cancer cell lines T24 and 5637 after treatment with cisplatin. (J) The inhibition ratio detection after treatment with sh-TUG1 or sh-TUG1 + cisplatin in bladder cancer cell lines T24 and 5637. $p < 0.01$. (K) Photographs of tumors excised 37 days after the inoculation of stably transfected T24 cells into nude mice. Mean tumor volume measured by caliper on the indicated days and tumor weight of each nude mice at the end of 37 days are shown. $p < 0.01$.

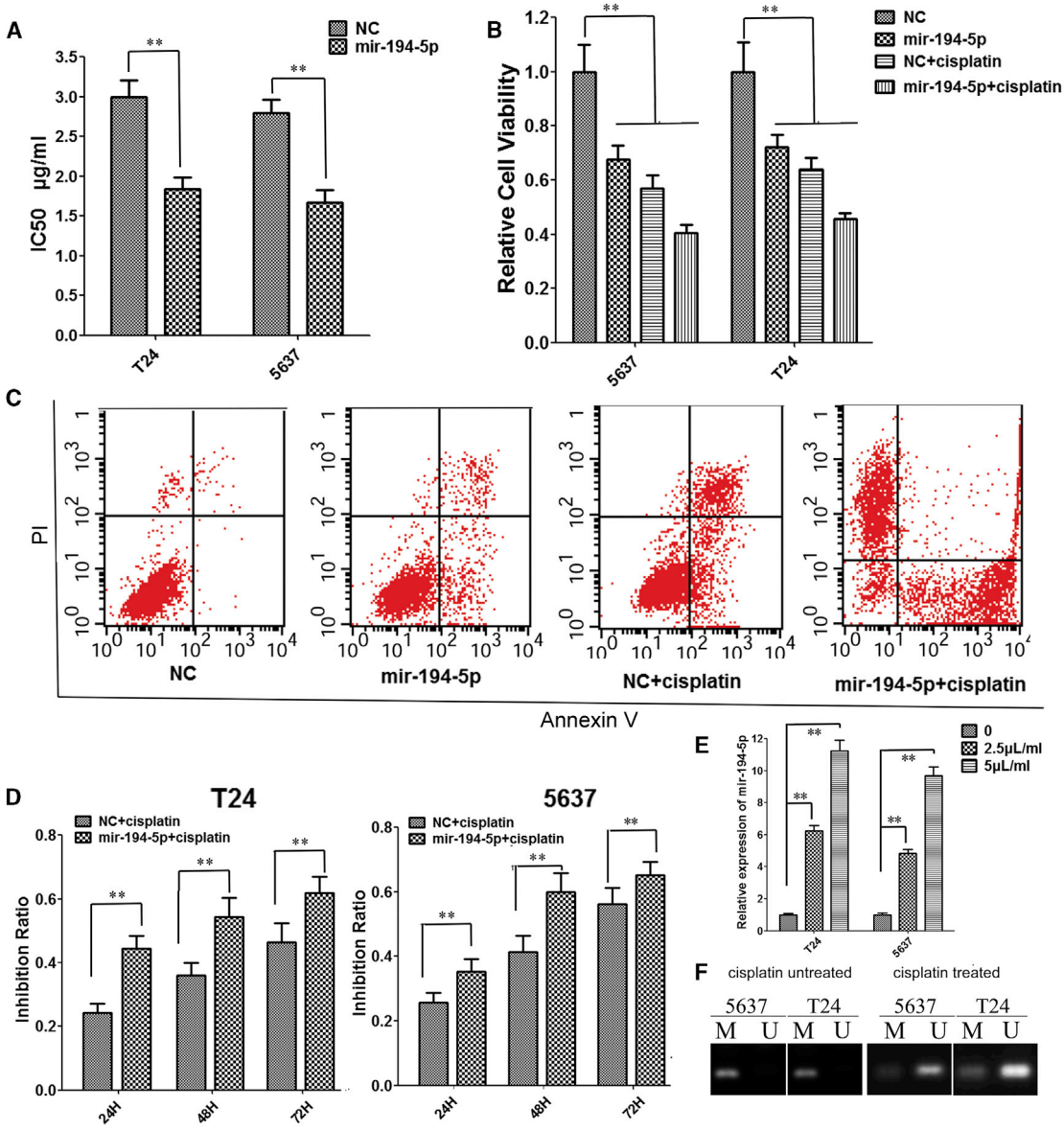

Figure 4. miR-194-5p Could Be Regulated by Cisplatin and Sensitizes Bladder Cancer Cells to Cisplatin

(A) The change of IC_{50} after transfecting miR-194-5p mimics into bladder cancer cell lines T24 and 5637. $p < 0.01$. (B) Cell viability was detected after treating with miR-194-5p mimics, cisplatin, and miR-194-5p mimics + cisplatin in bladder cancer cell lines T24 and 5637. $p < 0.01$. (C) Apoptosis detection after treatment with miR-194-5p mimics, cisplatin, and miR-194-5p mimics + cisplatin in bladder cancer cell lines T24 and 5637. $p < 0.01$. (D) The inhibition ratio detection after treatment with miR-194-5p mimics or miR-194-5p mimics + cisplatin in bladder cancer cell lines T24 and 5637. $p < 0.01$. (E) The expression of miR-194-5p was evaluated by real-time PCR in bladder cancer cell lines T24 and 5637 after treating with cisplatin at different concentrations. $p < 0.01$. (F) Methylation-specific PCR was performed using primers specific for the unmethylated (U) or methylated (M) miR-194-5p promoter region in the genomic DNA deriving from bladder cell lines T24 and 5637 after treating with cisplatin.

