TBX3 stimulates proliferation and stem cell self-renewal in bladder carcinoma

Lifu Huang[1,5], Wenfei Shao[2,5], Xiaohong Wang[3,5], Feiping Li[1,5] and Weijun Mao[4,5]

[1]Department of Urology, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, [2]Health Management Center, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, [3]Department of Obstetrical, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, [4]Department of Central Sterile Supply, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Linhai City and [5]Enze Hospital, Taizhou Enze Medical Center (Group), Taizhou City, Zhejiang Province, China

Summary. Background. With the change of people's lifestyle in recent years, bladder carcinoma has been the second leading cause of death for men. Nevertheless, surgical results of bladder carcinoma are unsatisfying with recurrence and distant metastasis. Therefore, it is urgent to find a new target for bladder carcinoma treatment.

Methods. The protein and mRNA expression levels of TBX3 in bladder carcinoma tissue samples and cells were tested using western blot and qRT-PCR assays, respectively. Cancer stem cells (CSCs) were separated with immunomagnetic beads. Expression levels of cell stemness-associated proteins CD44, CD24 and ESA in T24 CSCs and T24 cells were detected by western blot assay. Cell self-renewal ability was detected by stem cell sphere formation assay. CCK-8 and colony formation assays examined cell viability and proliferation. Cell apoptotic level was examined by flow cytometry.

Results. Elevated TBX3 expression in bladder carcinoma stimulated cell proliferation and inhibited cell apoptosis. Stemness-related proteins and TBX3 were highly expressed in T24 CSCs relative to those in normal bladder carcinoma cells. In addition, TBX3 promoted stem cell self-renewal and inhibited cell apoptosis. Finally, qRT-PCR, western blot and cell sphere formation assays revealed that the potential role of TGF-β1 in the regulation of TBX3.

Conclusion. TBX3, mediated by TGF-β1, can promote bladder carcinoma cell proliferation, inhibit apoptosis, and enhance cell stemness. Hence, TBX3 is a potential target to stem cells of bladder carcinoma.

Corresponding Author: Weijun Mao, Department of Central Sterile Supply, Taizhou Hospital of Zhejiang Province affiliated to Wenzhou Medical University, 150 Ximen Street, Linhai City, Zhejiang Province, 317000, China, Enze Hospital, Taizhou Enze Medical Center (Group), No. 1 East of Tongyang Road, Luqiao District, Taizhou City, Zhejiang Province, 318050, China. e-mail: maoweijun_mwj@163.com

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Introduction

Bladder carcinoma is common in men and is the second leading cause of cancer deaths (Murta-Nascimento et al., 2007; Torre et al., 2015). Currently, the incidence of bladder carcinoma has been the highest among tumors in the urinary system due to environmental deterioration, diet structure adjustment and lifestyle changes (Yang et al., 2004). Despite active treatment, 50-60% bladder carcinoma patients still develop recurrence or distant metastasis within 2 years of surgery (Hussain and James, 2003). Unravelling bladder carcinoma pathogenesis holds crucial value for clinical diagnosis and treatment presently.

Cancer stem cells (CSCs) are important for tumor progression (Nassar and Blanpain, 2016). CSCs generate two cells through asymmetric division: stem cells with self-renewal ability; progenitor cells that can differentiate into various cells to constitute tumors (Visvader and Lindeman 2008, 2012). Accumulating evidence has proved that CSCs can directly explain tumor occurrence, development, recurrence, and metastasis (Reya et al., 2001; Birkeland et al., 2015). For instance, studies illuminated that CK14 (a biomarker of bladder carcinoma stem cells) was abnormally highly expressed in muscle invasive bladder carcinoma, and positively correlated with tumor recurrence and deterioration, thus working as an independent risk factor to predict the recurrence risk of bladder carcinoma (Jung et al., 2020). $CD44^+CD24^{-/low}$ cells are identified as stem cells in breast cancer, which can lead to tumors in the immuno-compromised mouse (Al-Hajj et al., 2003). Results of CSCs research elaborate malignant behaviors like tumor progression, recurrence and metastasis in a new way. Besides, treatment targeting CSCs casts a

novel light for cancer patients. Hence, we hope to find a gene that can adjust bladder carcinoma cell stemness in this study.

