LncRNA MNX-AS1 combined with YBX1 to promote bladder cancer proliferation, migration, and invasion through activiting Wnt signaling pathway

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Abstract

Long non-coding RNAs (lncRNAs) govern fundamental biochemical and cellular processes. Bladder cancer is one of the most common genitourinary malignancies, and its proliferation, migration and invasion also regulated by many functional lncRNAs. In our study, we found that lncRNA MNX1-AS1 was highly-expressed in bladder cancer tissues and cell lines. Moreover, higher MNX1-AS1 expression was correlated with poor survival of MNX1-AS1 patients. Mechanistically, we showed that MNX1-AS1 could interact with YBX1 and promotes its location at the promoter region of β-catenin, then to promote transcriptional activation of β-catenin. In addition, MNX1-AS1/YBX1 could promote bladder cancer cells proliferation, migration and invasion through active Wnt/β-catenin signaling by induce β-catenin transcriptional activation. Consistently, we find a new functional lncRNA MNX1-AS1 can regulate bladder cancer cells proliferation, migration and invasion by target β-catenin transcriptional activation together with YBX1.

Introduction

Bladder cancer is one of the most common genitourinary malignancies, and most of them are transitional cell carcinomas, which begin in cells that normally make up the inner lining of the bladder, and 20–40% of the patients present with or develop invasive disease[1,2]. In total, approximately 50% bladder cancer patients die from their aggressive disease within 5 years, despite treatment with the gold standard strategy radical cystectomy for pelvic lymphadenectomy. While, cancer will recurs in 60–70% of patients, in which, 10–30% eventually develop muscle-invasive bladder cancer or metastatic bladder cancer[3]. For transitions, the lung is one of the most favorite metastatic sites and it connected with a high frequency of recurrence and mortality, which lead to patient poor prognosis. Therefore, it is imperative to clarify the mechanism of bladder cancer invasion and metastasis to develop better therapeutic strategies for prediction and treatment bladder cancer.

In recently, genome deep sequencing uncovers that nearly 75 percent of genome will transcribe into RNAs and a few of them translate to proteins with others as noncoding RNAs (ncRNAs)[4]. In general, ncRNAs including microRNAs (miRNAs), small interfering RNA (siRNAs), PIWI-interacting RNAs (piRNAs), housekeeping ncRNAs, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), with shorter than 200 nt RNAs, and long non-coding RNAs (lncRNAs),longer than 200 nt RNAs[5–7]. Previous studies find many functional lncRNAs linked with govern fundamental biochemical and cellular processes, such as chromosome inactivation, genomic imprinting and development. In recently, more and more researches revealed that lncRNAs are frequently dysregulated in various human cancers and involved in the progression and metastasis of multiple malignancies, including bladder cancer[8]. Of note, many dysregulated lncRNAs are involved in EMT and metastasis in human cancers. Such as, lncRNA PANDAR could promote metastasis of colorectal cancer[9], lncRNA H19 promotes EMT by functioning as a miRNA sponge to miR-138 and miR-200a in colorectal cancer[10]. Certainly, in bladder cancer, according to previous reports, lncRNA UCA1 could promote bladder cancer invasion and EMT through regulate miR-

143/HMGBI pathway[11]. Even so, more functional lncRNAs are urgently required for bladder cancer prediction and treatment.

Y-box binding protein-1 (YBX1) is an evolutionary conserved master regulator of transcription and translation[12]. In previous reports, YBX1 functional linked to pleotropic cellular processes, including DNA repair, cell proliferation and pre-mRNA splicing[12,13]. YBX1 is often found to be dysregulated in human malignancies, with overexpression in several cancers, including breast[14], gastric[15] and prostate[16] cancers. YBX1 overexpression is also closely associated with an unfavorable clinical outcome[17], and has also recently been shown to regulate cancer cell invasion[18]. According to previous researches, YBX1 have promotes malignant potentials in bladder cancer cell lines. In our study, we found that lncRNA MNX1-AS1 could interact with YBX1 and regulate transcription of β-catenin to active Wnt signaling pathway, which is reported relate to bladder cancer EMT process previously.

Results

LncRNA MNX1-AS1 is highly-expressed in bladder cancer and cell lines

Long non-coding RNAs (lncRNAs) govern fundamental biochemical and cellular processes, such as chromosome inactivation, genomic imprinting and development. To assess more important functional lncRNAs in bladder cancers, we checked the lncRNA-disease related database LncRNADisease v2.0 (http://www.cuilab.cn/lncrnadisease)[19] and found a latent, but not verified, functional lncRNA, MNX1-AS1. In addition, we researched another database platform ENCORI platform (http://starbase.sysu.edu.cn/)[20] and finding that lncRNA MNX1-AS1 is highly-expressed in bladder cancers (Fig. 1A). To further validate the results in database, we detect the expression of lncRNA MNX1-AS1 in bladder cancer tissue compared with adjacent tissues of bladder cancer patients, the results showing that the expressing of MNX1-AS1 is higher than adjacent tissues (Fig. 1B).

Accordingly, we detect the relationship of expression of MNX1-AS1 with prognosis of bladder cancer patients and results given that it was positive correlation with the poor prognosis (Fig. 1C). In addition, we collect bladder cancer cell lines and preformed qPCR analysis, the results showing that MNX1-AS1 is higher expressed in bladder cancer cell lines, for example, 5637, J82, ECV-304, BIU-87, H/RB-CL2, 253JB-V, HCV29, RT112, RT4, T24 and HTB-1, than human uroepithelial cell line SV-HUC-1 (Fig. 1D). In thus, these highly expressed indicating the lncRNA MNX1-AS1 might possess potential function in bladder cancer development and progression.

MNX1-AS1 is functional interact with YBX1 and YBX1 is highly expressed in bladder cancer tissues and cell lines

To further understand the mechanistic role of lncRNA MNX1-AS1 in bladder cancer development and progression, we analyzed the potential interact proteins with RBPDB database (http://rbpdb.ccbr.utoronto.ca/)[21] and find a classical RNA binding protein YBX1, there are two YBX1

binding motif sites, 568-573nt and 917-922nt, in the sequence of lncRNA MNX1-AS1. The binding motif is CCUGCG as showing in RBPDB database. To further identifying the interactions between MNX1-AS1 and YBX1, we performed RNA-pulldown assays using an in vitro transcribed MNX1-AS1 and cell lysates from T24 cells, as showing in Fig. 2A. Simultaneously, we employed an RNA immunoprecipitation (RIP) assays in T24 cell with anti-YBX1 antibody and followed by qRT-PCR assays (Fig. 2C), lncRNA HOXC-AS3 as positive control and these results potentiates the interaction of lncRNA MNX1-AS1 with YBX1.

