Activation of the NF-κB Signaling Pathway Promotes Malignancy in Bladder Cancer Cells in a Positive Feedback Manner

Zhenfeng guan (guanzhenfeng@163.com)
Department of Urology, Shaanxi Provicial People's Hospital, Xi'an, Shaanxi, China
https://orcid.org/0000-0002-5746-3319

Yi Sun
Shaanxi Provincial People's Hospital

YaZhuo Jiang
Shaanxi Provincial People's Hospital

Xinyang Wang
Xi'an Jiaotong University

Jinhai Fan
Xi'an Jiaotong University

Primary research

Keywords: Bladder cancer, NF-κB signal, Co-culture, HUVEC, Tumor progression, Positive feedback loop

Posted Date: August 21st, 2020

DOI: https://doi.org/10.21203/rs.3.rs-60078/v1

Abstract

Background: The main issue arising from bladder cancer (BCa) is the high relapse ratio and tumor progression, the mechanism of which remains to be elucidated. Interaction of tumor cells with the stroma of microenvironment promoting tumor progression warrants much attention from researchers. Among all stromal cells, endothelial cells (ECs) are exceptional. Numerous studies have investigated its role of angiogenesis, but have not studied immunocyte recruitment and chemokine secretion, the important significance of which in tumor progression has been proven. Meanwhile, to the best of our knowledge, few studies have focused on the direct interaction between tumor cells and ECs in BCa tissue, which was the aim of the present study.

Methods: In the present study, immunohistochemical staining is used for detecting the distribution of ECs in BCa tissue, and we use SPSS 19 to analysis the relationship between ECs distribution and tumor grade/stage; inadition, co-curlturing of tumor cell with ECs is usd to mimicking the interaction of tumor cell with ECs, followed by Chamber Assay, BrdU incorporoartion, WB, Qt-PCR, *ect*, to investiatin the mechanism.

Results: The distribution of ECs in BCa tissue is significantly increased according to BCa grade and negatively associated to the time from BCa diagnosis to progression, manifesting as an independent risk factor for BCa prognosis. The following *in vitro* experiment indicates that the conditional medium from co-culture of tumor cells (T24/J82) with ECs (human umbilical vein endothelial cells, which were used as ECs in the *in vitro* experiment) contributes to the activation of the NF-κB signaling pathway in tumor cells, leading to the upregulation of CXCL1/8. This further results in enhanced tumor cell malignancy and EC recruitment, manifested as a positive feedback loop.

Conclusions: The present study provided a further understanding on the role of ECs in BCa progression—not only by angiogenesis but also by interacting with tumor cell dirctly.

Background

Urinary bladder cancer is the ninth most common malignant disease and the thirteenth most common cause of cancer death worldwide. A total of 76,960 new cases of bladder cancer (BCa) and 16,390 deaths attributable to BCa were predicted to occur in 2016 in the USA alone [1], of which ~ 70% are non-muscle-invasive (stages Tis, Ta and T1) and 30% are muscle-invasive (stages T2, T3 and T4) types. Among the non-muscle-invasive tumors, 50–70% will recur despite conservative measures, such as transurethral and intravesical therapy [2]. However, the molecular mechanisms that induce or promote BCa progression are still poorly understood [3]. Key steps of cancer progression and therapy response depend upon interactions of cancer cells with the reactive tumor stroma of the microenvironment. The mechanistic understanding of these microenvironmental interactions can influence the evaluation and selection of candidate agents for various cancers, in both the primary site and metastatic setting [4]. The contemporary view of cancer envisions tumors as “ecosystems” [5], consisting not simply of proliferating

cells alone but of diverse collections of recruited stromal cells that regulate cancer behavior [6–10]. Endothelial cells (EC) which line the blood vessels are the first cells in contact with any blood-borne element and is especially prevalent in tumors [11], which plays a critical role in tumor progression, but has not been fully understood.

Previous reports showed that ECs played a vital role in tumor progression, including, but not limited to nutrient/oxygen supply, metabolite transport and also as a potential metastatic pathway [12]. The direct or indirect interactions of tumor cells with ECs resulted in alternative expression/activation of involved chemokines, cytokines, integrins and signaling pathways, contributing to tumor progression [13, 14]. However, the mechanisms of these interactions in BCa tissue have not been fully elucidated.

CXC chemokine is a family of heparin-binding protein and be characterized by the presence of four cysteine residues in conserved positions, of which the first two cysteines are separated by one amino acid [15–17]. These chemokines are further grouped based on the presence or absence of a 3-amino acid sequence, glutamic acid-leucine-arginine (known as the ELR motif), immediately preceding the CXC sequence [17–20]. The activation of CXCR2 by ELR^+CXC chemokines (including CXCL1, 2, 3, 5, 6, 7 and 8) or CXCR4 by CXCL12/SDF-1 causes angiogenesis, whereas CXCR3 is an “angiostatic” receptor for several ELR^-CXC chemokines, including CXCL4, 9, 10 and 11 [16, 17]. The production of these chemokines, including constitutive and inducible expression, is involved in all types of physiological or pathological processes, and various signaling pathways [17, 21]. Among these signaling pathways, the NF-κB signaling pathway is reported [22] to play an important role in the expression of CXC-chemokines. It was also shown that the promoters of several CXC-chemokines and/or their corresponding receptors contain an NF-κB binding site [19, 20, 23, 24].

