WITHDRAWN ARTICLE

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Fibroblasts from pBOO promote tumorigenesis by secreting TGFbeta1 to induce EMT in bladder urothelial carcinoma cells

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Running title: fibroblasts promoted tumorigenesis by secreting TGF-β1

Abstract:

Urinary bladder cancer (UBC) is one of the most common malignancies worldwide. UBC patients at muscle invasive stage have poor clinical outcome, due to high propensity for metastasis. Non-tumor activated fibroblasts, named α-SMA+Fs, is similar to carcinoma-associated fibroblasts (CAFs) which could express α-SMA. However, whether α-SMA+Fs patients could induce UBC cell invasion is unclear. Herein, we found that characterization of primary α-SMA+Fs separated from PBOO (partial bladder outlet obstruction) rats was fell in between normal fibroblasts (α-SMA-Fs) and CAFs. Additionally, the conditional medium from α-SMA+Fs enhanced the NBT-II cell invasion through inducing EMT, and the oncogenic function of mixed supernatant of α-SMA+Fs/CAFs was stronger than that of CAFs. Inhibition of TGF-β1 by TGF-β1 neutralizing antibody decreased the EMT-associated gene expression and NBT-II cell invasion, suggesting that α-SMA+Fs can induce tumor EMT through TGF-β1. Xenograft experiments showed that the tumorigenic effect of α-SMA+Fs in mice was also between CAFs and α-SMA-Fs, and α-SMA+Fs/CAFs also had a strong tumorigenic effect. We preformed rats with PBOO and found that the incidence of invasive bladder cancer in PBOO+BBN group was higher than in BBN group, suggesting the PBOO treatment contributed to tumorigenesis. Thus, α-SMA+Fs promoted tumorigenesis by secreting TGF-β1 to induce EMT.

Key words: Urothelial Bladder Carcinoma; Epithelial-Mesenchymal Transition; TGF-β1; Non-tumor activated fibroblasts

Introduction

Bladder tumor is the common cancer in the world [1]. About 95% of bladder tumors are urothelial tumors, which are the second most frequent malignancy of the urinary system and ranks ninth in worldwide cancer incidence [2]. Urinary bladder cancer (UBC) are divided into non-muscular invasive tumors and muscular invasive tumors. It is easy to relapse after surgical treatment in non-muscular invasive tumors, some cases would progress into muscular invasive tumors. The muscular invasive tumor is easy to transfer and have a poor prognosis. This study to investigate the development mechanism of bladder urothelial tumors which will be benefit to

improve therapy of urinary bladder cancer in the future.

Epithelial-mesenchymal transition (EMT) is a critical process occurring during embryonic development and in fibrosis and tumor progression [3]. EMT has been reported to be involved in the critical mechanism for the acquisition of the invasive phenotype in various type of tumor, including bladder cancer[4]. At the molecular level, EMT is characterized by loss of E-cadherin and increased expression of several transcriptional repressors of E-cadherin expression (Zeb-1, Zeb-2, Twist, Snail, and Slug) [5]. In addition, Twist1 is expressed in stromal fibroblasts in gastric cancer tissues and that Twist1 expressing fibroblasts possess CAF-like properties [6]. Transforming growth factor-β (TGF-β) is a potent pleiotropic cytokine that regulates mammalian development, differentiation, and homeostasis in essentially all cell types and tissues. TGF-β normally exerts anticancer activities by prohibiting cell proliferation and by creating cell microenvironments that inhibit cell motility, invasion, and metastasis. However, in tumor progression, tumor cells are resistant to TGF-β, which translates cytokine signaling to promote tumor growth and enhances tumor invasion and metastasis[7]. TGF-β1 plays an important role in the process of EMT, and it mediated urothelial EMT also play a key role in the process of the bladder fibrosis[8, 9]. Previous studies have reported that TGF-β1 was the major cytokine in the process of bladder fibrosis[10, 11].

Cancer associated fibroblasts (CAFs) are abundant in the stroma of desmoplastic cancers where they promote tumor progression, and interactions between cancer cells and CAFs play an important role in tumor development and progression [12, 13]. Trough secreting various cytokines, such as TGF-β1, CAFs could stimulate cancer cell growth and invasiveness[14], and the expression levels of CAF markers, such as FSP1 and fibroblast-activating protein (FAP), have been used to predict clinical outcomes in multiple cancer types[15]. A-SMA is the major cellular marker after CAFs activation[16]. Activated fibroblasts express several mesenchymal markers such as α-smooth muscle actin (α-SMA) and FAP [17]. Because of diabetes, bladder outlet obstruction or other factors, fibroblast proliferation and activation changes will occurred in the bladder tissue at the cellular level [18, 19]. Activated fibroblasts, named myofibroblasts, are involved in bladder fibrosis. In addition, these activated fibroblasts are similar to CAFs which could express α-SMA and secrete biologically active cytokines [10]. The tumor microenvironment theory deemed that the activation of fibroblasts promoted tumor generation, and we found that the

fibroblasts were activated in pBOO rats, thus, we inferred that pBOO might promoted tumor generation through promoting the activity of fibroblasts.

Partial Bladder Outlet Obstruction (PBOO) is a ubiquitous problem in urology, occurring secondary to spina bifida and posterior urethral valves which could result in significant renal injury[10]. PBOO patients have activated fibroblasts which were involved in the process of bladder fibrosis and a higher TGF-β1 concentration in urine[10, 20]. The clinical study has reported that the patients of lower urinary tract obstruction (LUTO) has much possibility to suffer the bladder cancer, which promoted us to study the relationship between pBOO and bladder cancer. In this study, we performed PBOO in rats to get non-tumor activated fibroblasts (α-SMA+Fs), and explored the mechanism of α-SMA+Fs on promoting tumorigenesis that α-SMA+Fs secreted TGF-β1 to induce EMT in bladder urothelial carcinoma cells.

Material and methods

To establish the rat bladder urothelial tumor model and PBOO model

Experiments were conducted in accordance with national guidelines and approved by the ethical committee of the Second Affiliated Hospital of Nanchang University. Wistar rats (6 weeks old) were housed in a room with 21-25°C and 45–65% relative humidity before the experiments, and with free access to food and water. The rats were divided into 5 groups: group 1 were given normal water (control); in group 2 , rats were given water for 12 weeks and received PBOO operation; in group 3, rats were with a 0.05% solution of n-butyl-butanol nitrosamine (BBN) in their drinking water every day for 8 weeks as is reported previously [21-23], and received normal water later; the rats in group 4 were received the injection of NBT-II combined with α-SMA-F/CAFAll; the rats in group 4 were received the injection the NBT-II combined with α-SMA-F/CAFAll and SB431542 (10 mg/kg). After surgery, all rats were intraperitoneal injected with 5 mg/kg Gentamicin once a day for 3 days, and cultured in a cozy environment and kept the cage clean and dry. All rats were killed humanely 20 weeks after the start of treatment (Fig. 1A).

Rats received PBOO operation, firstly, anaesthetized with diethyl ether and pentobarbital sodium, and then placed supine. Secondly, sterilization with an iodine/alcohol mixture, the abdominal cavity was opened by a midline incision to expose the urethrovesical junction. Thirdly, the proximal urethra was loosely tied with a 19 G needle using 2-0 silk thread, and the needle was

removed to produce PBOO. Lastly, the incision was closed and the rats were given penicillin G potassium.