effect on proliferation of miR-194-5p by transfecting the cisplatin-treated (2 μg/mL) cells with negative control or miR-194-5p. As shown in Figure 4D, the inhibition rate of bladder cancers was increased in both groups as time went by, while the inhibition rate of bladder cancer cells in the negative control + cisplatin group was significantly lower than that in the miR-194-5p + cisplatin group. Moreover, flow cytometry apoptotic detection also supported that miR-194-5p induction enhanced the cisplatin sensitivity of bladder cancer cells (Figure 4C). These results jointly indicated that miR-194-5p may function as a tumor suppressor gene in bladder cancer via sensitizing bladder cancer cells to cisplatin.

In addition, we detected the expression of miR-194-5p in bladder cancer cell lines 5637 and T24 after treating them with cisplatin.

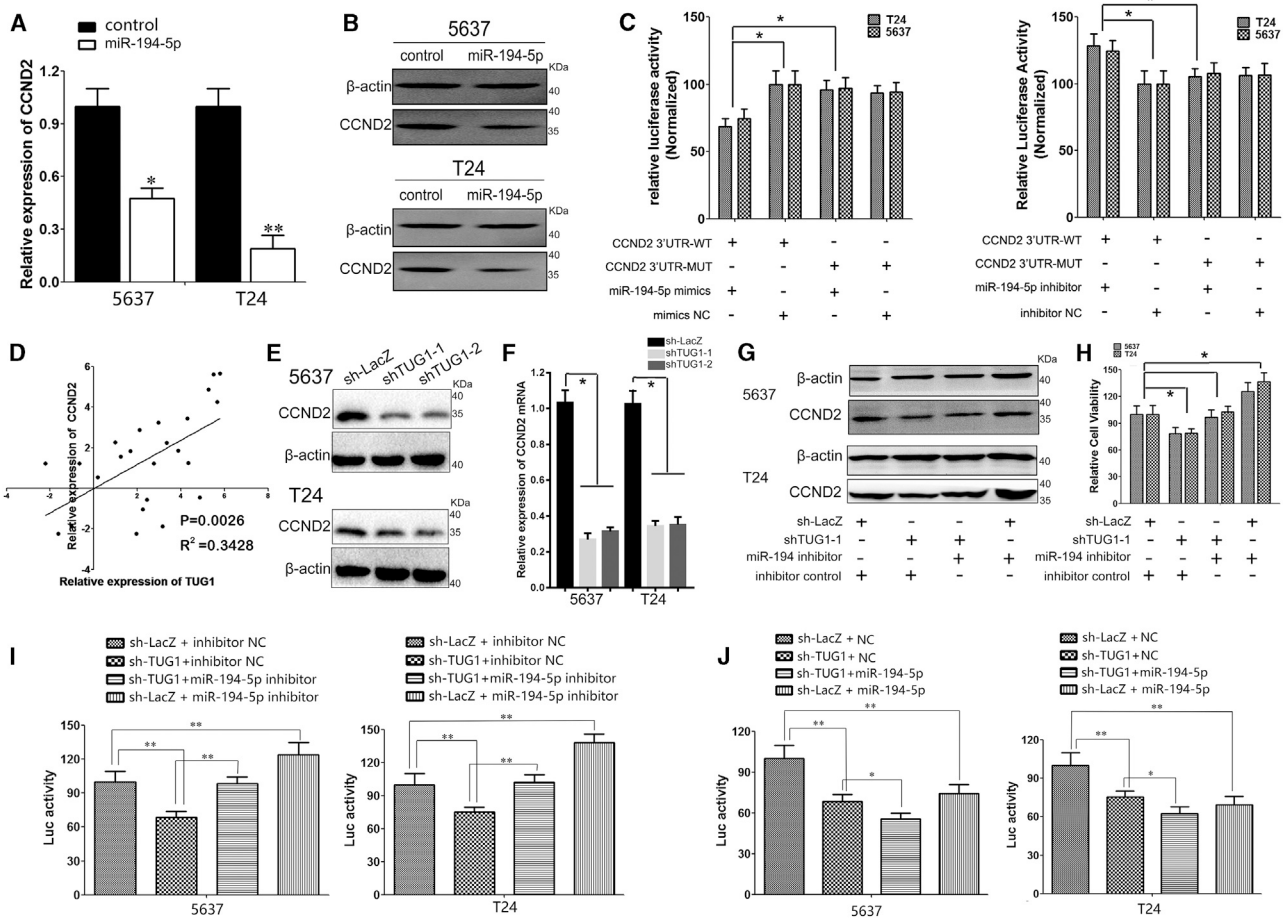

Figure 5. CCND2 Was a Bona Fide Target of miR-194-5p, and TUG1 Upregulated CCND2 Level in Bladder Cancer