To confirm the interaction of lncRNA MNX1-AS1 and YBX1, we employed another RIP assays in HTB-1 cells, this indicates the interaction of lncRNA CBR3-AS1 and β-catenin is prevalence in bladder cancer cells (Fig. 2C). In previous reports, bladder cancer patients with high YBX1 expression had poor prognosis[22]. While, YBX1 functional linked many biology progresses, for example cell growth, motility, invasion and resistance to cisplatin and doxorubicin[23]. However, the underlying mechanism of YBX1 promotes tumor progression is unclearly. Thus, we detect the expression of YBX1 in bladder cancer cell lines, and we find that YBX1 is highly expressed in these cell lines generally, such as 5637, J82, ECV-304, BIU-87, H/RB-CL2, T24, HCV29, RT112, RT4, 253JB-V and HTB-1 compared to SV-HUC-1 cell lines (Fig. 2D). In addition, YBX1 is highly expressed in bladder cancer tissues compared to its adjacent tissues (Fig. 2E). According to these results, the interactions between MNX1-AS1 and YBX1 and co-highly expressed in bladder cancer cells indicating possess potential function in bladder cancer development and progression

MNX1-AS1 promotes bladder cancer cell survival, invasion and migration along with YBX1

To further characterize the function of MNX1-AS1 in bladder cancer cell, we construct MNX1-AS1 sgRNAs according to Crispr Cas9 technology to knockout MNX1-AS1 in T24 cells and HTB1 cells through picking single cell colony, the expression of MNX1-AS1 is showing in Fig. 3A and Figure S1A. After that, we performed colony formation assays and the result showing that MNX1-AS1 knockout was associated with a reduced colony number of T24 cells (Fig. 3B). The patients of bladder cancer easily developed into muscle-invasive bladder cancer or metastatic bladder cancer and the cancer cells often appear an EMT character, so that, we performed transwell invasion assays, the results indicating that MNX1-AS1 depletion inhibited the invasion of T24 cells (Fig. 3C). In addition, wound healing scratch assays further consolidated the function of lncRNA MNX1-AS1 in promoting bladder cancer cell migration (Fig. 3D).

To further explore the function of YBX1 in bladder cancer cells, we transfect shRNA target YBX1 plasmids to T24 cells and performed colony formation assays and the result given that YBX1 knockdown decrease the colony number of T24 cells (Fig. 3F). Surely, our results consistent with recently report that YBX1 could promotes tumor growth through enhancing glycolysis of bladder cancer, but respect to the effect on EMT character of bladder cancer cells was not expounded. In thus, we performed transwell invasion assays upon knockdown YBX1 through shRNAs, the results indicating that YBX1depletion inhibited the invasion of T24 cells (Fig. 3G). Similarly, wound healing scratch assays further consolidated the function of YBX1 in promoting bladder cancer cell migration (Fig. 3H). To further character the relationship between lncRNA MNX1-AS1 and YBX1, we performed rescue assays through colony assays and transwell assays, the results showing that decreased colony number by MNX1-AS1 depletion could rescued by

overexpression of YBX1, the same function was explored in transwell assays (Fig. 3I, 3J and Figure S1B). In contrast, the reduced colony number and invasion ability of T24 cells induced by knockdown YBX1 could rescued by overexpressing MNX1-AS1 (Fig. 3K and 3L). In conclusion, MNX1-AS1 could interact with YBX1 and co-regulate bladder cancer cell survival, invasion and migration.

MNX1-AS1 combined with YBX1 to regulate WNT signaling pathway

In previous reports, YBX1 depletion attenuates the growth of basal-like breast cancer cell lines and some growth-promoting related genes was decreased according to chromatin immunoprecipitation (ChIP) analysis, such as, MET, CD44,CD49f, Wnt and NOTCH family members[24,25]. In thus, we examined the expression of Wnt signaling pathway related genes, the results given us that YBX1 depletion decreased the expression of Wnt signaling related genes, such as β-catenin, c-Myc, PYGO1, LGR5, AXIN2 and WNT1 genes, in T24 and HTB-1 cells (Fig. 4A and Figure S2A). In addition, to exploring the effect of MNX1-AS1 on Wnt signaling pathway, we analyzed the expression of these genes on MNX1-AS1 depleted genes, the results showing that down regulated MNX1-AS1 also could decreased the expression of Wnt signaling related genes, such as β-catenin, c-Myc, PYGO1/2, LGR5, AXIN2 and WNT1 genes, in T24 and HTB-1 cells (Fig. 4B and Figure S2B). Furthermore, the down regulated expression of β-catenin, c-Myc and AXIN2 genes by YBX1 depletion could rescued by overexpression MNX1-AS1 (Fig. 4C), in contrast, down-regulated β-catenin, c-Myc and AXIN2 genes expression by MNX1-AS1 depletion could rescued by overexpression YBX1 (Fig. 4D). These result indicated that MNX1-AS1 and YBX1 might cooperative regulate the expression of Wnt signaling pathway regulated genes. In thus, we performed Top/FopFlash reporter assays, in which pGL3–TopFlash reporter containing eight tandem repeats of TCF/LEF-binding elements and its counterpart pGL3–FopFlash reporter containing mutated TCF/LEF-binding elements (Figure. 4E), we co-transfected them into T24 cells together with YBX1 shRNA or MNX1-AS1 sgRNAs and Renila luciferase vector. Cells were cultured in the absence or presence of LiCl, which inhibits GSK3β and thus stabilizes β-catenin to activate the canonical Wnt pathway[26]. Reporter assays showed that YBX1 and MNX1-AS1 depletion resulted in a marked reduction of TCF/LEF reporter activity in responding to LiCl stimulation (Figure. 4E and Fig. 4F). Conclusion, these results suggest us that YBX1 and MNX1-AS1 could co-regulate Wnt signaling pathway.

MNX1-AS1 promote WNT signaling pathway through mediate YBX1 binding to promoter region of β-catenin

To further explore the effect of YBX1 on Wnt signaling pathway, we then performed hromatin immunoprecipitation (ChIP) assays through anti-YBX1 antibody. After that, precipitated DNAs were analyzed by quantitative PCR and the results showing that YBX1 was recruited to the promoter regions of β-catenin, Cyclin D1, c-Myc, AXIN2, WNT1 and WNT10B even bits of AXIN2 and WNT1 promoter region DNAs were immunoprecipitated (Fig. 5A). These given us YBX1 might act as a transcript factor to promoter these Wnt signaling pathway related genes. In addition, to further understand the function of MNX1-AS1 we compound antisense strand oligo of lncRNA MNX1-AS1 and it could effectively decrease the expression of MNX1-AS1 in T24 and HTB-1 cells (Fig. 5B and Figure S3A). In previous results, we verified the interaction between MNX1-AS1 and YBX1 and in order to explore the function of MNX1-AS1-

YBX1 axis in Wnt signaling pathway, we performed ChIP assays in MNX1-AS1 depleted cells and cultured in the absence or presence of LiCl. The results showed that YBX1 was recruited to the promoter regions of β-catenin genes under LiCl stimulation in control cells, whereas in MNX1-AS1-depleted cells, the recruitment of YBX1 on these promoters was significantly reduced (Fig. 5C). While, YBX1 was also recruited to the promoter regions of Cyclin D1, WNT10B, c-Myc, WNT1 and AXIN2 genes under LiCl stimulation in control cells, whereas in MNX1-AS1-depleted cells, the recruitment of YBX1 on these promoters was not changed (Fig. 5C and Figure S3B), these results given that knockdown MNX1-AS1 could inhibit the recruitment of YBX1 on the promoter region of β-catenin genes but not Cyclin D1, WNT10B, c-Myc, WNT1 and AXIN2 genes. Moreover, MNX1-AS1 might promote YBX1 binding to the promoter region of β-catenin to promote its transcription.

To further understand the function of MNX1-AS1, we transfect MNX1-AS1 ASO to knockdown MNX1-AS1 in T24 cells and we find that downregulated MNX1-AS1 could decrease the expression of β-catenin, but not CyclinD1, c-Myc and WNT10B, but the downregulation of β-catenin could rescued by overexpression of YBX1 (Fig. 5D). While, knockdown YBX1 decreased the expression of β-catenin, CyclinD1, c-Myc and WNT10B and these downregulation could not rescued by overexpression of MNX1-AS1, even overexpression MNX1-AS1 increased the expression of β-catenin, CyclinD1, c-Myc and WNT10B. Accordingly, these results strengthen the functional engagement of MNX1-AS1 promote YBX1 binding to the promoter region of β-catenin.