Rats were divided into 5 groups, and received control, pBOO, BBN, pBOO+BBN, pBOO+BBN+SB treatments (Fig 5A and supplementary figure 3). Firstly, rats were with a 0.05% solution of BBN in their drinking water for 8 weeks and received normal water later, while receiving PBOO operation at 12 weeks. **The pBOO+BBN+SB5 groups also received 10 mg/kg SB twice a week, for one week.**

Preparation of fibroblasts

Primary urinary bladder tissue were collected from bladder tissues of above three groups, respectively. Tumor and non-tumor specimens of the bladder were cut into small pieces, and placed into a matrix covered cell culture flask for 30 min in the incubator (37°C, 5% CO_2). And then adding 2 ml of Epilife media supplemented with HKGS, 1% of a 300mM glycine solution (Sigma–Aldrich), 1% MEM-NEAA, 10% FBS (Biochrom) and 2% antibiotic solution (Invitrogen). After 24 h, medium was changed by 6 ml of supplemented EpiLife media. When cell cultures reached subconfluence (60-80%), cells were detached by accutase for 1 to 2 min, and after elution, gain the fibroblasts that cultured in Dulbecco Minimum Essential medium (DMEM; Invitrogen, Paisley, UK) containing 10% FBS and 1% antibiotic solution. The obtained cells from three groups named α-SMA-F, α-SMA+F and CAF cells, representing non-activated fibroblasts, activated fibroblasts, and tumor-associated fibroblasts, respectively[24].

Conditioned media (CM), that was obtained from 48-hour serum-starved cellular supernatant of α-SMA-F, α-SMA+F and CAF cells, was filtrated and clarified by centrifugation [25]. CAFs (0.5×10^5), α-SMA+F cell (0.5×10^5), α-SMA-F cell (0.5×10^5) or CAFs and α-SMA+F cell (0.5×10^5 +0.5×10^5) were inoculated in the culture dish, respectively. After being adhered to the wall, the culture medium changed to DMEM (without FBS). After the culture medium was replaced for 48 h, the supernatant was collected and centrifuged.

Cell culture and treatment

Rat-derived bladder cancer cell line NBT-II cell was purchased from ATCC (CRL-1655) and cultured in the DMEM supplemented with 2mM glutamine and antibiotics and 10% FBS. NBT-II

cells were treated with the CM from α-SMA-F, α-SMA+F and CAF cells, as well as an equal volume of complete culture medium. TGFβ1 blocking antibody (R&D Systems, MAB240) was added to the media to neutralize TGFβ1, the dosage of neutralizing Ab is 50 μg/ml.

Transwell assay

The invasion and migration capacities of cells in vitro was evaluated by transwell assay. Before the Transwell assay, the NBT-II cells were cultured using serum-free medium for 6 h to eliminate the disturb of FBS. 1×10^5 cells were inoculated in the upper chamber, coated with Growth factor reduced (GFR) Basement Membrane Matrigel for invasion assay or without for migration assay, and the obtained different media containing 10% FBS was added into the lower chamber as a chemoattractant. After incubation for the appropriate time, cells on the upper surface of the membrane were removed, and the cells on the lower surface of the membrane were the invaded cells. The membrane containing invasive cells were fixed with formaldehyde and stained using 0.5% crystal violet. The numbers of invaded cells were counted in five randomized fields using a microscope.

Western blot analysis

After various treatments, fibroblasts or NBT-II cells were collected and lysed in protein lysis buffer. Proteins samples (30μg) were separated on SDS-12% PAGE and then PAGE transfer onto PVDF membranes (Thermo). Primary mouse monoclonal antibodies against a-SMA (Abcam), FAP (1:500, Abcam), E-cadherin (1:50, Abcam), vimentin HIF 1α (Abcam), b-FGF (Abcam) and their respective β-actin (1:500, Abcam) at 4°C for 24 h, and secondary antibody were used in western blot analysis at room temperature for 1 h. Band intensity was quantified by Quantity one software. The protein expression were normalized to β-actin levels.

Real-time PCR (RT-qPCR)

After various treatments, total RNA was extracted from fibroblasts or NBT-II cells using Trizol (Invitrogen) according to the manufacturer's instructions. The level of a-SMA, FAP, TGF-β1, HIF 1α and b-FGF were calculated relative to β-actin (internal control) using the $2^{-\Delta\Delta Ct}$ method using real-time PCR system according to manufacturer's instructions in SYBR green

master mix (Applied Biosystems). The primers used in the present study were as follows:

Snail: Forward 3′-TCTTCCACCTCGGCCTCATC-5’

Reverse: 5’-TTCGGATGTGCATCTTCAGAGC-3’

ACTA2: Forward 3′-GCCACTGCTGCTTCCTCTTC-5’

Reverse 5’-CCGCCGACTCCATTCCAATG-3’

FAP: Forward 3′-ACACAGCGACATACTACATCTACG-5’

Reverse 5’-AGCACAGATACTGAATTGGACGAG-3’

TGF-beta1: Forward 3′-CAACAATTCCTGGCGTTACCTTG-5’

Reverse: 5’-CCTGTATTCCGTCTCCTTGGTTC-3’

E-cadherin: Forward 3′-TCAGATCAGGACCAGGACTACG-5’

Reverse 5’-CGCCGCCACCATACATATCG-3’

Vimentin: Forward 3′-TAGCCGCAGCCTCTATTCCTC-5’

Reverse 5’-GTGTTCTTGAACTCGGTGTTGATG-3’

ELISA assay

The contents of TGF-β1 in mediums were measured by TGFβ1 ELISA kit (BOSTER, EK0514). Mediums were co-incubated with plates for 90 min, and the plates were tapped dry. Biotin labeling TGFβ1 antibody were added and incubated for 60 min, washed three times using Tris-buffered saline (TBS) followed by adding Affinity Biotin-peroxidase Complex (ABC). Next, incubation on an orbital shaker for 30 min, plates were washed using TBS. Tetramethylbenzidine (TMB) color-substrate solution was added to each well, and incubation in the dark for 30 min. Lastly, TMB stop buffer was used to stop reaction, and the plates were read at 450 nm on a tunable microplate reader. The incubation temperature was 37°C.

Xenograft experiments

Approximately 1.0×10^6 NBT-II cells suspended in 100μl PBS were injected subcutaneously into the right side of the posterior flank of female BALB/c nude mice (n=6 in each group). For co-injection with fibroblasts, 1×10^6 NBT-II cells and 0.5×10^6 fibroblasts were combined, re-suspended in 100μL of PBS, and injected in mice. Tumor growth was examined every 5 day with a vernier caliper. Tumor volumes were calculated using the equation: $V=A\times B^2/2$ (mm^3),

where A is the largest diameter and B is the perpendicular diameter.

Statistical analysis

The SPSS 17.0 software (SPSS Inc., USA) was applied for statistical analyses. All experiments were repeated for three independent times, and all data were presented as means ± standard deviation (SD). Statistically significant differences between groups were defined as p-values less than 0.05.

Result

The Characterization of primary α-SMA-F, α-SMA+F and CAF cells.