(A) The expression of CCND2 mRNA in bladder cancer cell lines T24 and 5637 after transfection with miR-194-5p was detected by real-time PCR. (B) The expression of CCND2 protein in bladder cancer cell lines T24 and 5637 after transfection with miR-194-5p was detected by western blot. (C) Luciferase reporter assay was conducted to determine if miR-194-5p could bind to the wild-type or mutant 3′ UTR of CCND2. Relative luciferase activity was plotted as the mean ± SEM of three independent experiments. (D) The positive correlation between the expression level of TUG1 and CCND2 in bladder cancer tissues. $p < 0.05$. (E) Western blot results showed the protein expression of CCND2 in bladder cancer cell lines T24 and 5637 after transfecting with shRNAs for TUG1 or sh-LacZ. (F) The expression of CCND2 in bladder cancer cell lines T24 and 5637 after transfection with shRNAs for TUG1 or sh-LacZ by real-time PCR. $p < 0.01$. (G) The expression of CCND2 in bladder cancer cell lines T24 and 5637 after co-transfecting with sh-TUG1 or sh-LacZ or miR-194-5p inhibitor or negative control for inhibitor by western blot. (H) The proliferation assays were performed to evaluate the effect of TUG1 on the miR-194-5p-CCND2 axis. (I) Luciferase reporter assay showed that knockdown of the expression of TUG1 partially abrogated the promotion of the luciferase signal by miR-194-5p inhibitor. (J) Luciferase reporter assay showed that knockdown of the expression of TUG1 and overexpression of miR-194-5p decreased the luciferase signal further.

qRT-PCR demonstrated that miR-194-5p could be upregulated by cisplatin treatment in a dose-dependent manner (Figure 4E). To determine if this induction of miR-194-5p involved the epigenetic regulation, we carried out bisulfite modification of the genomic DNA from cisplatin-treated or non-cisplatin-treated bladder cell lines 5637 and T24. Methylation-specific PCR proved that cisplatin treatment induced miR-194-5p promoter demethylation, as both the methylated and unmethylated PCR products were detected in cisplatin-treated cells (Figure 4F). Taken together, these results provided us with the evidence that cisplatin induced miR-194-5p promoter demethylation and further increased miR-194-5p expression in bladder cancer cell lines.

CCND2 Is a Bona Fide Target of miR-194-5p and Mediates the Oncogenic Roles of TUG1

Protein is the direct performer of all cellular processes, so it's imperative to identify the downstream protein molecule of the TUG1-miR-194-5p pathway. The StarBase website also indicated that CCND2 might be a novel target gene of miR-194-5p. First, we observed that CCND2 mRNA and protein were both suppressed after miR-194-5p overexpression via qRT-PCR and western blot, respectively (Figures 5A and 5B). To determine whether CCND2 was a direct target of miR-194-5p, we constructed reporter plasmids by cloning the wild-type or mutant 3′ UTR of CCND2 bearing the putative binding sites of miR-194-5p into the downstream of a luciferase reporter