The TBX gene family, also called T-box, is an essential gene family in the early stages of embryonic development that regulates development-related transcription factors (Herrmann et al., 1990). TBX3 (T-box3) is a research concern because of its carcinogenesis and ability to maintain stem cells (He et al., 1999). A report showed that TBX3 upregulation was closely associated with metastasis of cervical cancer (Lyng et al., 2006). TBX3 was also closely related to pancreatic cancer progression because of its high expression in metastatic pancreatic endocrine tumors (Hansel et al., 2004). TBX3 expression was higher in melanoma cell lines relative to that in normal melanocytes (Peres and Prince, 2013; Peres et al., 2015). The above studies indicated that TBX3 is closely relevant to tumor occurrence. Furthermore, TBX3 also plays a significant role in CSCs development. TBX3 participates in stem cell development and stimulates cell stemness through the NODAL/ACTIVIN signaling pathway in pancreatic cancer (Perkhofer et al., 2016). However, the impact of TBX3 on CSCs and biological functions of bladder carcinoma has been less well defined.

CSCs take their strong oncogenic ability as a representative feature, which is similar to the clinical feature of bladder carcinoma. In addition, TBX3 was reported to be highly expressed in tumor tissue in bladder carcinoma, and affected the biological function of tumors. Based on the previous known background, we researched here whether TBX3 correlates with malignant behaviors of bladder carcinoma and regulates stem cell properties.

Materials and methods

Clinical samples

Altogether, all samples were frozen in liquid nitrogen immediately following isolation. Inclusion criteria: (1) Resected samples were pathologically and histologically identified as bladder carcinoma; (2) with no other treatments before surgery and with signed informed consent; (3) with specific pathological stage. This project had been approved by ethic committee of Taizhou hospital before conduct. The Medical Ethics Committee of Taizhou Enze Medical Center (Group) Luqiao Hospital unanimously agreed that it was in line with medical ethics and approved it.

Cell culture

Human bladder carcinoma cells T24 (BNCC311582) and BIU-87 (BNCC100982) and immortalized bladder epithelial cells SV-HUC-1 (BNCC100273) were offered by BeNa Culture Collection (China). Human bladder carcinoma cells UMUC3 (ATCC® CRL-1749™) were supplied by American Type Culture Collection (ATCC;

USA). T24 and BIU-87 cells were preserved in RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA). UMUC3 cell line was cultured in DMEM (Gibco, USA) containing 10% FBS supplemented with 2 mM L-glutamine and 1% streptomycin/penicillin. Immortalized bladder epithelial cells SV-HUC-1 were cultured in CM7-1 medium (Thermo Scientific, USA) containing 90% F-12K+10% FBS. All cell cultures were performed in a temperature incubator under common culture conditions. For TGF-β1 treatment, we used 5 ng/ml rhTGF-β1 (R&D Systems, USA) diluted in 4 mM HCl and 1 mg/ml bovine serum albumin.

CSCs sorting and culture

T24 cells were digested and prepared into single cell suspension until cultured to 80% confluence. Then, cells were counted and suspended in buffer containing phosphate buffer saline (PBS), 2 mM edetic acid and 0.5% bovine serum albumin (BSA) at pH 7.2. Cells were then grown with rabbit anti-CD44 (1:1000, ab189524, abcam, UK), rinsed and resuspended. Goat anti-rabbit secondary antibody (Miltenyi Company, Germany) was added for further incubation. A separation column was placed in the magnetic field, and cells following buffer suspension were loaded to the column. Following that, the separation column was rinsed with buffer to elute negative cells. Then, the magnetic field was removed and the separation column was washed quickly. The eluted cells were CD44^{+} cells. CD44^{+} cells were grown in RPMI-1640 not supplemented with serum. Every 3 d, the medium was changed once. The obtained cells were T24-derived CSCs.