The α-SMA-F, α-SMA+F and CAF cells were isolated from three bladder tumor tissues. As shown in Fig1A, The rats were divided into three groups, normal control group, PBOO (Partial Bladder Outlet Obstruction) group and BBN group, receiving different treatments respectively. After 20 weeks, all rats were sacrificed and the fibroblasts were separated from the bladder tissues of the three groups, named α-SMA-F, α-SMA+F and CAF cells, representing non-activated fibroblasts, activated fibroblasts, and CAFs, respectively. The mobility of cells were detected by transwell assay and the number of migrated α-SMA+Fs was significantly less than CAFs, but more than α-SMA-Fs (Fig 1B). The ACTA2 (coding α-SMA) and FAP were CAF-specific genes, and their mRNA levels in α-SMA-F, α-SMA+F and CAF cells have a similar tendency to the mobility of the three cells. As Fig 1C shown that, ACTA2 and FAP expression of α-SMA+F cell were significantly lower than that of CAF cells, but higher than α-SMA-F cells. The α-SMA and FAP protein levels of the three cells were in accordance with their mRNA levels (Fig 1D and 1E). In addition, N/E-cadherin (epithelial cell marker), Vimentin (mesenchymal cell marker) and Twist protein expression were analyzed by western blot. As Fig 1D showed that the expression levels of N/E-cadherin, Vimentin and Twist in α-SMA-F cells fell in between α-SMA-F cells and CAF cells. Thus, the characterization of primary α-SMA+F, α-SMA+F and CAF cells indicated that the degree of α-SMA+F cells activation was between α-SMA-F cells and CAF cells.

Medium from α-SMA-F, α-SMA+F and CAF cells induced EMT in NBT-II cell

To investigated whether conditional medium from α-SMA-F, α-SMA+F and CAF cells could increase bladder cancer cell motility, rat bladder cancer cell line (NBT-II cell) was treated with

culture medium, CM-α-SMA-F, CM-α-SMA+F and CM-CAF. As is shown in Fig 2A and 2B, cells treated with CM-α-SMA+F greatly accelerates cell migration and invasion comparing with control and CM-α-SMA-F, and the number of NBT-II cell migration and invasion in mixed supernatant of α-SMA+F/CAF cells was maximum, suggesting that the effect of supernatants of α-SMA+F/CAFs was strongest than any other groups. Next, the TGF-β1 levels were detected by RT-PCR and ELISA in cell and their respective supernatants, respectively. The TGF-β1 levels of cell were kept consistent with their respective supernatants. TGF-β1 was the most highly expressed cytokine in CAFs than those in the α-SMA-F, α-SMA+F and NBT-II cell (Fig 2C). The higher level of TGF-β1 was detected in the CM-CAF than those in the α-SMA-F, α-SMA+F and NBT-II cell (Fig 2D).

Epithelial-mesenchymal transition (EMT) is a key step toward cancer metastasis, and snail is a major transcription factor governing EMT [26]. Moreover, some EMT-related molecules protein expression were detected by western blot in NBT-II cell that co-cultured with conditional medium from α-SMA-F, α-SMA+F and CAF cells. The results showed that the treatment with α-SMA+F/CAFs supernatants led to the decrease of E-cadherin expression along with the increase of vimentin and snail expression (Fig 2E and 2F), which indicated that the effect of α-SMA+F/CAF cell on EMT was better than single effect.

TGF-β1 induce EMT and invasion of NBT-II cell

Transforming growth factor-β1 (TGF-β1) secreted by CAFs and present in tumor microenvironment acts in a coordinated fashion to promote tumor development [27]. To identify whether α-SMA-F, α-SMA+F and CAF cell-secreting TGF-β1 induce EMT and invasion of NBT-II cell, we performed TGF-β1 blocking assays using a neutralizing TGFβ1 antibody. Transwell assay showed that anti-TGF-β1 adding into conditional medium obvious reduced the number of migrated and invasive NBT-II cell (Fig 3A and 3B). 20 nM SB431542 was added 1 h prior to conditional medium treatment to block TGFβ signaling in NBT-II. The western blotting showed that abrogation of TGF-β1 increased the CM-α-SMA+F, CM-CAF and CM-α-SMA+F/CAF-suppressed E-cadherin expression and suppressed the expression of vimentin and snail (Fig 3C and 3D). These data implied that α-SMA+F and CAF cell promoted NBT-II cell motility and EMT via secreting TGF-β1. Non-tumor activated fibroblasts (α-SMA+F) fell in

between normal fibroblasts (α-SMA-F) and tumor-associated fibroblasts (CAFs), and its function was similar to CAFs, which could secrete TGF-β1 to promote tumorigenesis, invasion and metastasis. Also, α-SMA+Fs combined with CAFs, have a stronger effect on promoting tumorigenesis.

The effect of non-tumor activated fibroblasts on tumor formation in nude mice

In order to verify the effect of activated fibroblasts and cancer-associated fibroblasts (α-SMA+Fs and CAFs) on tumor formation, we performed xenograft experiments. And the results of tumor incidence and tumor volume showed that the tumorigenic effect of α-SMA+F in mice was between CAFs and α-SMA-F cells, and CAF/α-SMA+F have the strongest promotion of tumor formation (Fig 4A and 4B). Also, the TGF-β1 and EMT-related molecules expression was detected in the tumor tissues. The expression of TGF-β1, vimentin and snail was highest while the E-cadherin expression was lowest in group of NBT-II cells combined with CAF/α-SMA+F, followed by group of CAF, α-SMA+F, indicating that α-SMA+F cell and CAFs could both promote tumor formation through TGF-β1, and the combination of them had a stronger effect (Fig 4C and 4D). In addition, the TGF-β1 knockdown combined with α-SMA+Fs and CAFs decreased the EMT compared with normal CM-(α-SMA+F) +CM-CAF (Supplementary figure 2).

The effect of PBOO treatment on tumor incidence in rats

As we have known that non-tumor activated fibroblasts, named α-SMA+F cell, was isolated from bladder tissues of PBOO rats and promoted EMT of bladder cancer cells in vitro and in vivo. To identify whether the PBOO operation contributed to tumorigenesis, rats were divided into three groups, normal control group (n=3), BBN group (n=10) and BBN +PBOO group (n=10), which were treated according to the method shown in Fig 5A. While normal control group was the rats without bladder cancer, the incidence of tumor were 60% and 100% in BBN group and PBOO+BBN group, respectively. Moreover, the occurrence of tumor invasion in BBN group and PBOO+BBN group were 50% and 70%, respectively, suggesting that PBOO operation contributed to tumorigenesis (Fig 5B). As is shown in Fig 5C, bladder weight was detected in the three groups, combination of BNN and pBOO resulted in the heaviest bladder quality. The HIF1a and b-FGF genes were tumor markers, and their expression were detected by RT-PCR and western blot,

respectively. As shown in Fig 5D and 5E, the expression of HIF1a and b-FGF in BBN+PBOO group was higher than that in BBN group. These results showed that combination of BNN and pBOO could result in the highest tumor formation rate, the strongest tumor invasive, and the heaviest bladder quality.