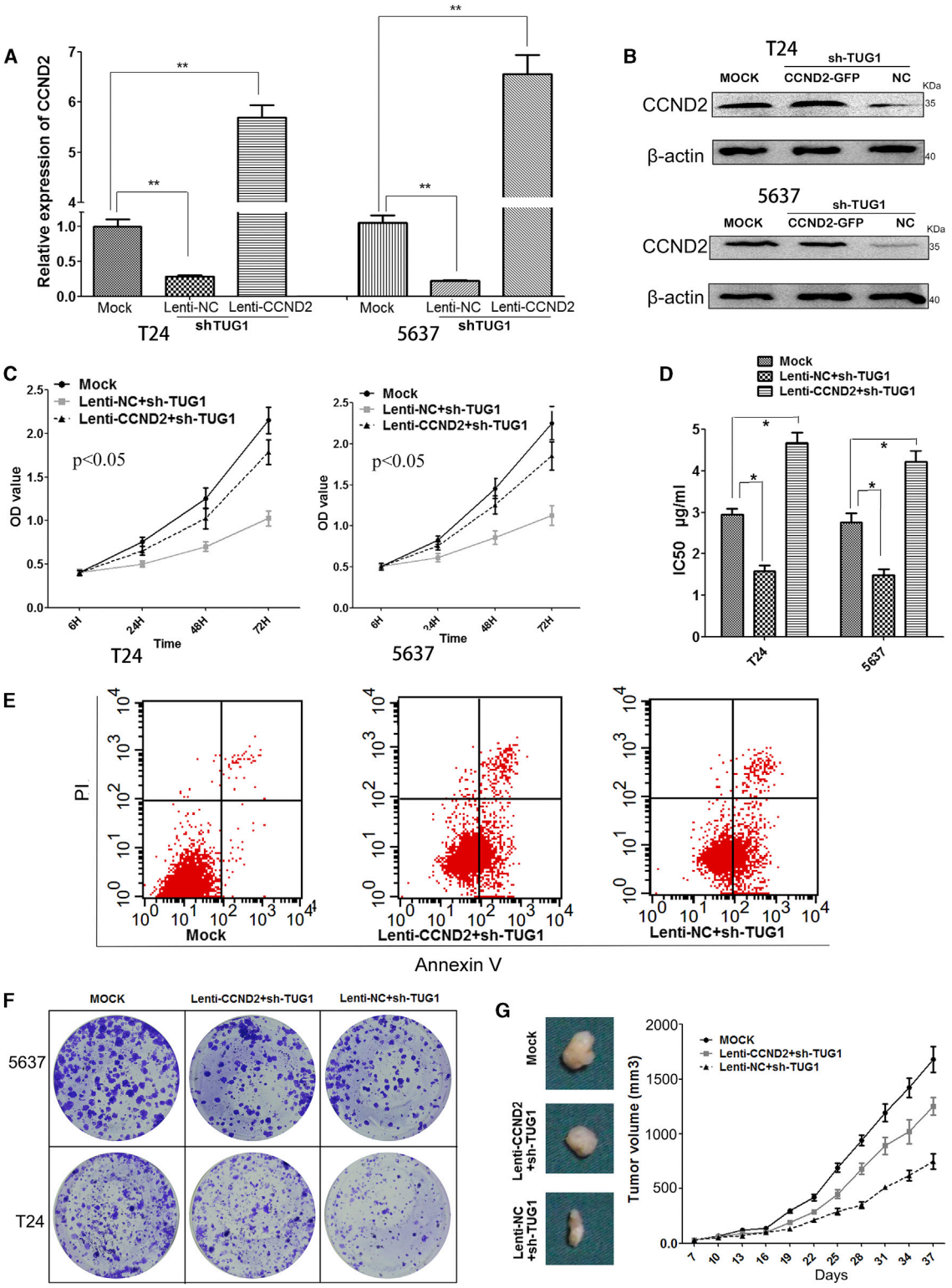

Figure 6. TUG1 Positively Regulated the Expression of CCND2 by Rescue Assays

(A) The expression of CCND2 in bladder cancer cell lines T24 and 5637 after co-transfection with sh-TUG1 and Lenti-CCND2 was detected by real-time PCR. $p < 0.01$. (B) The expression of CCND2 in bladder cancer cell lines T24 and 5637 after co-transfection with sh-TUG1 and Lenti-CCND2 was detected by western blot. (C) MTS assays

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psiCHECK2 (Figure S3). Co-transfection of the wild-type reporter plasmid and miR-194-5p mimics in both T24 and 5637 cells resulted in a decrease in the luciferase signal, while co-transfection of the wild-type reporter plasmid and miR-194-5p inhibitor resulted in an increase in the luciferase signal when compared with the vector control (Figure 5C). However, we did not detect a significant change in the luciferase signal in the mutant-type groups (Figure 5C). These results proved that CCND2 was a bona fide target of miR-194-5p.

We further evaluated whether CCND2 expression might be regulated by TUG1. Using the Pearson correlation analysis, we found that the expression of TUG1 was positively correlated with the expression of CCND2 ($p = 0.0026$, $R^2 = 0.3428$) (Figure 5D). Furthermore, the effects of TUG1 expression on endogenous CCND2 protein were detected. We observed the downregulation of CCND2 in bladder cancer cell lines 5637 and T24 following TUG1 knockdown (Figure 5E), which was in accordance with the positive correlation between CCND2 and TUG1. In addition, the mRNA expression of CCND2 was attenuated after the knockdown of TUG1 (Figure 5F).

To further establish a functional regulatory pathway among TUG1, miR-194-5p, and CCND2, we first evaluated whether TUG1 might regulate the expression of CCND2 via downregulating miR-194-5p. Western blot showed that sh-TUG1-1 could inhibit the expression of CCND2, whereas miR-194-5p inhibitor could rescue the inhibition of CCND2 by TUG1 (Figure 5G). We then tested whether TUG1 deregulation was required for the regulation of miR-194-5p on cell proliferation. We transfected miR-194-5p inhibitor or negative control to bladder cancer cells stably transfected with sh-TUG1 or sh-LacZ. The pro-proliferative effect of miR-194-5p inhibitor was partially attenuated by sh-TUG1 (Figure 5H). Besides, using a luciferase reporter containing the 3′ UTR of CCND2, we found that knockdown of the expression of TUG1 partially abrogated the promotion of the luciferase signal by miR-194-5p inhibitor (Figure 5I). Knockdown of the expression of TUG1 and overexpression of miR-194-5p decreased the luciferase signal further (Figure 5J). Combined with the above results, we concluded that CCND2 was directly regulated by miR-194-5p, while TUG1 could bind to miR-194-5p and regulate their expression. These data also demonstrated that, by binding with miR-194-5p, TUG1 acted as a ceRNA to modulate CCND2.

CCND2 Mediates the Oncogenic Roles of TUG1 in Bladder Cancer by Rescue Assays

We previously established the interactions among TUG1, miR-194-5p, and CCND1; however, their interactions' impacts on the proliferation, apoptosis induction, and drug sensitivity of bladder cancer remained unsolved. To determine whether TUG1 regulated bladder cancer cell proliferation, apoptosis induction, and colony formation by regulating CCND2 expression, rescue assays were performed. Bladder cancer cells transfected with both empty lentiviral plasmids (Lenti-negative control [NC] and sh-LacZ) were used as a control and named the mock group.