To investigate whether the influence of YBX1 on the transcriptional activity of the Wnt signaling was dependent on MNX1-AS1, we transfect Flag–YBX1 into MNX1-AS1-depleted T24 cells, then, luciferase reporter assays results showed that MNX1-AS1 depletion associated inhibition of reporter activity could be rescued YBX1 (Fig. 5E). In addition, overexpression YBX1 could also active luciferase reporter in control cells. Furthermore, the same results were verified in HTB-1 cells (Fig. 5F). These results support the notion that MNX1-AS1-promoted YBX1 recruitment onto the promoters of β-catenin to active the transcript of Wnt target genes.

MNX1-AS1/YBX1 signaling promote proliferation and invasion of bladder cancer through induce expression of β-catenin

It has been well known that the Wnt signaling pathway is implicated in cell proliferation, migration, invasion, and metastasis in various types of cancer[27], and we reported previously that YBX1 and MNX1-AS1 is up-regulated in bladder cancer. Thus, we next investigated whether MNX1-AS1/YBX1 signaling contributes in any way to bladder carcinogenesis. To this end, we developed T24 cells transcript with control or MNA1-AS1 ASO stably expressing control vector or β-catenin. Apoptosis assays showed that MNX1-AS1 knockdown was associated with an increased apoptosis percentage and an effect that could be ameliorated by overexpression of β-catenin (Fig. 6A). Similarly, the increased apoptosis percentage by YBX1 shRNAs could also rescued by overexpression of β-catenin (Fig. 6B). According to previous results, MNX1-AS1/YBX1 could promote the expression of β-catenin, the increased apoptosis percentage by MNX1-AS1 knockdown could also rescued by overexpression of YBX1 (Fig. 6C). In addition, we transwell assays further consolidated the functional link between MNX1-AS1/YBX1 promoted β-catenin

transcription and bladder cancer cell invasion (Fig. 6D and E), supporting a notion that MNX1-AS1/YBX1 promotes bladder cancer cell invasion through targeting β-catenin. Furthermore, cell growth viability assay further consolidate the results of MNX1-AS1/YBX1 promote the transcription of β-catenin to promote bladder cancer cell proliferation (Fig. 6F).

Discussion

Here, we find a new highly-expressed functional lncRNA in bladder cancer cells and bladder cancer patient tumor tissue. We identified that MNX1-AS1 could promotes the transcription of β-catenin to active the canonical Wnt signaling pathway and inducts the expression of its target genes, thus, regulates the proliferation, migration and invasion of bladder cells. In our results, RNA-pulldown and RIP assays identified the bona fide the interactions between MNX1-AS1 and YBX1, interestingly, this interaction could promote YBX1 binding to the promoter region of β-catenin to promote the transcription of β-catenin, furthermore, up-regulate the activating of Wnt signaling pathway. After that, activated Wnt signaling pathway increased the expression of its target genes, such as Cyclin D1, c-Myc and WNT10B, and to regulate the proliferation, migration and invasion ability of bladder cancer cells (Fig. 7).

LncRNAs are relatively mature characterized class of ncRNAs and functions in facilitating or inhibiting the development and progression of tumors, without doubt including bladder cancer cells[28,29]. In previous reports, even lncRNA MNX1-AS1 was be reported associate the proliferation and cell cycle of bladder cancer cell[30], but we find a novel mechanism of lncRNA MNX1-AS1 in regulating proliferation, migration and invasion through promote YBX1 binding to the promoter region act as a transcript factor to activating transcription of β-catenin, further activating Wnt signaling pathway. Refreshingly, we discovered a novel regulator of β-catenin in transcription level and enriched the Wnt signaling pathway in bladder carcinogenesis.

YBX1 is a versatile molecule that possesses multiple biological functions in both the nucleus and cytoplasm and regarded as a potential prognostic factor in bladder cancer[22,31]. According to previous reports, knock down YBX1 could decrease the expression of Myc and HIF1α in different mechanism[32], in transcriptional or post-transcriptional level, thus promote glycolysis of bladder cancer cells[33]. In our results, we uncovered the transcriptional regulation of YBX1 on c-Myc genes through associated with MNX1-AS1 to regulate Wnt signaling pathway and promote bladder cancer proliferation, migration and invasion. In thus, we are exploring the function of MNX1-AS1 in regulating bladder cancer glycolysis and there are many intrinsic mechanism need to be uncovered in bladder cancer diagnoses and treatments.

Methods

Antibodies and Reagents

The sources of antibodies against the following proteins were: β-actin (A1978, 1:10,000 for WB) from Sigma; β-catenin (51067-2-AP, 1:1,000 for WB); c-Myc (10828-1-AP, 1:1,000 for WB), Cyclin D1 (26939-1-AP,

1:1,000 for WB), WNT10B (67210-1-Ig, 1:1,000 for WB) from proteintech.

Plasmids

FLAG-YBX1, lncRNA MNX1-AS1 and FLAG-β-catenin were carried by pcDNA3.1 vector. TopFlash DNA fragment coding eight tandem repeats of TCF response elements, and FopFlash DNA fragment as a negative control, carrying eight mutant TCF tandem repeats, were synthesized and introduced to pGL3-promoter vector upstream of the SV40 promoter to generate pGL3-TopFlash and pGL3-FopFlash respectively.

Cell Culture

5637, J82, ECV-304, BIU-87, H/RB-CL2, 253JB-V, HCV29, RT112, RT4, T24, HTB-1 and SV-HUC-1 were got from the American Type Culture Collection (Manassas, VA) and cultured under the manufacturer's instructions. All of the cultured cells were authenticated by examination of morphology and growth characteristics, and were confirmed to be mycoplasma-free.

Western Blotting

Cells were lysed by Laemmli sample buffer (161–0737, BioRad), and re-suspending in 5 × SDS-PAGE loading buffer. The boiled protein samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane incubated with appropriately primary antibodies and secondary antibodies.

RNA-pulldown

The RNA pull-down assay was modified from previous studies [34]. Substrate RNAs were in vitro transcribed in a 200 μl reaction mix containing 1 μg of pcDNA3.1-CBR3-AS1 DNA detailed protocol as RiboMAX™ Large Scale RNA Production Systems–T7 manufacturer's instructions (P1300, Promega). In addition, transcribed RNAs produced 3' End Desthiobiotinylation using Pierce™ RNA 3' End Desthiobiotinylation Kit (20163, Invitrogen) and incubated with A549 cell lysed by IP lysis buffer (87787,Invitrogen) for RNA-pulldown assays as Pierce™ Magnetic RNA-Protein Pull-Down Kit (20164, Invitrogen).

RNA immunoprecipitation (RIP).

For A549 cells, 1×10^7 cells were harvested and crosslinked with 0.3% formaldehyde for 10 min at RT and quenched with 0.125 M glycine for 5 min. Nuclei were extracted and lysed in RIP Cross-Linked Lysis Buffer and product protocol supplied by Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit (17-10520, Millipore). In addition, RNAs were extracted with TRIzol and detected by qRT-PCR.

RNA Interference

In our studies, all ASOs were transfect to cells using Lipofectamine RNAiMAX (Invitrogen) reagents following the manufacturer's recommendations. The final concentration of the oligo molecules is 10 nM and cells were harvested 72 hours. Control oligo and the individual oligos against CBR3-AS1 were

chemically synthesized by GenePharma. The short hairpin RNAs (shRNAs) against YBX1 were expressed as lentiviral, purchased from GenePharma, transfected into appropriate cells. MNX1-AS1 sgRNAs were cloning into Cas9 sgRNA vector (68463, addgene). The sequences of ASOs, sgRNAs and shRNAs are provided in Supplementary Table 1.

qRT-PCR

Total cellular RNAs were isolated by TRIzol reagent (Invitrogen) and transcribed by the Reverse Transcription System (Roche). Quantitation of all gene transcripts was done by qPCR using a Power SYBR Green PCR Master Mix (Roche) and Q5 detection system (Thermo) with the expression of ACTB as the internal control. The primers used were listed in Supplementary Table 2.

Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed according to the procedure described previously. About 10 million cells were cross-linked with 1% formaldehyde and resuspended in SDS lysis buffer (1% SDS, 50 m M Tris-HCl, 5 m M EDTA, pH 8.1) in the presence of protease inhibitors and subjected sonication (Bioruptor, Diagenode) to generate chromatin fragments. Then the fragments were immunoprecipitation with indicated antibodies, after gently washed with washing buffer, the DNA were collected and performed qPCR analysis. The primers used were listed in Supplementary Table 3.

Colony Formation Assay

T24 cells stably expressing indicated genes or/and shRNAs or sgRNA were cultured for two weeks. After two weeks, the cells were washed and fixed with PBS and methyl alcohol respectively, then, stained with crystal violet (0.5% wt/vol). The number of colonies per well was counted.

Cell invasion assay

Transwell chamber filters (Chemicon Inc.) were coated with Matrigel. After transfection, cells were resuspended in serum free media, and 2.5×10^4 cells in 0.5 ml of serum-free media were placed in the upper chamber of the transwell. The chamber was then transferred to a well containing 500 µl of media containing 10% FBS. Cells were incubated for 18 h at 37 °C. Cells in the upper well were removed by wiping the top of the membrane with cotton swabs. The membranes were then stained, and the remaining cells were counted. Representative fields were captured and counted for each membrane.

Statistical Analysis

Experimental data from biological triplicate experiments are presented with error bar as mean ± S.D.. Two-tailed unpaired Student’s t-test was used for comparing two groups of data. Analysis of variance (ANOVA) with Bonferroni’s correction was used to compare multiple groups of data. A P value of less than 0.05 was considered significant and higher than 0.05 was considered no specific differences. All of the statistical testing results were determined by SPSS 22.0 software diagrams were conducted using GraphPad prism 8.0. Before statistical analysis, variation within each group of data and the assumptions of the tests were checked.

Declarations

DATA AVAILABILITY

All relevant data are available from the authors on request.

AUTHOR CONTRIBUTIONS

Hailong Hu and Dawei Tian conceived this project; Hailong Hu, Zhouliang Wu, Chong Shen, La Da, Gangjian Zhao and Songlin Zuo conducted experiments; Hailong Hu, Zhouliang Wu, Chong Shen, La Da, Gangjian Zhao, Songlin Zuo and Dawei Tian acquired and analysed data; Hailong Hu wrote the manuscript.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted with the approval of the ethics comittee of The Second Hospital of Tianjin Medical University. Documented informed consents were obtained from all patients.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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Figures

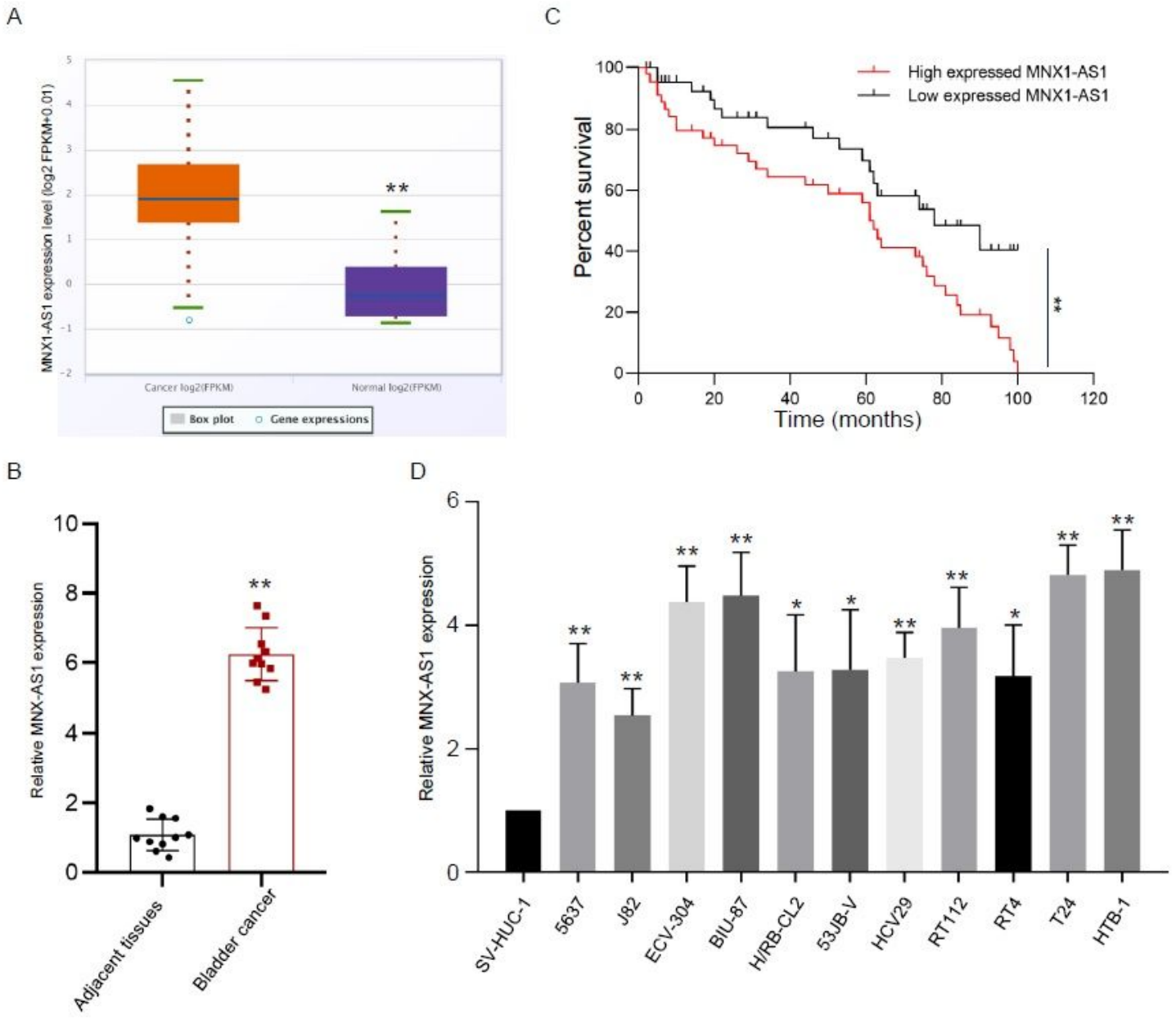

Figure 1

LncRNA MNX1-AS1 is highly-expressed in bladder cancer and cell lines. (A) LncRNA MNX1-AS1 is highly-expressed in bladder cancer tissues compared with normal bladder tissues data from LncRNADisease database. n=167 cancer and 10 normal samples. **P<0.01, one-way analysis of variance (ANOVA). (B) qRT-PCR analysis expression of LncRNA MNX1-AS1 in bladder cancer tissues compared to normal bladder tissues. n=10, **P<0.01, one-way analysis of variance (ANOVA). (C) Bladder cancer patients with lncRNA MNX1-AS1 high-expression have shorter overall survival compared to patients with lncRNA MNX1-AS1 low-expression. n = 46, **P<0.01, two-way ANOVA. (D) Levels of lncRNA MNX1-AS1 are elevated in bladder cancer cell lines compared with human uroepithelial cell line. 0.01<*P<0.05, **P<0.01, one-way ANOVA.