Discussion

Tumor microenvironment, also known as “Tumor Stroma”, includes extracellular matrices and different types of cells, such as inflammatory cells, vascular smooth muscle cells, endothelial cells, pericytes and fibroblasts. Tumor microenvironment provides a basis for tumor cell survival, proliferation, migration, and infiltration, promoting tumor formation and doing changes with tumor progression. Previous studies on malignant transformation of tumors have focused on tumor cells themselves, but more and more genes and cell biology studies have shown that the development of tumors not only depends on the malignant proliferation of tumor cells themselves, but also on the interaction relationship between tumor cells and the microenvironment. Tumor-associated fibroblasts (CAFs) are the major cellular components of tumor stroma. The effect of CAFs on tumor progression become more and more important. CAFs regulated tumor microenvironment through directly contacting with other cells, secretion of soluble factor and modification of extracellular matrix. In the process of tumor formation, these CAFs precursor cells are recruited into tumor tissue, interacting with tumor cells, and transforming into CAFs that promoted tumor progression.

EMT is a process in which polar epithelial cells transformed into interstitial cells with invasion and migration ability. EMT are involved in multiple signal transduction pathways and complex molecular mechanisms, and have a close correlation to growth factors, transcription factors, micro-environmental hypoxia. EMT is also closely related to the progression and metastasis of bladder urinary tract epithelium. CAFs derived from urinary tract epithelium can secrete high concentrations of TGF-β1 in vitro, and TGF-β1 induces urinary tract epithelial tumor cell line EMT. Wu [28] and Shintani [29] reported that CAFs enhanced the migration and EMT of cancer cells through the secretion of IL-6. Chong and his colleagues [30]found that CAFs induced EMT through β1 integrin-mediated upregulation of Gli1 in gastric cancer. Inhibition of CAFs could suppress pancreatic cancer cells EMT[31], while activation of CAFs could promote

pancreatic cancer cells EMT[32]. This study demonstrated that CAFs and its culture fluid both could promote NBT-II cell EMT, and non-tumor activated fibroblasts, named α-SMA+Fs in this paper, that was similar to CAFs which could express α-SMA, also promote NBT-II cell EMT.

In recent years, it has been identified that TGF-β expressing in a variety of cells could enhance tumor invasion and metastasis ability in the process of tumor progression. TGF-β-mediated signaling pathways play an important role in the process of interstitial and epithelial interactions. For example, TGF-β1-mediated urinary tract epithelium EMT promote fibrosis of the bladder. TGF-β1 can induce EMT changes in urothelial cells, and the E-cadherin expression was decreased and the expression of N-cadherin and α-SMA were up-regulated. Yu et al [33] have shown that CAFs promoted aggressive phenotypes of breast cancer cells through EMT induced by paracrine TGF-β1. Zhuang et al [14]reported that CAFs induces EMT and invasion of human UBC cells through the TGFβ1-ZEB2NAT-ZEB2 axis. In this study, the α-SMA+Fs separated from primary urinary bladder tissue of PBOO rat model was activated. The cell migration experiment and nude mouse tumorigenicity assay demonstrated that α-SMA+Fs could promote NBT-II cell EMT and contributed to tumor formation in nude mice by secreting TGF-β1. In addition, our study showed that anti-TGF-β1 adding into conditional medium obvious reduced the number of migrated and invasive NBT-II cell, suggesting that α-SMA+Fs promoted NBT-II cell motility and EMT via secreting TGF-β1. This study has found that abrogation of TGF-β1 increased the CM-α-SMA+Fs, CM-CAFs and CM-α-SMA+Fs/CAFs-suppressed E-cadherin expression and suppressed the expression of vimentin and snail. In addition, the activated fibroblasts that derived from pBOO could promote the EMT in NBT-II via TGF-β1 and further promote tumor generation.

The urinary bladder is responsible for storage and expulsion of urine. When diabetes, bladder outlet obstruction and other factors affect the bladder, the fibroblasts of bladder submucosa and detrusor muscle bundle between the loose connective tissue are activated, and these activated fibroblasts express α-SMA with myofibroblast phenotype. The cell and molecular level of the bladder with Bladder Outlet Obstruction have a series of changes. In the BOO early phase, fibroblasts are activated and involved in the process of bladder fibrosis. TGF-β1 is the major cytokine in the process of bladder fibrosis and the higher TGF-β1 concentration were found in BOO patients' urine. Thus, the bladder cancer were induced in rats by BBN to obtain CAFs and

the α-SMA+Fs were derived from PBOO Rat model in our study. Our data presented herein demonstrate that the degree of α-SMA+Fs activation was between normal fibroblasts (α-SMA-Fs) and CAFs, and the conditional medium from α-SMA+Fs and CAFs that containing TGF-β1 could induced NBT-II cell EMT. We investigated the effect of α-SMA+Fs on tumor formation in nude mice, and the results showed that α-SMA+F cell could promote tumor formation, and the combination of α-SMA+Fs and CAFs had a stronger oncogenic function. In the end, we also investigated the effect of PBOO treatment on tumor incidence in rats, and the data suggested that PBOO operation contributed to tumorigenesis, which might related to theα-SMA+Fs and CAFs.

In this study, we have found that bladder outlet obstruction accelerated bladder carcinogenesis, which was reported by Matsumoto and his coworkers in 2008[34]. Non-tumor activated fibroblasts was similar to CAFs which could express α-SMA and secrete TGF-β1. The α-SMA+F cells were separated from primary urinary bladder tissue of PBOO rats and could induce EMT in NBT-II cell and tumor formation in nude mice. We also found that α-SMA+Fs activation was between normal fibroblasts and tumor-associated fibroblasts and promoted tumorigenesis by secreting TGF-β1 to induce EMT.

Conflict of Interest

The authors declare no conflict of interest.

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Authors' contributions

RK put forward the concept of the study, designed the study, prepared the manuscript and contributed to the statistical analysis. LD contributed to the data acquisition. JZ contributed to the quality control of data and algorithms. ZZ analyzed the data and interpretation. XL edited the manuscript. RK put forward the concept of the study, contributed to the data analysis and interpretation and reviewed the manuscript. All authors read and approved the final manuscript

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Figure legend:

Figure1. The Characterization of primary α-SMA+F, α-SMA+F and CAF cells

(A) The rats were randomly divided into three groups: normal control group, PBOO group and BBN group, which were established according to the method shown in Fig 1A. After 20 weeks, all rats were sacrificed and the fibroblasts were isolated from the bladder tissues of the three groups (n=3 in each group), named α-SMA-F, α-SMA+F and CAF cells, representing non-activated fibroblasts, non-tumor activated fibroblasts, and cancer-associated fibroblasts, respectively. (B) The mobility of α-SMA+F, α-SMA-F and CAF cells. (C) α-SMA (ACTA2) and FAP mRNA expression of α-SMA+F, α-SMA-F and CAF cells. (D) α-SMA, FAP, E/N-cadherin, Vimentin and Twist protein expression in α-SMA+F, α-SMA-F and CAF cells. (E) α-SMA, FAP, E/N-cadherin and Vimentin mRNA expression in α-SMA+F, α-SMA-F and CAF cells.* P <0.05 vs α-SMA-F, # P <0.05 vs CAFs.

Figure 2. Medium from α-SMA-F, α-SMA+F and CAF cells induced EMT in NBT-II cell.