The qRT-PCR and western blot findings showed that ectopic expression of CCND2 induced the expression of CCND2 at the transcription and translation levels when compared with the corresponding control, and ectopic expression of CCND2 increased the CCND2 protein level, which was attenuated by TUG1 silencing (Figures 6A and 6B). Besides, the results of MTS, flow cytometry, and colony formation assays demonstrated that reintroduction of CCND2 could partially rescue the impaired cell growth, the enhanced apoptosis, and compromised colony formation capacity in 5637 and T24 cells following TUG1 knockdown (Figures 6C, 6E, and 6F). What's more, we observed that CCND2 upregulation also reversed the bladder cancer cells' capacities for resistance to cisplatin, as the IC_{50} was increased following CCND2 overexpression (Figure 6D). Finally, we validated our assumption that CCND2 mediated the oncogenic roles of TUG1 in the nude mouse model experiment, as reintroduction of CCND2 partially restored the oncogenic roles of TUG1 (Figure 6G). These data indicate that TUG1 promotes bladder cancer cell proliferation and chemoresistance to the cisplatin through the upregulation of CCND2 expression.

DISCUSSION

In this study, we provide solid evidence that lncRNA TUG1 and miR-194-5p exert pivotal roles in the chemoresistance to cisplatin in bladder cancer. Higher level of TUG1 can directly bind to and act as a biological sponge for mature miR-194-5p, which reduces the expression level of miR-194-5p. Besides, miR-194-5p expression is regulated by TUG1-induced EZH2-associated promoter methylation, which lessens the cellular concentration of miR-194-5p. Moreover, a lower level of miR-194-5p leads to higher levels of CCND2 mRNA and protein, which promote the chemoresistance of bladder cancer cells to cisplatin. Taken together, these results elucidate the regulatory mechanism of the TUG1-miR-194-5p-CCND2 pathway on cisplatin sensitivity in bladder cancer cells (Figure 7). Besides, we proved that this regulatory network also plays a role in the proliferation and apoptosis of bladder cancer. Additionally, we identify TUG1 as a promising therapeutic target and useful biomarker for bladder cancer.

For the past few years, many investigations indicate that the dysregulation of lncRNAs contributes to the pathogenesis of almost all human cancers, stating that lncRNAs can act as oncogenes or tumor suppressors in the carcinogenesis and progression of human cancers.[15–17] TUG1 was first discovered and considered as a novel retinal noncoding RNA;[14] further studies also claimed that TUG1 could interact with the relocation of growth-related genes and function as a pivotal oncogene in various malignant tumors.[18–21] In particular,

revealed cell growth curves of the indicated cells. (D) The change of IC_{50} after co-transfection of sh-TUG1 and Lenti-CCND2 in bladder cancer cell lines T24 and 5637. $p < 0.01$. (E) Apoptosis detection after co-transfection of sh-TUG1 and Lenti-CCND2 in bladder cancer cell lines T24 and 5637. $p < 0.01$. (F) Representative micrographs of crystal violet-stained cell colonies analyzed by clongenic formation. (G) Photographs of tumors excised 37 days after inoculation of stably transfected T24 cells into nude mice. Mean tumor volume was measured by caliper on the indicated days. $p < 0.01$.