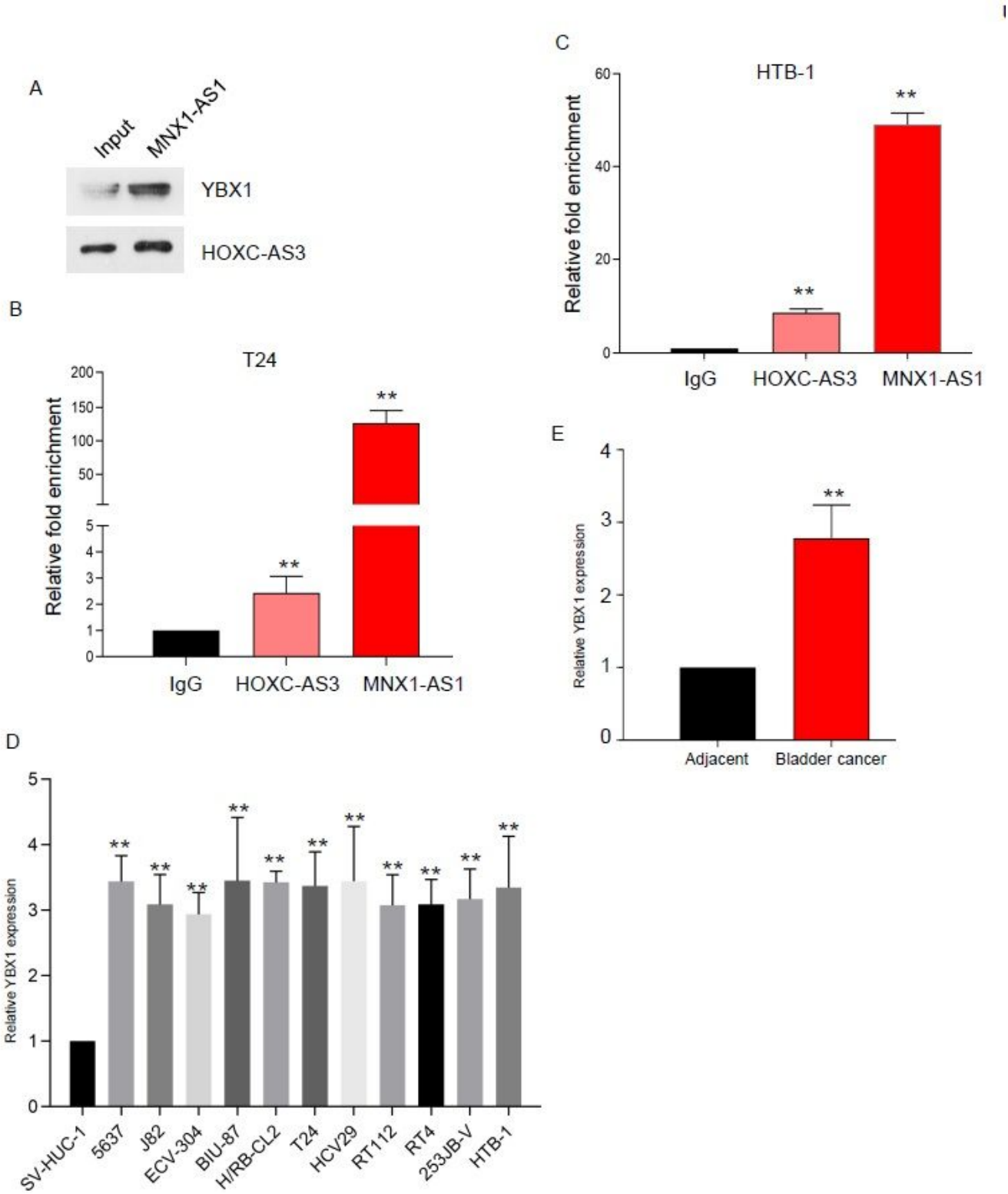

Figure 2

MNX1-AS1 is functional interact with YBX1 and YBX1 is highly expressed in bladder cancer tissues and cell lines. (A) LncRNA MNX1-AS1 RNA pull-down assay was examined by western blotting with antibody against YBX1. (B) RNA immunoprecipitation (RIP) assays with anti-YBX1 antibody and followed by qRT-PCR assays in T24 cells. **P<0.01, one-way ANOVA. (C) RNA immunoprecipitation (RIP) assays with anti-YBX1 antibody and followed by qRT-PCR assays in HTB-1 cells. **P<0.01, one-way ANOVA. (D) Levels of

YBX1 are elevated in bladder cancer cell lines compared with human uroepithelial cell line. 0.01<*P<0.05, **P<0.01, one-way ANOVA. (E) qRT-PCR analysis expression of YBX1 in bladder cancer tissues compared to normal bladder tissues. n=10, **P<0.01, one-way analysis of variance (ANOVA).

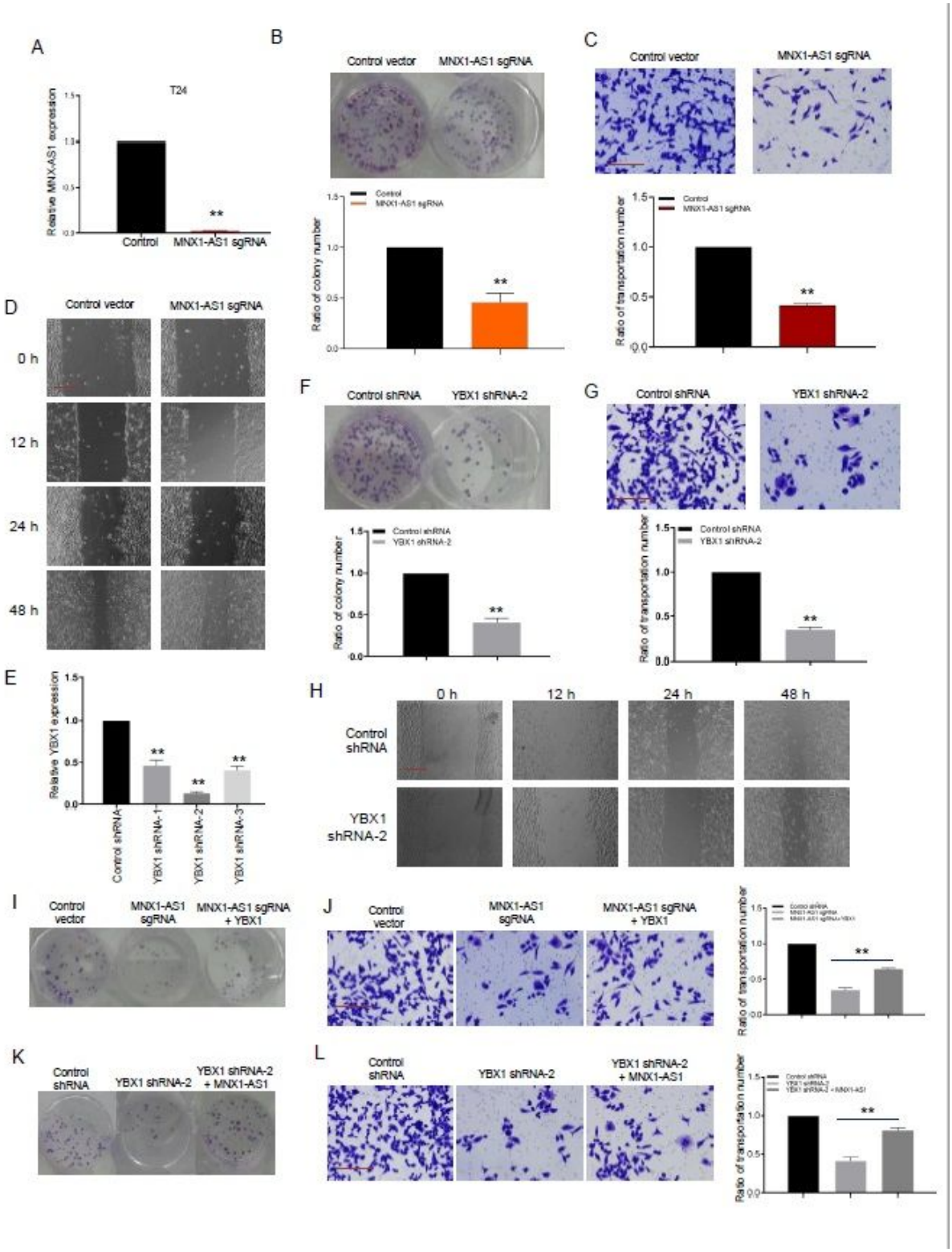

Figure 3

MNX1-AS1 promotes bladder cancer cell survival, invasion and migration along with YBX1. (A) qRT-PCR analysis the expressing of MNX1-AS1 in stable expressed MNX1-AS1 sgRNA in T24 cells, **P<0.01, one-