After the above three kinds of fibroblasts (α-SMA+F, α-SMA+F and CAF cells) were cultured for 48 h, the culture supernatants of the three cells were collected and the mixed supernatant of α-SMA+F/CAF were used to co-culture with NBT-II cells.

(A and B) NBT-II invasion and migration by conditional medium from above three kinds of fibroblasts. (C and D) The level of TGF-β1 in NBT-II cells and above three kinds of fibroblasts and their respective culture medium. (E and F) Conditional medium from above three kinds of fibroblasts induced EMT phenotypes in NBT-II cell. * P <0.05 and ** P <0.01.

Figure 3 TGF-β1 induce EMT and invasion of NBT-II cell

TGF-β1 neutralizing antibody was added into α-SMA+F and CAF cell medium, and supernatant were collected and co-cultured with NBT-II.

(A and B) NBT-II cell invasion and migration were both inhibited by TGF-β1 neutralizing antibody. (C and D) the EMT-related molecules protein expression changes in NBT-II cell. * P <0.05 and **P <0.01.

Figure 4. The effect of non-tumor activated fibroblasts on tumor formation in nude mice

(A and B) tumor incidence and tumor volume. The tumorigenic effect of α-SMA+F in mice was between CAF and α-SMA-F cells. (C and D) TGF-β1 and EMT-related molecules expression. The expression of TGF-β1 in CAF/α-SMA+ F group was the highest, followed by CAF, α-SMA + F. (E) the expression of E-cadherin .*P <0.05 and ** P <0.01.

Figure 5. The effect of PBOO treatment on tumor incidence in rats

(A) Rats were divided into three groups: normal control group (n=3), BBN group (n=10) and BBN +PBOO group (n=10), while the rats used for the western blot was also n=3 in BBN group and BBN+PBOO group. The following experiments were based on the (B) Normal control group without bladder cancer, and the incidence of bladder cancer in PBOO+BBN group was higher than that in BBN group. (C) The bladder weight in the three groups. (D and E) tumor markers molecule (HIF1a and b-FGF) mRNA and protein expression. ** P <0.01.

Supplementary figure 1. The invasive and migration of NBT-II cell were regulated by exogenous TGF-β1.

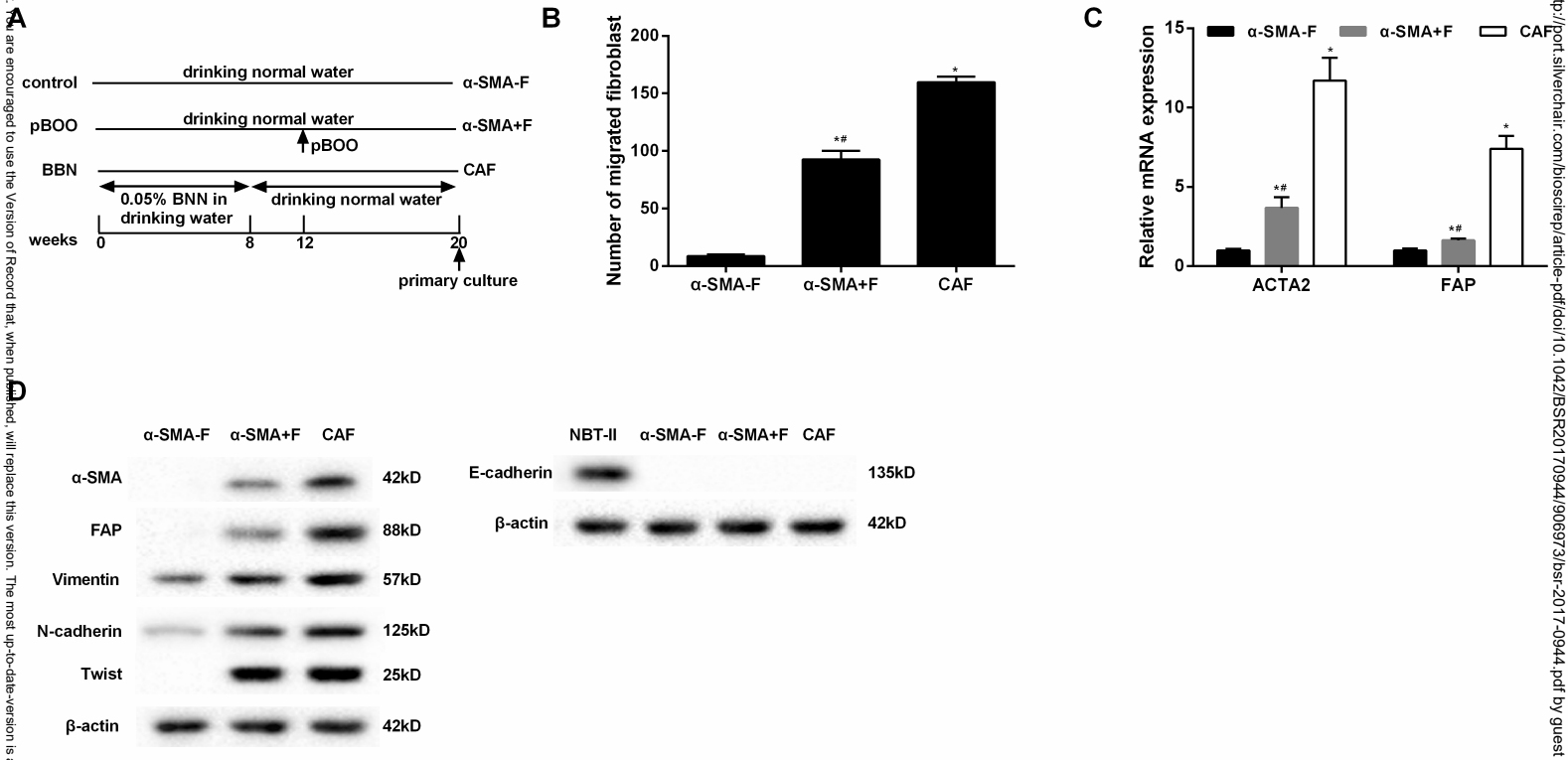
(A and B) The invasive and migration of NBT-II cell were both increased with the increasing of TGF-β1 concentration. (C) The E-cadherin protein expression was downregulated with the increasing of TGF-β1 concentration, while the Vimentin and Snail protein expression were upregulated with the increasing of TGF-β1 concentration .