Plasmids construction and cell transfection

Geneseed Biotech (Guangzhou, China) provided lentivirus-mediated TBX3 short hairpin RNA (sh-TBX3) and negative control (sh-NC). T24 cells and T24 CSCs were infected with lentivirus with polybrene (8 ng/ml, Sigma). PCR amplification of the complete sequence of TBX3 (TBX3iso 1) cDNA used Phusion Flash High Fidelity PCR Master Mix (Thermo Scientific, USA). Next, the sequence was subcloned to pcDNA3.1 plasmid to build overexpressed TBX3 (oe-TBX3), and its NC vector (vector) was sequentially established. After lentivirus infection, T24 and T24 CSCs were treated with 1 mg/ml puromycin to get stably transfected cell lines. There were 24h of cell cultures before cell transfection. Cells were transfected when cells grew to 70-80% fusion. After 48h, cells were collected.

Total RNA extraction and qRT-PCR

Total RNA was extracted from cells through RNAiso Plus (Takara, Japan). cDNA was synthesized by reverse transcription kit (Takara, Japan) after RNA concentration was determined. qRT-PCR was conducted on 7500 Real-

Time PCR Systema by SYBR Green Master Mix Kit. Target TBX3 mRNA expression was standardized by GAPDH. Melting curve was applied to monitor non-specific amplifications. The $2^{-\Delta\Delta Ct}$ method calculated the relative expressions. Each PCR amplification was repeated 3 times in a bid to testify the results. Primers of target gene TBX3 and internal reference GAPDH in this study are in Table 1.

Western blot assay

Transfected T24 cells and T24 CSCs were washed with cold PBS twice. Afterwards, cells were exposed to 500 μL RIPA buffer containing PMSF and placed on ice for 30 min. Next, 15 min centrifuge running of cell lysates were conducted at 14000 g and then the supernatant was maintained at -80°C. Concentration of proteins was measured by BCA test kit (Thermo Scientific, USA). The prepared proteins were boiled for 5 min and sequentially separated through 12% SDS-PAGE. Next, proteins were transferred onto a PVDF membrane activated in methanol. Western blot was undertaken on the membrane firstly by adding rabbit anti-TBX3 (1:1000, ab154828, abcam, UK), rabbit anti-CD44 (1:1000, ab189524, abcam, UK), rabbit anti-CD24 (1:1000, ab179821, abcam, UK), rabbit anti-ESA (1:1000, ab181988, abcam, UK) and rabbit anti-GAPDH (1:2500, ab9485, abcam, UK) at 4°C overnight. Thereafter, the membrane was cultured with specified horseradish peroxidase (HRP) conjugated with secondary antibody goat anti-rabbit IgG (1:5000, ab205718, abcam, UK) for 1-2h. Protein abundance was standardized by GAPDH. Enhanced chemiluminescence (ECL, Beyotime, China) was applied on the membrane and autoradiography was performed. The experiment was repeated three times. Band density was determined by optical density analysis.

Colony formation and CCK-8 assays

Following 0, 24, 48, 72 and 96h of transfection, viability of cells was measured by CCK-8 (Dojindo, Japan). The transfected T24 cells were added to plates (2×10^3 cells/well). Meanwhile, 10 μL CCK-8 reagent was filled in each well for cell incubation. Then, absorbance at 450 nm was measured by a microreader (Thermo Fisher Scientific, USA).

For colony formation examination, the transfected T24 cells (5×10^2 cells/well) were first inoculated in

Table 1. Primer sequence.

Target gene	Primer (5'-3')
TBX3	F: TTCCACATTGTAAGAGCCAATG
	R: CTTTGAGGTTCGTTGTCCCTAC
GAPDH	F: TGCACCACCAACTGCTTAGC
	R: GGCATGCACTGTGGTCATGAG

plates for further standard culture. Two weeks later, 4% paraformaldehyde and 0.5% crystal violet (Meilunbio, China) were applied for fixation and staining at room temperature for 30 min, respectively. The remaining crystal violet was washed off and cell colonies were counted under a microscope (Colonies ≥50 cells were counted).