way ANOVA. (B) Colony formation assays with T24 cells stably expressing control sgRNA or MNX1-AS1 sgRNA and cultured for two weeks. Each bar represents the mean ± S.D. for biological triplicate experiments. ** P<0.01, one-way ANOVA. (C) T24 cells stably expressing control sgRNA or MNX1-AS1 sgRNA followed by transwell invasion assays. Each bar represents the mean ± S.D. for biological triplicate experiments. ** P<0.01, one-way ANOVA. Scale bar, 50 μm. (D) Wound healing scratch assays with T24 cells stably expressing control sgRNA or MNX1-AS1 sgRNA. Scale bar, 50 μm. (E) qRT-PCR analysis the expressing of YBX1 in stable expressed YBX1 shRNA in T24 cells, **P<0.01, one-way ANOVA. (F) Colony formation assays with T24 cells stably expressing control shRNA or YBX1 shRNA and cultured for two weeks. Each bar represents the mean ± S.D. for biological triplicate experiments. ** P<0.01, one-way ANOVA. (G) T24 cells stably expressing control shRNA or YBX1 shRNA followed by transwell invasion assays. Each bar represents the mean ± S.D. for biological triplicate experiments. ** P<0.01, one-way ANOVA. Scale bar, 50 μm. (H) Wound healing scratch assays with T24 cells stably expressing control shRNA or YBX1 shRNA. Scale bar, 50 μm. (I) Colony formation assays with T24 cells stably expressing control sgRNA or MNX1-AS1 sgRNA or co-transfected with FLAG-YBX1 and cultured for two weeks. (J) T24 cells stably expressing control sgRNA or MNX1-AS1 sgRNA or co-transfected with FLAG-YBX1 followed by transwell invasion assays. Each bar represents the mean ± S.D. for biological triplicate experiments. ** P<0.01, one-way ANOVA. Scale bar, 50 μm. (K) Colony formation assays with T24 cells stably expressing control shRNA or YBX1 shRNA or co-transfected with MNX1-AS1 and cultured for two weeks. (L) T24 cells stably expressing control shRNA or YBX1 shRNA or co-transfected with MNX1-AS1 followed by transwell invasion assays. Each bar represents the mean ± S.D. for biological triplicate experiments. ** P<0.01, one-way ANOVA. Scale bar, 50 μm.

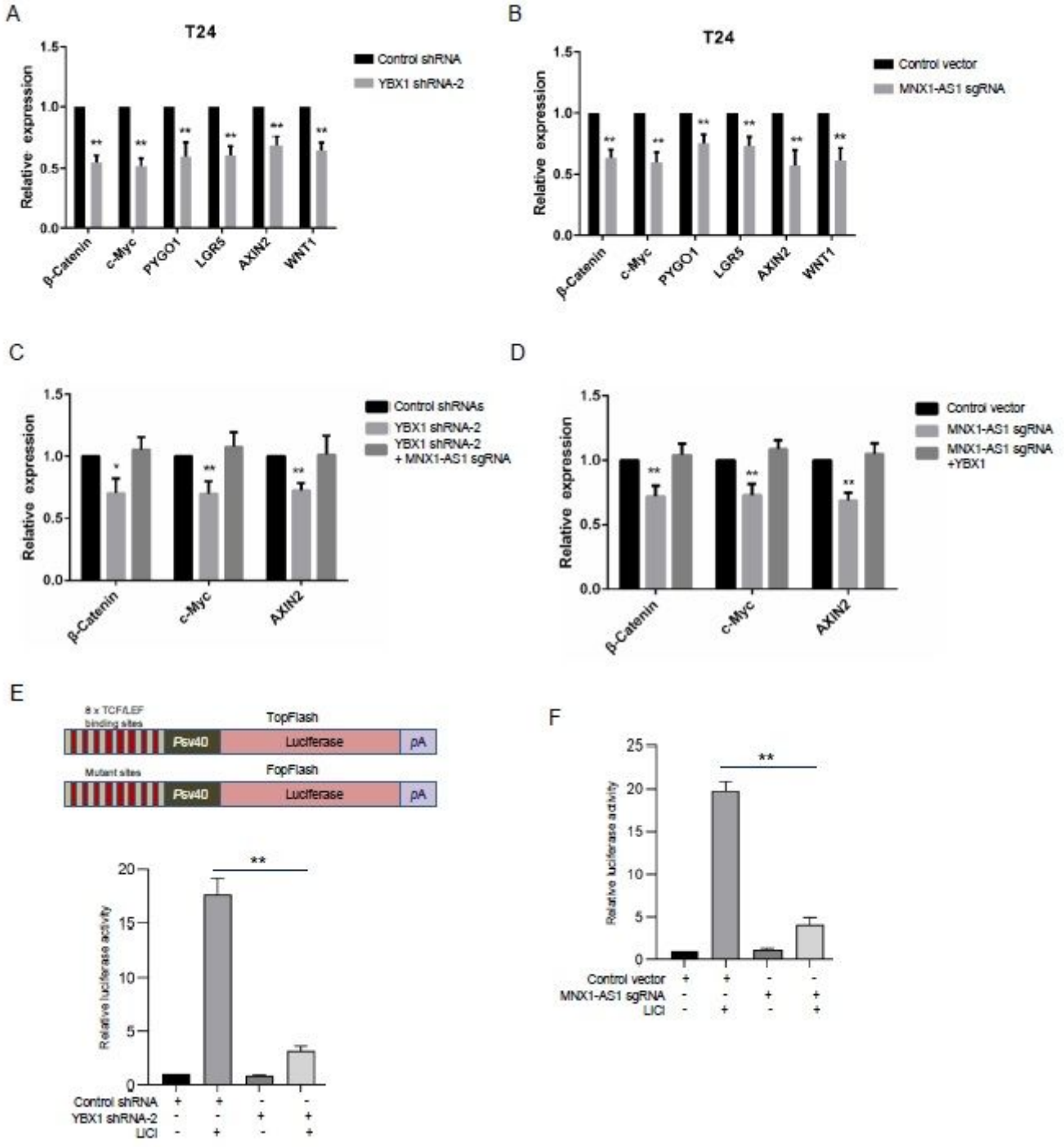

Figure 4

MNX1-AS1 combined with YBX1 to regulate WNT signaling pathway. (A) T24 cells were stably transfected with control shRNA or YBX1 shRNA and examined the expression of β-catenin, c-Myc, PYGO1, LGR5, AXIN2 and WNT1 genes by qRT-PCR assays. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (B) T24 cells were stably transfected with control sgRNA or MNX1-AS1 sgRNA and examined the expression of β-catenin, c-Myc, PYGO1, LGR5, AXIN2 and WNT1 genes by qRT-PCR assays. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (C) T24 cells were stably transfected with control shRNA or