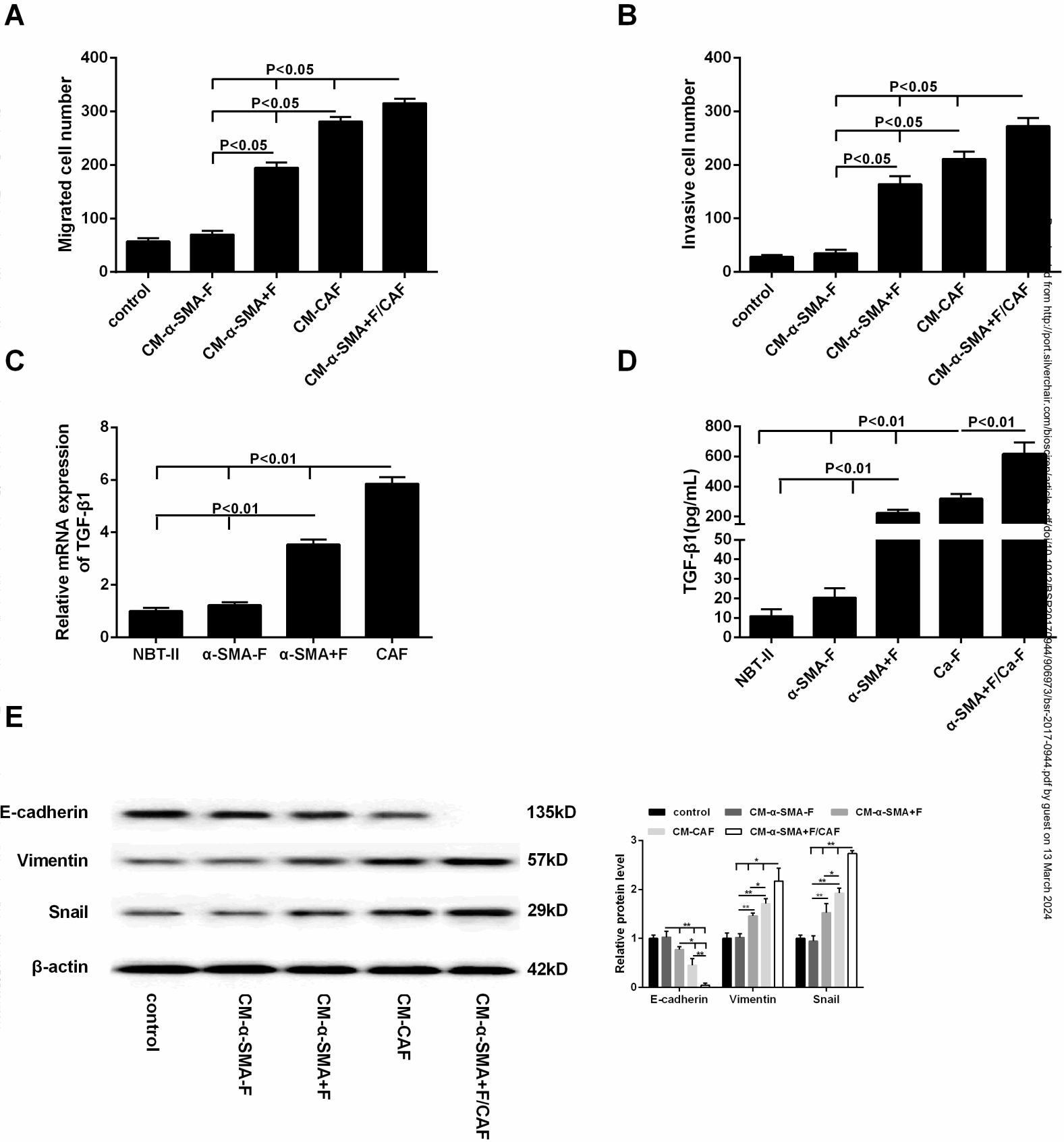
Supplementary figure 2. α-SMA+F combined with CAF promoted NBT-II EMT via TGF-beta1.

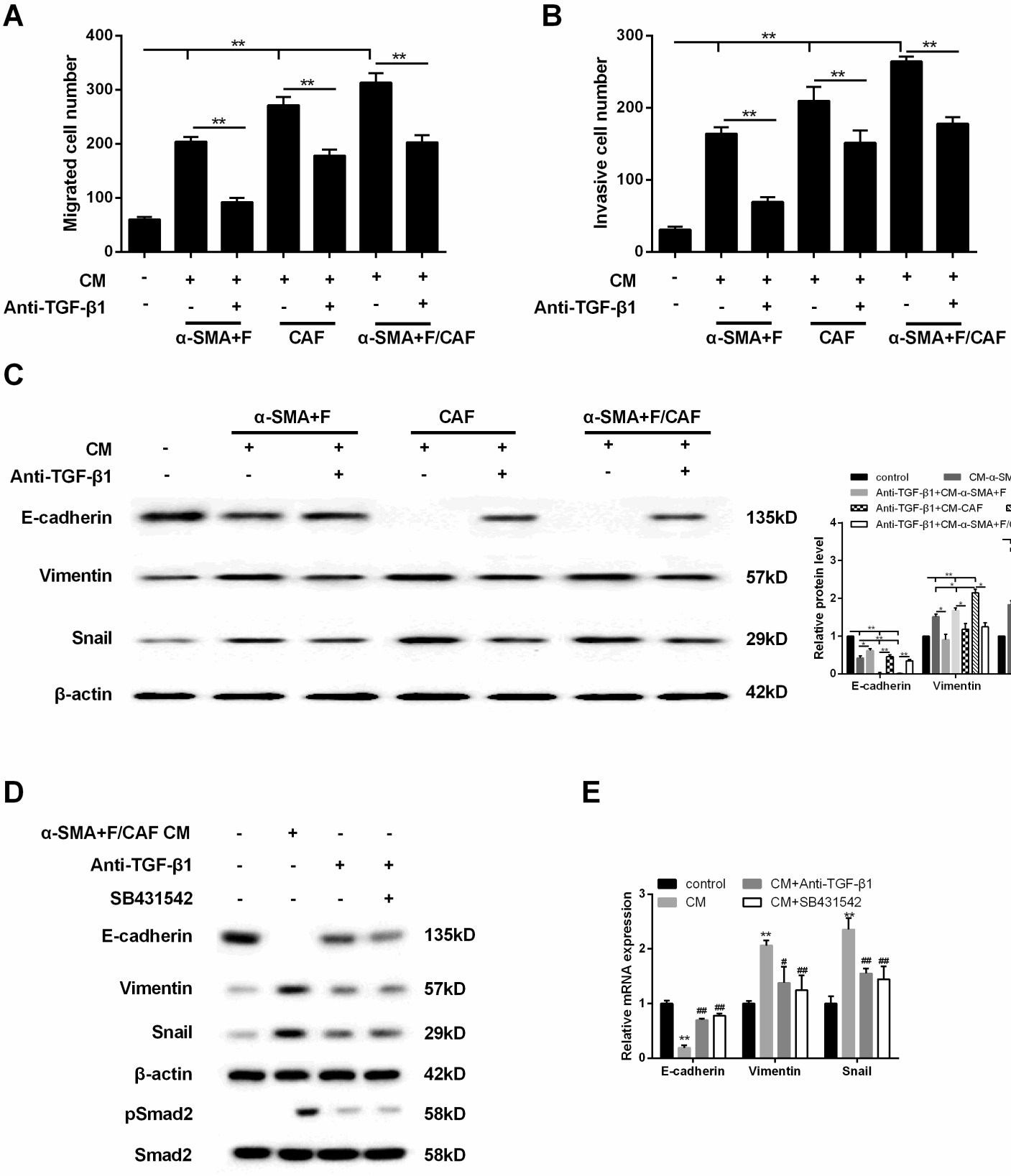
(A) The knockdown of TGF-beta1 on α-SMA+F; (B and C) α-SMA+F combined with CAF under TGF-beta1 knockdown on the effects of EMT; (D) The micrograph of normal fibroblast.