Flow cytometry

T24 bladder carcinoma cells and T24 CSCs were stained with Annexin PI kit/V-FITC (BD, USA). FACS Calibur (BD, USA) was used to detect cell apoptosis. Flowjo software (BD, USA) was applied for analysis of apoptosis rate.

Stem cell sphere formation assay

Transfected T24 CSCs (2×10^3 cells/well) were cultured in DMEM/Ham's F12 50/50 mixture (Mediatech, China), supplemented with 20 ng/mL fibroblast growth factors (Peprotech, USA), B-27 (Life Technologies, USA), and 20 ng/mL epidermal growth factors (Peprotech, USA). Cells were cultured for 14 d in an ultra-low absorption plate (Corning, USA). Every other day, the medium was changed. An inverted phase contrast microscope was applied for evaluation of sphere formation. Lastly, NIS Elements viewer (Nikon Instruments, Japan) was applied to count spheres with a diameter more than 100 μm.

Statistics processing

SPSS 17.0 software (SPSS Inc, USA) and GraphPad Prism 6 software (San Diego, CA) were utilized for analyzing data (mean ± standard deviation). Differences between two groups were analyzed by two-tailed t test. All data were presented as $p<0.05$ stood for statistical significance.

Results

TBX3 is highly expressed in bladder carcinoma tissue and cells

Previous studies reported that TBX3 was significantly highly expressed in various cancers (Fischer and Pflugfelder, 2015). However, little has been reported about it in bladder carcinoma, and thus TBX3 was chosen for research in our study. Firstly, qRT-PCR was performed on 10 pairs of collected normal bladder tissue and bladder carcinoma tissue samples, and it was discovered that the mRNA expression level of TBX3 in bladder carcinoma tissue was higher than that in normal bladder tissue (Fig. 1A). The results of qRT-PCR and western blot revealed that TBX3 mRNA and protein expression levels were significantly elevated in bladder carcinoma cells (Fig. 1B,C). Moreover, differential expression of TBX3 was the most significant in T24

cells. Therefore, T24 cells were selected for following experiments. These results indicated TBX3 was remarkably upregulated in bladder carcinoma.

TBX3 stimulates cell proliferation while hampers cell apoptosis of bladder carcinoma

To investigate the biological functions of TBX3 in bladder carcinoma, T24 cell line was transfected with overexpressed TBX3 (oe-TBX3) or silenced TBX3 (sh-TBX3). qRT-PCR and western blot examined transfection efficiency (Fig. 2A,B). Cell proliferation in each transfection group was measured by CCK-8 and colony formation. It was found that TBX3 overexpression stimulated cancer cell proliferation, while inhibiting TBX3 expression suppressed cancer cell proliferation (Fig. 2C,D). Apoptotic rate of bladder carcinoma cells was detected by flow cytometry. It was shown that high TBX3 expression reduced cell apoptotic rate while low TBX3 expression elevated the cell apoptotic rate (Fig. 2E). All the above experiments confirmed that TBX3 worked as a cancer promoter in bladder carcinoma.

TBX3 expression is upregulated in CSCs and promotes cell stemness in bladder carcinoma

Firstly, we obtained CD44^{+} marked T24 CSCs through immune magnetic activated cell sorting. It was illuminated that stemness-related protein was significantly upregulated in T24 CSCs (Fig. 3A). This suggested that the separation of T24 CSCs was successful, and the CSCs could be used for following experiments. Afterwards, TBX3 mRNA expression level in T24 CSCs and normal T24 cells was tested by qRT-PCR. It was illustrated that mRNA expression of TBX3 was upregulated in T24 CSCs (Fig. 3B). T24 CSCs were then subjected to transfection with oe-TBX3 or sh-TBX3. As analyzed, TBX3 mRNA and protein expression in the oe-TBX3 group was remarkably upregulated, while in sh-TBX3 group TBX3 expression was downregulated relative to the control group (Fig. 3C,D). The result of colony formation assay showed that overexpression of TBX3 was able to promote the colony formation ability of T24 CSC, while knockdown TBX3 exhibited the opposite effect (Fig. 3E). Self-renewal of stem cells in each transfection group was detected by stem cell sphere formation assay. It was found that tumor sphere formative rate was higher in oe-TBX3 group relative to NC group, while the rate was markedly inhibited in the sh-TBX3 group (Fig. 3F). Additionally, CD44, CD24 and ESA protein expression levels were found to be upregulated while apoptosis was inhibited upon TBX3 overexpression. However, sh-TBX3 transfection caused the opposite results (Fig. 3G,H).