YBX1 shRNA or YBX1 shRNA and MNX1-AS1 sgRNAs, examined the expression of β-catenin, c-Myc and AXIN2. Each bar represents the mean ± S.D. for biological triplicate experiments. 0.01<*P<0.05, **P<0.01, one-way ANOVA. (D) T24 cells were stably transfected with control vector or MNX1-AS1 sgRNAs or MNX1-AS1 sgRNAs, and YBX1, examined the expression of β-catenin, c-Myc and AXIN2. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (E) schematic diagrams of the TopFlash/FopFlash luciferase reporter constructs are as shown (upper panel). For reporter assays, T24 cells were co-transfected with control shRNA or YBX1 shRNA together with Renilla and pGL3–TopFlash or pGL3–FopFlash. These cells were cultured in the absence or presence of 25 m M LiCl for 6 h before measuring luciferase activity. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (F) T24 cells were co-transfected with control vector or MNX1-AS1 sgRNA together with Renilla and pGL3–TopFlash or pGL3–FopFlash. These cells were cultured in the absence or presence of 25 m M LiCl for 6 h before measuring luciferase activity. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA.

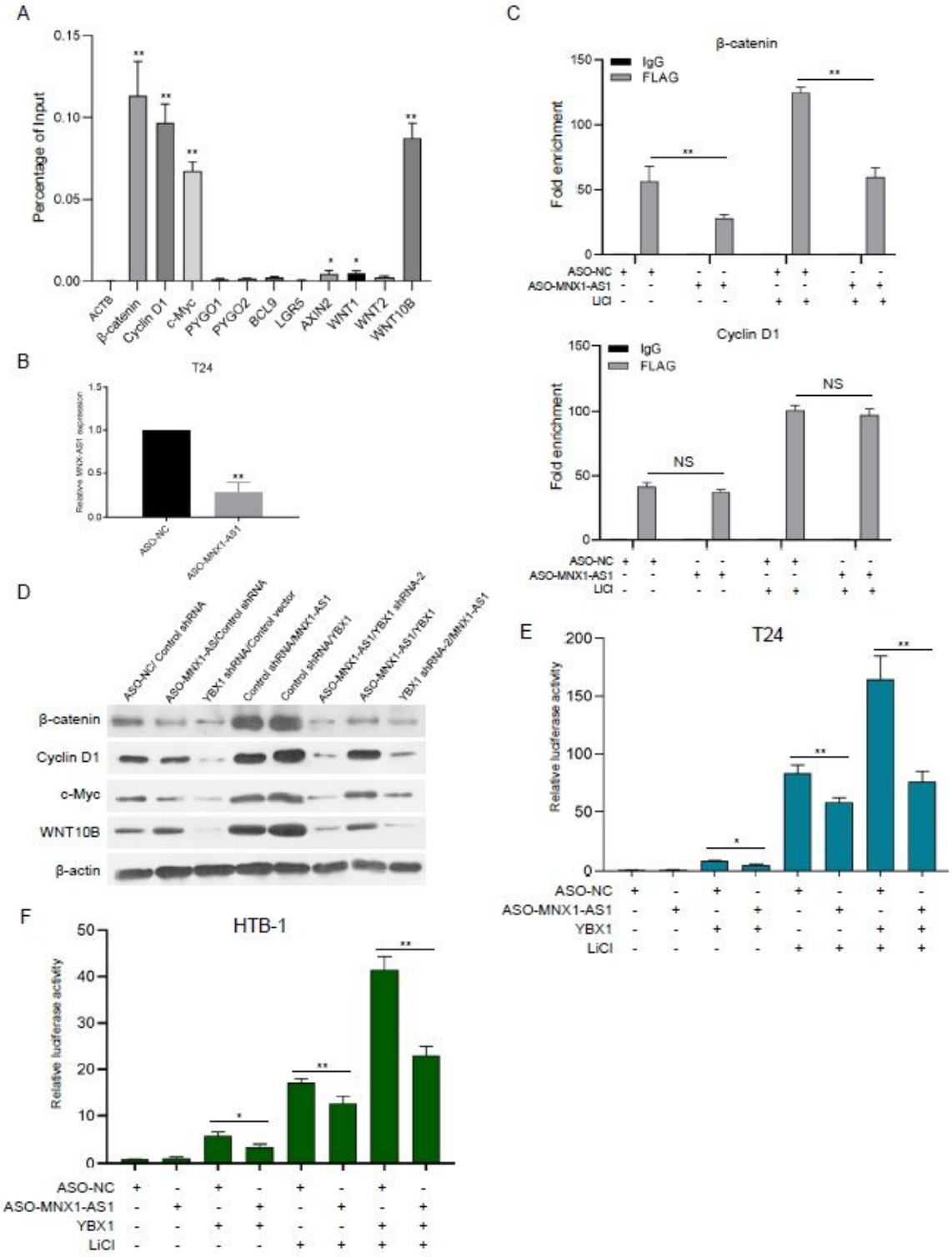

Figure 5

MNX1-AS1 promote WNT signaling pathway through mediate YBX1 binding to the promoter of β-catenin. (A) ChIP assays through anti-YBX1 antibody, the precipitated DNAs were analyzed by indicated genes qChIP primer. Each bar represents the mean ± S.D. for biological triplicate experiments. 0.01<*P<0.05, **P<0.01, one-way ANOVA. (B) qRT-PCR analysis the expression of MNX1-AS1 gene in T24 cells. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (C) Stably

expressed Flag-YBX1 T24 cells were transfected with control ASO or MNX1-AS1 ASO and cultured in the absence or presence of 25 m M LiCl for 6 h before followed by ChIP assays. Indicated gene primer were used. Each bar represents the mean ± S.D. for biological triplicate experiments. NS: nonsense. **P<0.01, one-way ANOVA. (D) T24 cells were transfected with indicated plasmids followed by Western blotting analysis through against with indicated antibodies. (E-F) T24 or HTB-1 cells were transfected with control or MNX1-AS1 ASO or Flag-YBX1 together with Renilla and pGL3–TopFlash or pGL3–FopFlash, and cultured in the absence or presence of 25 m M LiCl for 6 h before measuring luciferase activity. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA.