Supplementary figure 3. The western blot detection of pSmd2, HIF-1α and β-FGF in control, pBOO, BBN, pBOO+BBN and pBOO+BBN+SB. The pBOO group showed no tumor generation, and two rats (a total of five rats in this group) showed tumor generation in pBOO+BBN+SB group(the data was not shown). The pSmad2 signaling was strengthen in pBOO group rather than that in control, while HIF-1α and β-TGF showed no dramatically change. While

HIF-1α and β-TGF was significantly decreased in **pBOO+BBN+SB group.**





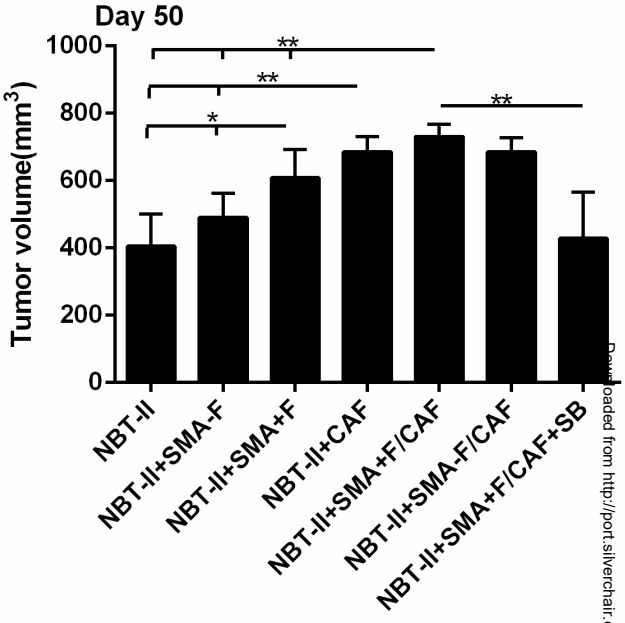


A

Tumor incidence

Group	Days after injection			
	5	10	30	50
NBT-II	0/6	2/6	6/6	6/6
NBT-II+SMA-F	0/6	3/6	6/6	6/6
NBT-II+SMA+F	3/6	5/6	6/6	6/6
NBT-II+CAF	5/6	6/6	6/6	6/6
NBT-II+SMA+F/CAF	6/6	6/6	6/6	6/6
NBT-II+SMA-F/CAF	6/6	6/6	6/6	6/6
NBT-II+SMA+F/CAF+SB	1/6	4/6	6/6	6/6

B



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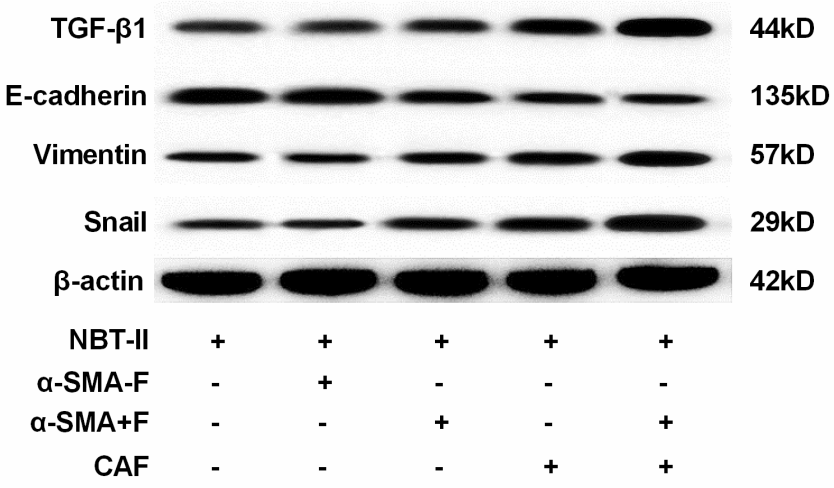

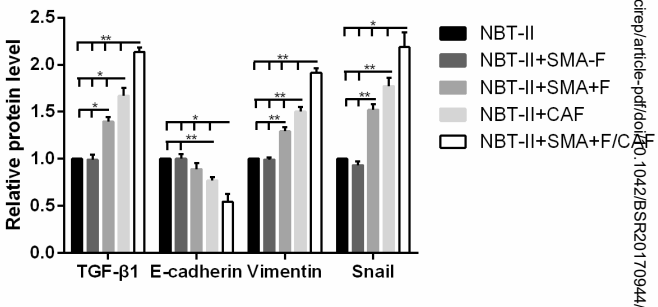

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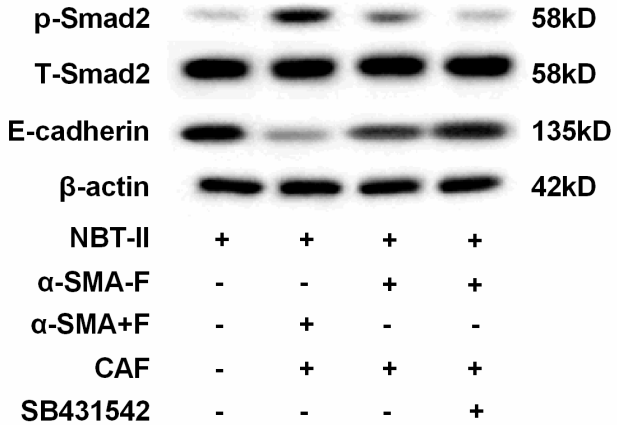

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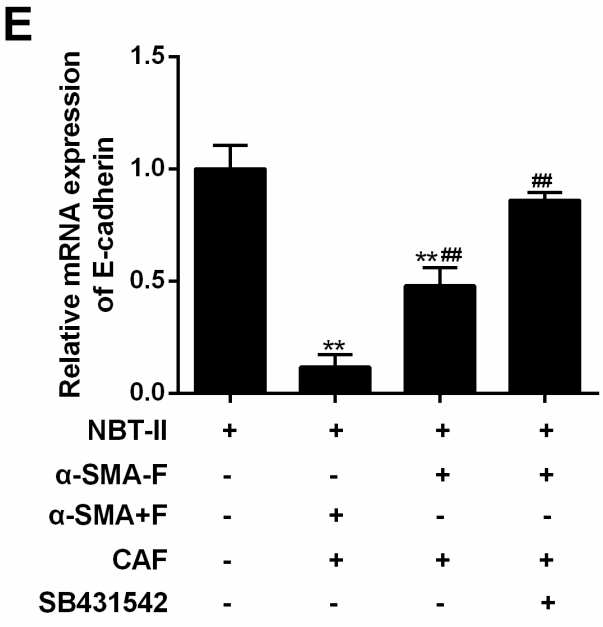

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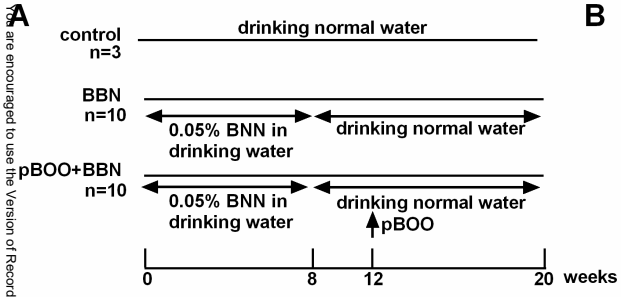
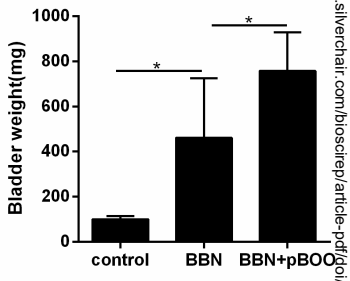