The expression of various stemness markers have already been identified in bladder cancer (Amini et al., 2014). Li et al. (2013) demonstrated that the TGF-β1 pathway up-regulates TBX3 protein and mRNA levels in breast epithelial cells. To elucidate whether transcription factors regulate TBX3 in bladder carcinoma, we devised a series of experiments. The results of TGF-β1 treatment showed that the mRNA and protein expression levels of TBX3 were significantly increased (Fig. 3I,J). It was found that tumor sphere formative rate was higher in TGF-β1 treatment group relative to NC group, while the
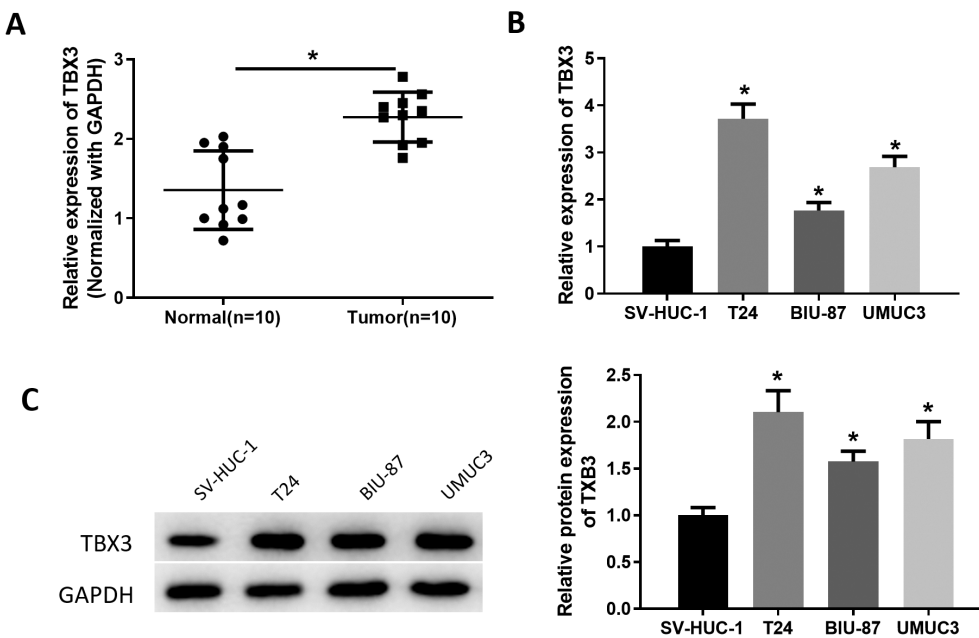

Fig. 1. TBX3 is highly expressed in bladder carcinoma tissue and cells. **A, B.** qRT-PCR detected TBX3 mRNA expression level in tissue and cells. **C.** Western blot detected TBX3 protein expression in bladder carcinoma and normal bladder cells, and the quantification of the blots. *$p<0.05$.

rate was markedly inhibited in the sh-TBX3 group (Fig. 3K). The above results suggested that the expression of TBX3 was higher in bladder carcinoma stem cells. Meanwhile, TBX3 mediated by TGF-β1 promoted the self-renewal capacity of bladder carcinoma stem cells while inhibiting their apoptosis level.