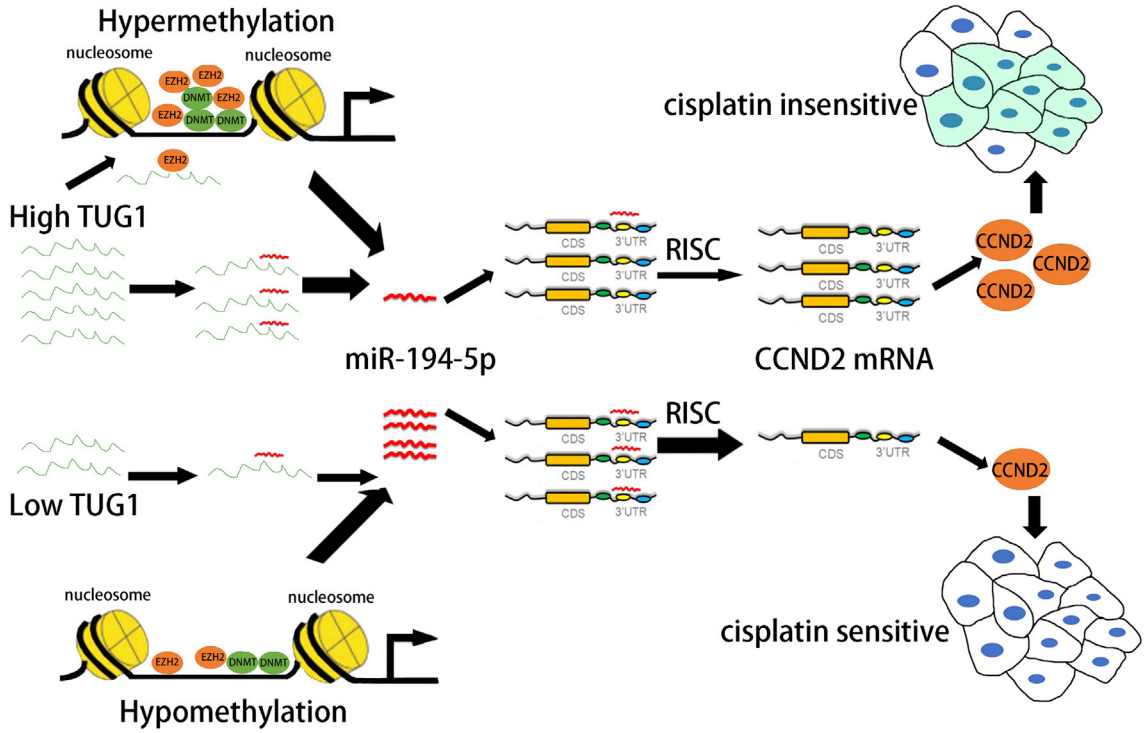

Figure 7. Model of TUG1- and miR-194-5p-Dependent Regulation of the Chemoresistance

A higher level of TUG1 can directly bind to and function as a biological sponge for mature miR-194-5p, which lowers the expression level of miR-194-5p. Besides, miR-194-5p expression is regulated by promoter methylation; hypermethylation of miR-194-5p promoter also lowers its cellular concentration. TUG1 also induces the expression of EZH2 and further lowers the miR-194-5p via promoter hypermethylation. Moreover, a lower level of miR-194-5p results in higher levels of CCND2 mRNA and protein, which promote the chemoresistance of bladder cancer cells to cisplatin. This could also explain why miR-194-5p is induced by cisplatin treatment.

Liu et al.[22] claimed that the downregulation of TUG1 inhibited proliferation and induced apoptosis through the TUG1-miR-142/Zeb2 axis in bladder cancer cells. However, there are sparse studies to evaluate the effects of TUG1 on the chemotherapy sensitivity of bladder cancer.

In this study, we also validated that TUG1 was upregulated in bladder cancer and played pivotal roles in bladder cancer proliferation, apoptosis, and cisplatin resistance. Besides, TUG1 was increased in bladder cancer patients with higher tumor stage and lymph node metastasis, and it also possessed the predictive value for overall survival of patients. Interestingly, cisplatin could induce the expression of TUG1, while knockdown of the expression of TUG1 could, in turn, reinforce the cisplatin sensitivity of bladder cancer cells *in vitro* and *in vivo*. These results supported that TUG1 silencing could efficiently decrease tumorigenesis of bladder cancer cells, which was further supported by its effect on cisplatin chemosensitivity. It is noteworthy that knockdown of TUG1 also impacts tumor proliferation, so the net effects of TUG1 on cisplatin sensitivity may be weaker after deducing the pro-proliferation effects *in vitro* and *in vivo*. Since cisplatin-based chemotherapy is widely used and cisplatin resistance is one of the clinical obstacles for prolonging the survival of bladder cancer patients, our results have important implications for identifying a novel therapeutic target and predictive biomarker and overriding the chemotherapy resistance to facilitate the clinical management of bladder cancer.

TUG1 has been suggested to play parts in the carcinogenesis and progression of diverse human cancers by functioning as a ceRNA to regulate certain targets, such as miRNAs. As a miRNA sponge, TUG1 could bind with several miRNAs, such as miR-9-5p, miR-455-3p, and miR-335-5p, to further impact the expression of certain target genes of these miRNAs, thus affecting the biological behavior of tumor cells.[23–27] In this study, we confirmed that miR-194-5p expression was frequently decreased and exerted tumor-suppressive roles in bladder cancer cell lines and tumor tissues, which was consistent with other cancers.[11,12,28] Besides, the expression of TUG1 and miR-194-5p was negatively correlated, while luciferase reporter assays and RNA immunoprecipitation assay validated the interaction between miR-194-5p and TUG1.

Furthermore, our data also validated that lncRNA TUG1 increased cell growth and cisplatin chemoresistance by regulating CCND2 via downregulating miR-194-5p in bladder cancer. CCND2 is one of the cell cycle proteins expressed in a wide variety of tumors, and it plays important parts in their carcinogenesis and progression.[29–31] This cyclin was best known for its roles in cell cycle G1/S transition via interacting with CDK4 or CDK6. Previous studies have shown

that TUG1 can act as an oncogenic gene in bladder cancer,[32] although its underlying mechanisms are largely elusive. In keeping with these studies, our research also suggested that the expression of CCND2 was elevated in bladder cancer and its expression was positively correlated with that of TUG1. Through the luciferase reporter assay and western blot, we confirmed that miR-194-5p could simultaneously bind to the RNA sequence of TUG1 and CCND2 3′ UTR, which supported that TUG1 regulated the bladder cancer proliferation and chemoresistance by interacting with miR-194-5p and CCND2. In addition, rescue assays were performed to validate that introduction of CCND2 could partially mimic the effect of TUG1 on bladder cancer cells. Taken together, these findings further supported that TUG1 could function as a ceRNA to regulate CCND2 via miR-194-5p.