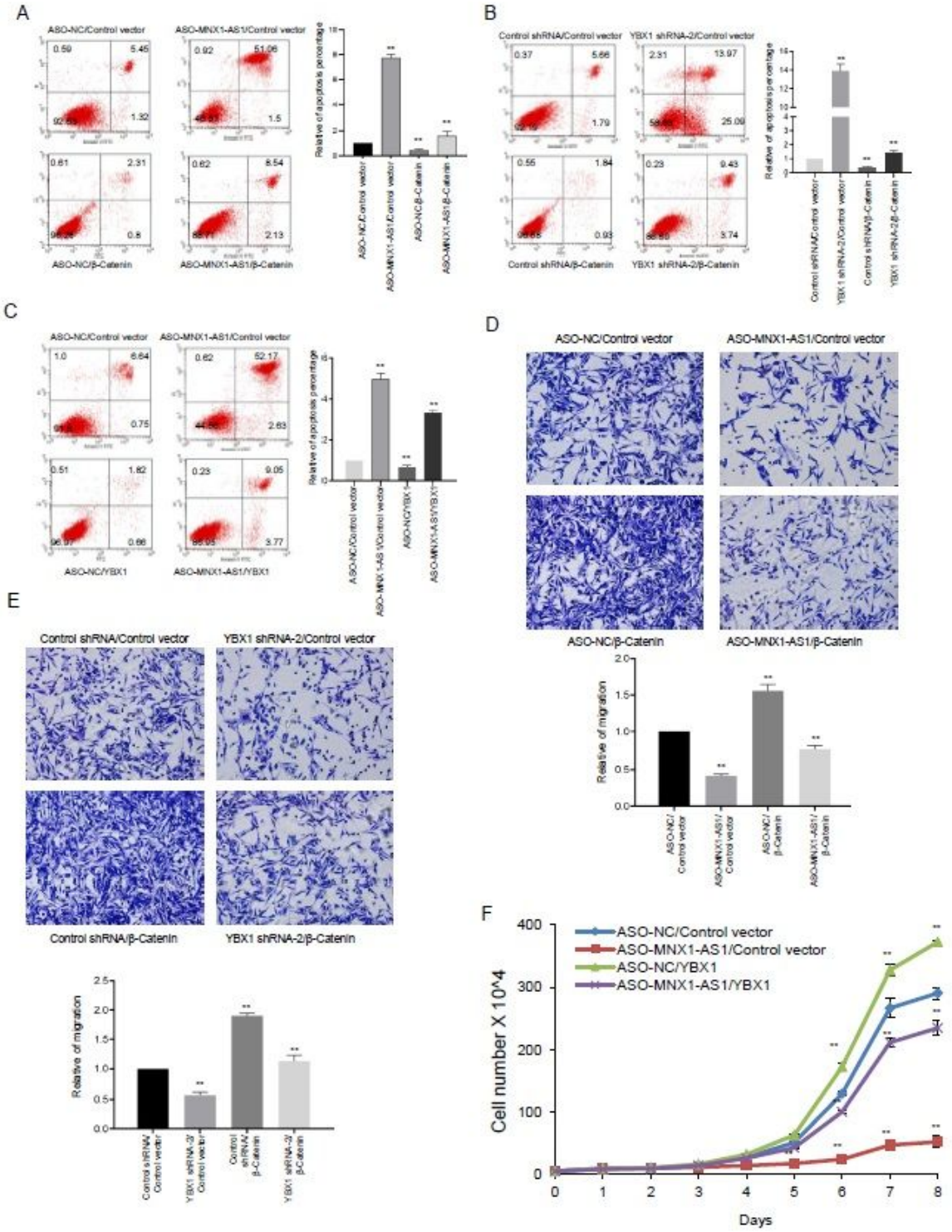

Figure 6

MNX1-AS1/YBX1 signaling promote proliferation and invasion of bladder cancer through induce expression of β-catenin. (A) T24 cells were transfected with control or MNX1-AS1 ASO or co-transfected with control vector or β-Catenin followed by apoptosis assays against with Annexin V and PI for flow cytometry. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (B) T24 cells were stable transfected with control or YBX1 shRNAs or co-transfected with control

vector or β-Catenin followed by apoptosis assays against with Annexin V and PI for flow cytometry. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (C) T24 cells were transfected with control or MNX1-AS1 ASO or co-transfected with control vector or YBX1 followed by apoptosis assays against with Annexin V and PI for flow cytometry. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (D) T24 cells were transfected with control or MNX1-AS1 ASO or co-transfected with control vector or β-Catenin followed by followed by transwell invasion assays. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA. (E) T24 cells were transfected with control or MNX1-AS1 ASO or co-transfected with control vector or YBX1 followed by cell growth assays. Each bar represents the mean ± S.D. for biological triplicate experiments. **P<0.01, one-way ANOVA.

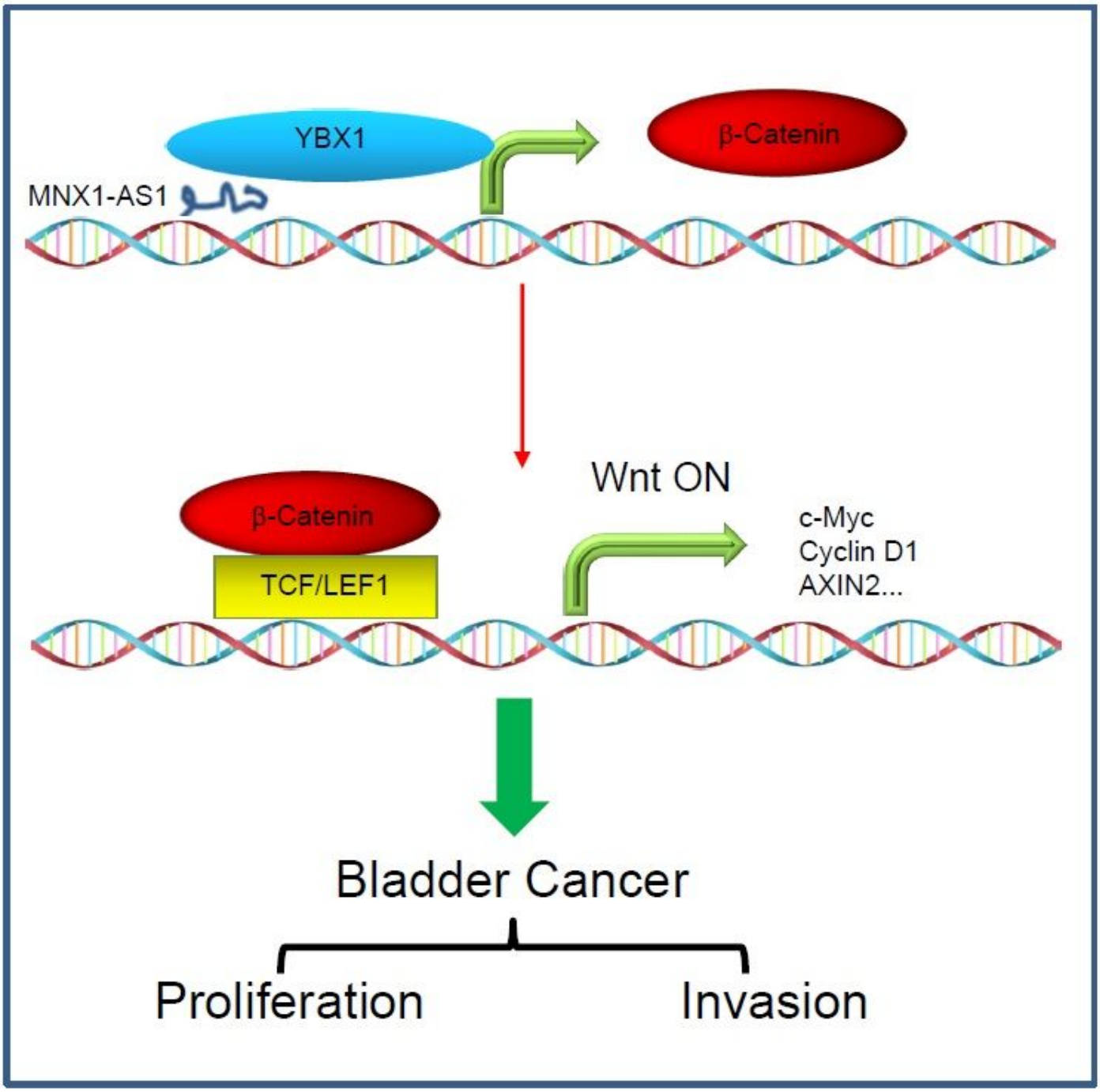

Figure 7

MNX1-AS1 and YBX1 in Wnt/β-catenin signaling and bladder cancer prolifereation and invasion. lncRNA MNX1-AS1 interacts with YBX1 target promoter region of β-catenin genes. Then they promote the transcriptional activation of β-catenin and lead to active Wnt signaling pathway target genes, thereby potentiating bladder cancer cell proliferation and invasion.

Supplementary Files

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