Discussion

Bladder carcinoma is the main threat for male health worldwide and has annually increasing incidence and mortality (Ploeg et al., 2009). Surgical resection is a preferred option of most bladder carcinoma patients, accompanied with regional infusion chemotherapy. However, bladder carcinoma can still recur up after 2 years of surgery, and more than half of the patients will undergo recurrence after initial lesion resection (Kaufman et al., 2009). Hence, it is of clinical significance to identify and screen biomarkers that can predict the recurrence of bladder carcinoma through molecular biology.

TBX3 is normally involved in transcriptional regulation of embryonic stem cell development, but is also abnormally hyper-expressed in some tumors and associates with malignant behaviors (Amini et al., 2014). Wang et al. (2015) elaborated that TBX3 overexpression was significantly correlated with poor prognosis of pancreatic cancer patients. Wu et al. (2017) proved that TBX3 protein expression was downregulated and markedly inhibited cell proliferation, invasion and metastasis in lung cancer. Since TBX3 is rarely studied in bladder carcinoma, we illustrated that TBX3 was highly expressed in bladder carcinoma tissue and cells. Cell functional assay further demonstrated that TBX3 acts as a cancer promoter in bladder carcinoma. The results were consistent with previous studies.

Moreover, TBX3 expression is also closely related to stem cells. For example, TBX3 was reported to maintain self-renewal of embryonic stem cells (Ivanova et al., 2006). Similar to the feature of stem cells, non-
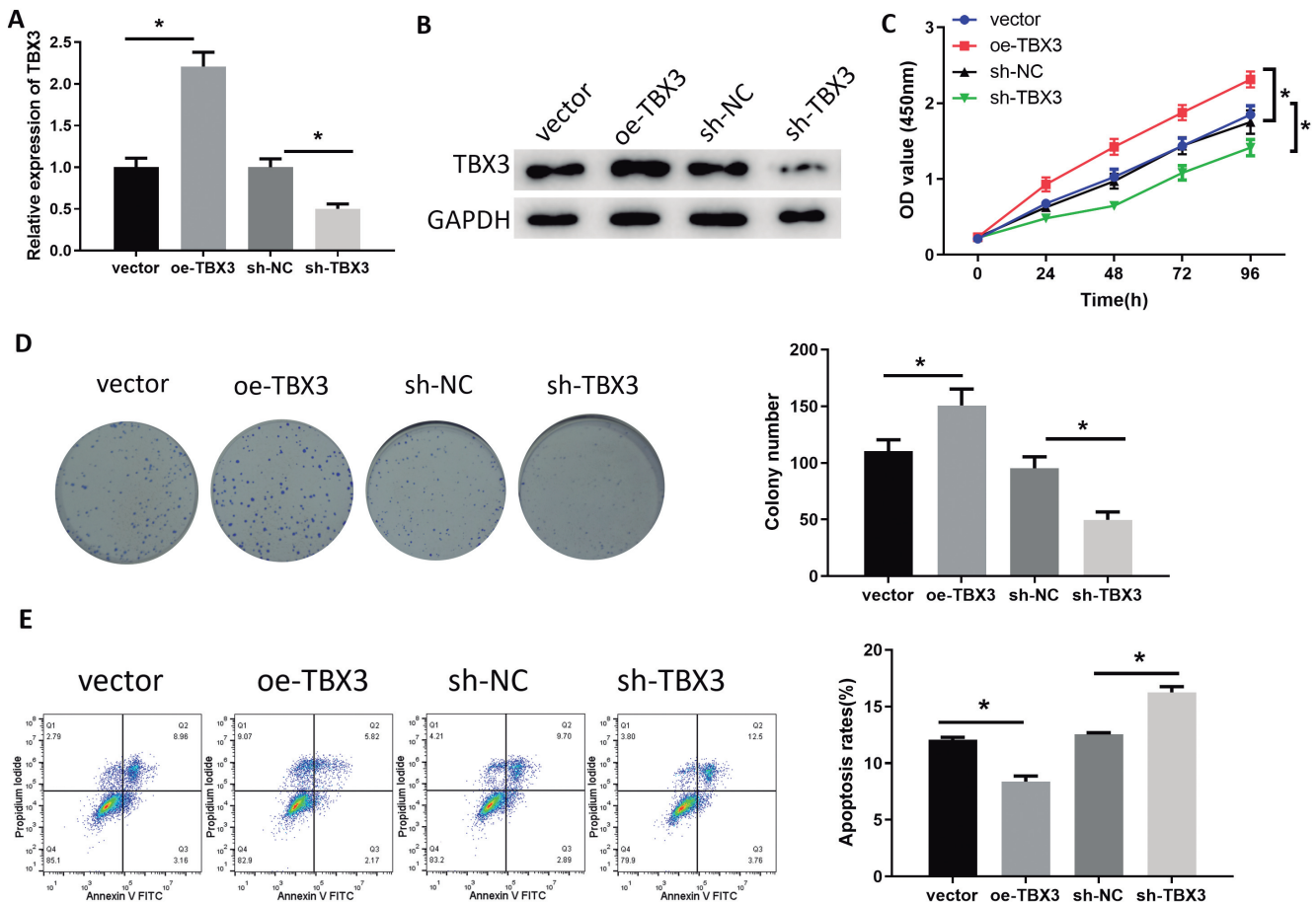