Promoter hypermethylation could be an important mechanism for the decreased miRNA expression in some human cancers, including urothelial cancer.[33] Previous studies also reported that the expression of small molecule RNA and chemoresistance are regulated by epigenetic regulation.[34–37] We then ascertained if promoter hypermethylation could be the underlying mechanism that may help to explain the decrease in miR-194-5p in bladder cancer. Here, combined with the methylation-specific PCR results and the data of 5-Aza-DC demethylation treatment, we clearly found that promoter hypermethylation also contributed to the decrease in miR-194-5p expression in bladder cancer. Previous studies proved that TUG1 could recruit and bind PRC2 and promote the binding of EZH2 to the promoter region of target genes, causing H3K27 trimethylation and, thereby, downregulating specific target genes.[10] In our study, we also validated that knockdown of TUG1 attenuated the expression of EZH2, alleviating the promoter hypermethylation of miR-194-5p and inducing the expression level of miR-194-5p. This epigenetic regulatory pathway helps propose additional mechanisms for how TUG1 could regulate the expression of miR-194-5p. Furthermore, miR-194-5p expression was also regulated by cisplatin treatment, and increased miR-194-5p induced by cisplatin in turn improved the cisplatin sensitivity of bladder cancer cells *in vitro* and *in vivo*. Consequently, we concluded that cisplatin-induced epigenetic alteration could be a plausible explanation for the downregulation of miR-194-5p expression.

Taken together, our research has suggested that TUG1 is significantly upregulated in bladder cancer and the activation of TUG1 may be involved in bladder cancer progression. The expression of miR-194-5p is frequently reduced in bladder cancer tissues and cell lines partially through EZH2-mediated promoter hypermethylation, while it is epigenetically increased in bladder cancer cells following cisplatin treatment. miR-194-5p overexpression and TUG1 knockdown can inhibit bladder growth, induce apoptosis, and sensitize bladder cancer cells to cisplatin. Furthermore, TUG1 induces cell growth and chemoresistance by regulating CCND2 via functioning as miRNA sponge to silence miR-194-5p in bladder cancer. Our study helps elucidate the precise roles and molecular mechanisms of the TUG1-miR-194-5p-CCND2 pathway in tumorigenesis and the progression of bladder cancer.

MATERIALS AND METHODS

Patients and Samples

All of the fresh tissues and matched noncancerous adjacent tissues were resected from patients who were diagnosed pathologically with bladder transitional cell carcinoma and underwent surgical resection of tumors at the Department of Urology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All the samples were immediately frozen and stored in liquid nitrogen. The clinicopathological data were retrieved and patients' survival data were collected during regular follow-up. Written informed consent was obtained from each patient, and the study was approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology.

Cell Culture, Lentiviral Infection and Transfection, and Cisplatin Treatment

Bladder cancer cell lines T24, 5637, HT1376, and J82 and the corresponding noncancerous bladder epithelial cell line SV-HUC-1 were purchased from ATCC. 5637, J82, and T24 were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS); HT1376 was maintained in minimum Eagle's medium (MEM) containing 10% FBS, while SV-HUC-1 was maintained in DMEM and F-12 medium. All of the cell lines were cultured in a humidified atmosphere of 5% CO_2 maintained at 37°C. The chemically modified short single- or double-stranded RNA oligonucleotides (miR-194-5p mimics, mimic NC, miR-194-5p inhibitor, and inhibitor NC) were purchased from Genechem (Shanghai, China). Prevalidated siRNA specific for TUG1 and corresponding siRNA control were purchased from Genechem (Shanghai, China). The shRNA plasmids were constructed by inserting the DNA sequence targeting TUG1 into the pLKO.1 empty plasmid (sh-LacZ). The target sequence for silencing TUG1 was as follows: shTUG1-1, 5′-GACTACC TTCCCTGTGCTATT-3′; and shTUG1-2, 5′-CTGATTGCTGAGT GTTCAC-3′.

All cell transfection was performed using Lipofectamine 3000 following the instructions of the manufacturer (Thermo Fisher Scientific, CA, USA). Lentiviral vectors were transfected into HEK293T cells in combination with lentiviral packaging vectors pRSVRev, pMD2.G, and pCMVVSVG using Lipofectamine 3000; after transfection for 72 h, supernatants containing lentiviruses were collected and filtered through a 0.22-μm filter, and they were infected into T24 cells to generate stably transfected cells.

Cisplatin (HY-17394, MedChemExpress) was dissolved as stock solution at the concentration of 1 mg/mL with sterile water under ultrasonic and warming, and then the stock solution was added to cells with the indicated volume. Cisplatin was prepared in sterile PBS and used at the final concentration of 25 mg/kg for *in vivo* experiments.

RNA Extraction, Real-Time qRT-PCR, and MSP

Total RNA was extracted from bladder cancer tissues or cell lines using TRIzol reagent (Invitrogen, USA) and then reverse transcribed

using Rever Ace qPCR RT Kit (TOYOBO, Shanghai), according to the manufacturer's instructions. Real-time PCR of TUG1 or mRNAs was performed using FastStart Universal SYBR Green Master (Roche, IN, USA) with the ViiA 7 Dx PCR System (Applied Biosystems, USA), while the expression of mature miRNA was determined by PCR with All-in-One miRNA qRT-PCR reagent kit (GeneCopoeia, USA).