B

Incidence	Group		
	control	BBN	pBOO+BBN
Carcinoma	0/3	6/10 (60%)	10/10 (100%)
Invasive carcinoma	0/3	3/6 (50%)	7/10 (70%)

C



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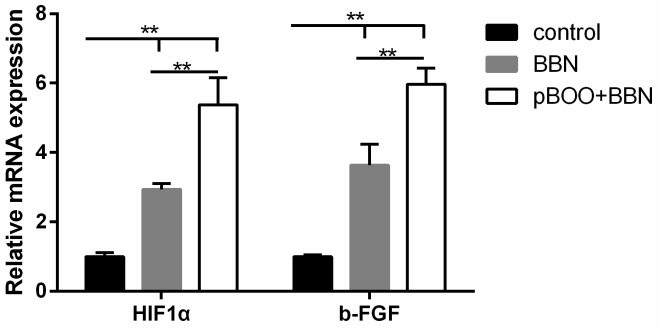

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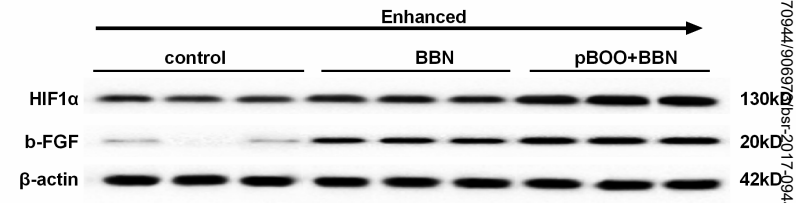

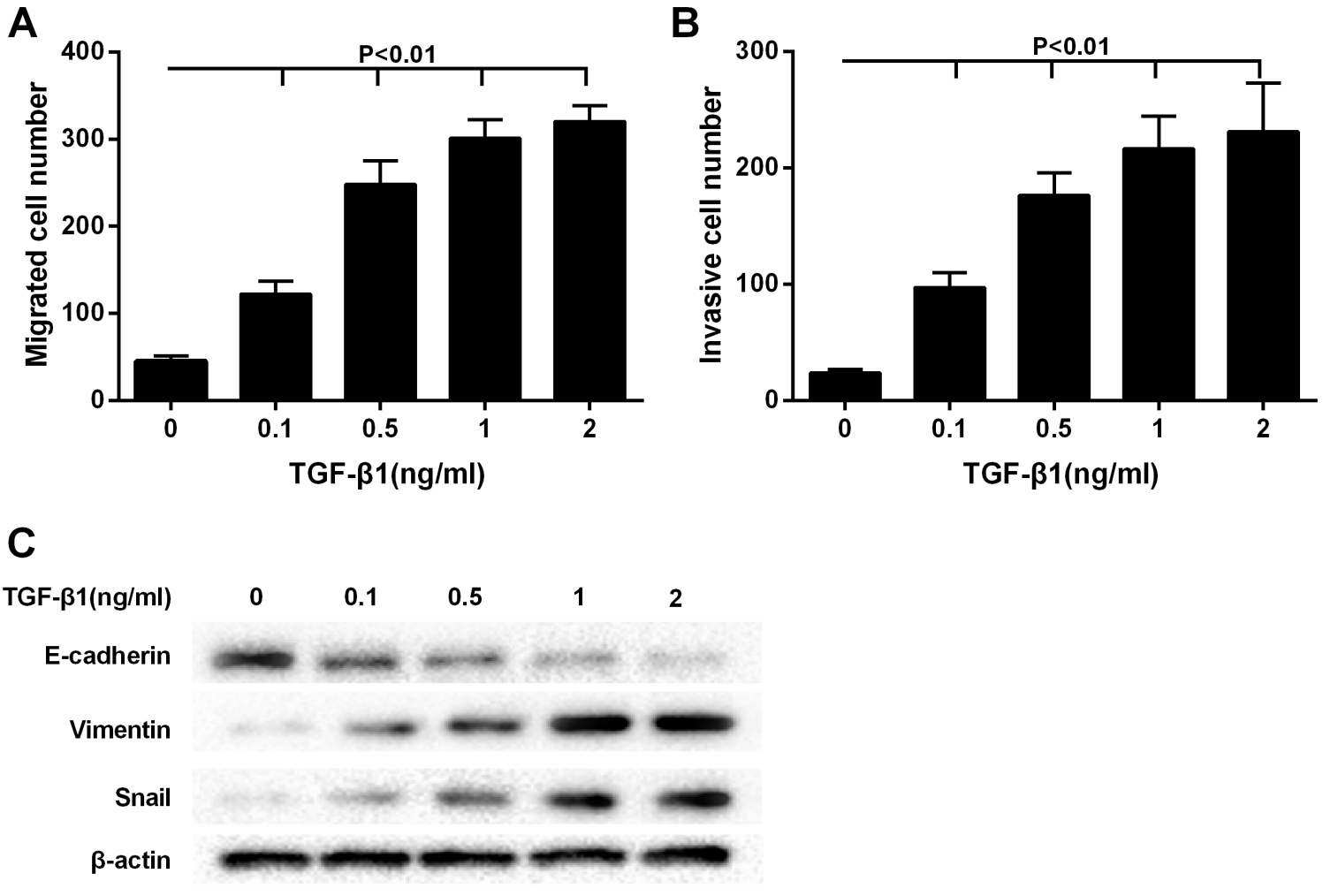

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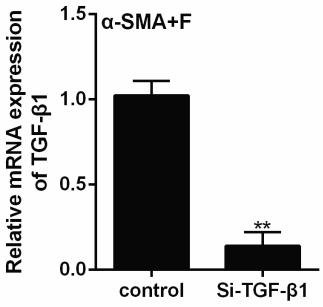

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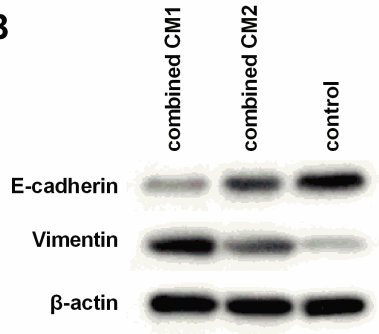
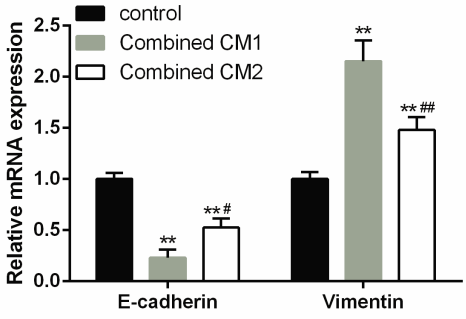


combined CM1 group: NBT-II were treated with the combined CM from α-SMA+F and CAF;
combined CM2 group: NBT-II were treatd with the combined CM from TGF-β1 knock down α-SMA+F and CAF;
control group: NBT-II were treated with normal culture medium

C



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