Fig. 2. TBX3 stimulates cell proliferation while hampers cell apoptosis of bladder carcinoma. **A, B.** Transfection efficiency in vector, oe-TBX3, sh-NC and sh-TBX3 groups was tested by qRT-PCR and western blot. **C.** CCK-8 detected the proliferative viability of bladder carcinoma cells in each treatment group. **D.** Colony formation assay detected the colony formative ability of cells in each treatment group. **E.** Flow cytometry detected cell apoptotic level in each treatment group. *$p < 0.05$.

controlled self-renewal is a crucial mechanism of tumor occurrence (Cho and Clarke, 2008). Therefore, it is posited that stem cells may be an initial cause of bladder carcinoma recurrence, metastasis and infinite proliferation. Here, the mRNA expression level of TBX3 in T24 CSCs and common T24 cells was detected, and

we found TBX3 was highly expressed in stem cells in bladder carcinoma. Furthermore, it was confirmed that high TBX3 expression could stimulate the self-renewal of stem cells while inhibiting their apoptosis in bladder carcinoma. A study elaborated that there are various tumor-related proteins on the surface of CSCs to be
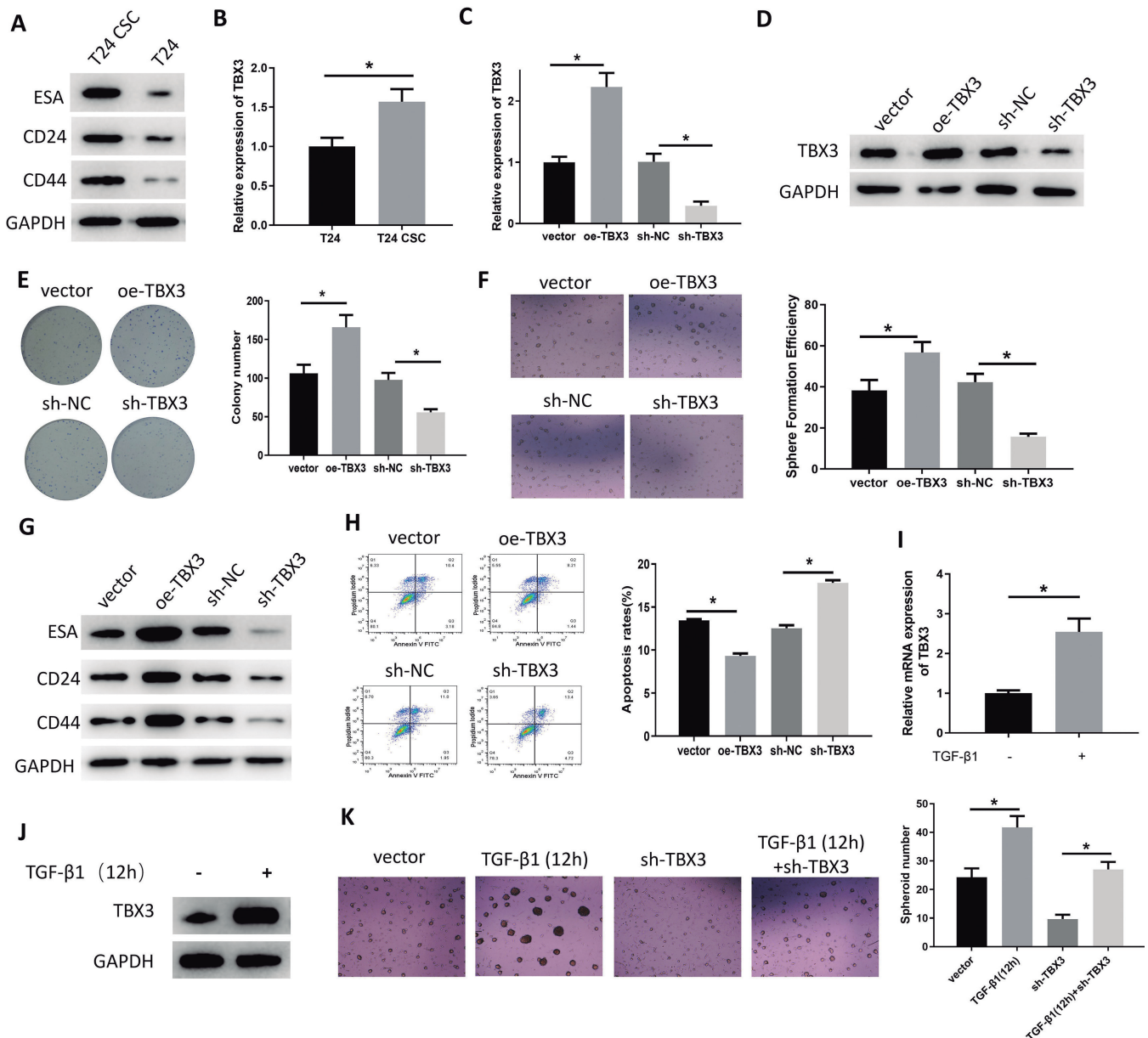
Fig. 3. TBX3 is highly expressed in CSCs and promotes cell stemness in bladder carcinoma. **A.** Western blot assay detected stemness-related protein expression in T24 CSCs and T24 cells. **B.** qRT-PCR showed elevated TBX3 expression in T24 CSCs and T24 cells. **C.** TBX3 mRNA expression level in T24 CSCs after transfection tested by qRT-PCR. **D.