Genomic DNA was isolated using QIAamp DNA Mini Kit (QIAGEN), and bisulfite modification of the genomic DNA was carried out using an Epitect Bisulfite Kit (QIAGEN), according to the manufacturer's instructions. Methylation-specific PCR (MSP) primers for miR-194-5p gene promoter were designed with Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi), using the same methods as Zhou et al.[38] Methylation or unmethylation primers for MSP were as follows: methylation, 5′-GGTTATGAGTAGAAGGGGTTGAC-3′ (forward), 5′-TCAATCTTAAACACTATCCGAACG-3′ (reverse); and unmethylation, 5′-GTTATGAGTAGAAGGGGTTGATG-3′ (forward), 5′-CAATCTTAAACACTATCCAAACACC-3′ (reverse).

Western Blot

First, protein was extracted by NP40 from bladder cancer cells, and it was separated on 10% SDS-PAGE gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad). Nonspecific binding was blocked by incubating the PVDF membranes with 5% nonfat milk for 90 min. The membrane was then incubated with primary antibodies, including anti-CCND2 (1:1,000 dilution; ab226972, Abcam), anti-EZH2 (1:1,000 dilution; 21800-1-AP, Proteintech), anti-β-actin (1:2,000 dilution; 23660-1-AP, Proteintech), and anti-GAPDH (1:2,000 dilution; 10494-1-AP, Proteintech) in TBST solution at 4°C overnight. After washing with TBST, the PVDF membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:4,000 dilution; Boster Biological Technology) for 1.5 h at 37°C. At last the proteins were visualized using ECL-plus detection system (Pierce).

Cell Proliferation Assay

Treated 5637 and T24 cells were digested and transferred to 96-well microplates, and they were replanted at a density of approximately 1,500(5637)/2,000 (T24) cells per well. Cell proliferation was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega), according to the manufacturer's instructions, and we measured the absorbance by using a Micro-plate reader (Thermo Fisher Scientific) at 12, 24, 48, and 72 h after seeding cells. For the colony formation assay, approximately 1,000 transfected cells were seeded in single wells of 6-well plates; cells were maintained in complete medium for 14 days and finally stained with crystal violet. All experiments were performed in triplicate.

Flow Cytometry for Cell Apoptosis Analysis

To detect apoptosis by flow cytometry, cells transfected with the indicated plasmids or siRNAs were digested, washed, and then stained for fluorescence with propidium iodide and APC Annexin V Apoptosis Detection Kit (BD Pharmingen). DNA content and annexin V-positive cells were measured using a FACSCalibur flow cytometer and CellQuest software. Apoptosis was evaluated by quantifying the percent of cells with hypodiploid DNA as an indicator of DNA fragmentation or the percent positively stained with annexin V. For all assays 10,000 cells were counted.

Dual Luciferase Assay

Briefly, 5637 and T24 cells were seeded in 24-well plates (8,000 cells/well) and co-transfected with 100 ng psiCHECK2 Luciferase vectors (Promega) containing wild-type or mutant 3′ UTR of CCND2 with 100 nM miR-194-5p mimic, mutant mimic, or negative control. At 48 h after transfection, dual luciferase reporter assay was performed according to the manufacturer's instructions; the Renilla luciferase signal was normalized to the firefly luciferase signal for each individual analysis. All experiments were repeated twice.

Animal Experiment

Bladder cancer xenograft in nude mice was conducted as described previously.[39] Each group of three mice each was injected subcutaneously with prepared cells at a single site. Tumor onset was measured with calipers at the site of injection weekly at different times on the same day. Animals were sacrificed 37 days after injection. To evaluate the chemosensitivity effect of TUG1, nude mice were injected subcutaneously with T24 cells at a single site. After an appreciable tumor was formed subcutaneously (approximately 2 weeks), cisplatin (25 mg/kg) was administered for mouse via intraperitoneal injection once every 2 days. Animals were sacrificed 37 days after injection. Tumor volume was calculated using the formula, $V = 0.5ab^2$, where "a" and "b" represent the largest and shortest diameters, respectively. Nude mice were manipulated and cared for according to NIH Animal Care and Use Committee guidelines in the Experiment Animal Center of the Tongji Medical College (Wuhan, Hubei, China).

Statistical Analysis

Continuous data were presented as mean ± SD and compared using Student's two-tailed t test, while categorical data were compared with chi-square anslysis. Kaplan-Meier curves and the log rank test were used for the survival analysis. A p value < 0.05 was considered statistically significant ($^*p < 0.05$ and $^{**}p < 0.01$). Data sharing is not applicable to this article as no datasets were generated or analyzed during this study.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.02.017.

AUTHOR CONTRIBUTIONS

G.Y., H.Z., and B.L. designed the study. G.Y., H.Z., and W.Y. conducted the experiments and G.Y. wrote the manuscript. G.Y., L.M., and B.L. supported the experiments, contributed in reagents/materials, and provided the financial support. H.Z. and W.Y. performed the data analysis and helped to draft the manuscript. L.M.

and B.L. revised the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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