** TBX3 protein expression in each group after transfection tested by western blot. **E.** Colony formation assay detected the colony formative ability of cells in each treatment group. **F.** The self-renewal ability of T24 CSCs in each group examined by stem cell sphere formation assay. **G.** Western blot detected stemness-related proteins in each transfection group. **H.** Flow cytometry detected stem cell apoptotic level in each group. **I.** Total RNA extracted from T24 cells after 12h TGF-β1 (5 ng/mL) treatment was reverse transcribed and subjected to qRT-PCR using primers specific to TBX3. **J.** Western blot was used to measure the TBX3 protein expression after 12h TGF-β1 treatment. **K.** The self-renewal ability of T24 cells in each group examined by stem cell sphere formation assay. *$p<0.05$.

involved in tumor cell proliferation, differentiation, invasion, migration, helping immune escape and tumor recurrence (Ciurea et al., 2014). Hence, we posited based on our experimental results and published references that TBX3 exacerbated the malignant progression of bladder carcinoma by regulating cell stemness.

Currently, this study proved that TBX3 deteriorated malignant progression and promoted stem cell self-renewal in bladder carcinoma. We hope to confirm the effect of cell stemness on bladder carcinoma malignancy through *in vivo* experiments, thereby further validating whether TBX3 affects tumor cell stemness to promote cancer progression. This study provided evidence for TBX3 as a potential target of stem cells in bladder carcinoma.

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