Levidou *et al* [25] showed that the nuclear expression of NF-κB, which indicates the activation of this pathway, is an independent prognosticator of adverse significance in BCa. However, the mechanism remains to be elucidated.

In the present study, immunohistochemical (IHC) staining of BCa sections for EC indicated the increased pattern of EC distribution from grade I to grade III, followed by worse prognosis. To mimic the microenvironment of the tumor niche, conditional medium from the co-culture of tumor cells with human umbilical vein endothelial cells (HUVECs) was used to culture the bladder tumor cell lines T24/J82. The present results indicated that T24/J82 cells cultured in conditional medium resulted in the activation of the NF-κB signaling pathway, leading to enhanced malignancy and HUVEC recruitment, the latter of which is mediated by upregulation of CXCL1/8, which manifested as a positive feedback loop. Thus, we provide evidence that in BCa tissues, the activation of the NF-κB signaling pathway is induced by the interaction of tumor cells with ECs, plays a role in BCa progression. The results may provide us an avenue for understanding another role of EC, apart from angiogenesis, in the progression of BCa.

Materials And Methods

Preparation of tumor tissues. A total of 77 tissue samples of bladder urothelial were obtained by cystectomy between January 2006 and March 2011 at the Department of Urology, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University. The samples included 48 males and 29 females, with an age range between 39–78 years (mean age, 50.7 years). Samples were fixed in 4% formalin and then paraffin-embedded. All experiments involving human tissue were approved by the Ethics Committee of Xi'an Jiaotong University.

IHC staining of ECs. IHC was performed using a Dako Autostainer Plus System (Dako; Agilent Technologies, Inc.). Tissues were de-paraffinzed, rehydrated and subjected to 5 min pressure-cooking antigen retrieval, 15 min endogenous enzyme blocking, 1 h primary antibody (CD31 antibody; 1:200; Santa Cruz Biotechnology, Inc.) incubation and 30 min Dako Cytomation EnVision-horeseradish peroxidase reagent incubation for rabbit antibody. Signals were detected by addition of substrate hydrogen peroxide using diaminobenzidine as a chromogen, followed by hematoxylin counterstaining. Negative control slices were prepared by omitting the primary antibody. Stained cells (brown) were quantified using the following formula: Number of positive cells x 100/total number of cells. A total of 10 randomly-selected fields in each slice were detected by microscopy (magnification, x400)

Patient follow-up. Among the 77 patients, 49 patients were followed up from the period cancer diagnosis to progression (relapse or death). The relationship between the number of ECs and the time from cancer diagnosis to relapse was evaluated. The Kaplan-Meier estimate was used for survival analysis.

Western blotting. Cells were harvested at 80% confluence and washed thrice with cold PBS. Total cellular protein lysates were prepared using RIPA buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP40 and 0.5% sodium deoxycholate] containing proteinase inhibitors (1% cocktail and 1 mM PMSF, both from Sigma-Aldrich, Merck KGaA). Nuclear protein was prepared using a kit (cat. no. BSP001; Sangon Biotech Co., Ltd.) according to the manufacturer's protocol. A total of 30 µg protein was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% skim milk in TBS (pH 7.6). Blots were incubated with polyclonal antibodies at different dilutions (Table I) dissolved in 5% skim milk in TBS overnight at 4˚C, followed by washing with TBS-Tween-20 (TBS-T; pH 7.6). Membranes were incubated with secondary antibodies (LI-COR Biosciences) coupled to the first antibody in the dark at room temperature for 1 h, followed by washes with TBS-T in the dark. Membranes were dried using neutral absorbent paper and scanned using the Odyssey Detection system (LI-COR Biosciences). MG-132 (10 µM; Sigma-Aldrich; Merck KGaA) was used to inhibit proteasome-dependent degradation at 4 h before the protein harvest. GAPDH (for total cell fractions) and Histone H1 (for nuclear fractions) were used as loading controls.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from each cell group was isolated using Trizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and quantified by measuring the absorbance at a wavelength of 260 nm. RNA (2 µg) was reverse transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using the SYBRR Premix Ex Taq II system (Takara Biotechnology Co., Ltd) on a

CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). A total of 12.5 µl SYBR Premix Ex Taq II, 1 µl primer (10 µM; primers listed in Table II), 200 ng cDNA and 9.5 µl double distilled water were mixed together. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 30 sec; 95˚C for 5 sec; 35 cycles of 60˚C for 30 sec; and dissociation at 95˚C for 15 sec, 60˚C for 30 sec and 95˚C for 15 sec. GAPDH was used as the loading control for mRNA normalisation.

Cell culture and preparation of conditional medium (CM). The human BCa cell lines T24 and J82 and HUVECs were purchased from American Type Culture Collection. Cells were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) at 5% CO_2 and 37˚C.

To mimic the interaction between tumor cells and ECs in the tumor niche, following 24 h of co-culturing of T24/J82 cells with HUVECs, the supernatant was extracted and supplemented with 10% FBS and used as the CM to culture the tumor cell lines.

Boyden chamber assay. The malignant abilities (migration and invasion) of cells were assessed using a Boyden chamber assay. The chambers (8 µm diameter) were purchased from EMD Millipore. For the migration assay, 1×10^4 cells/well in a 24-well plate cultured in 200 µl serum-free DMEM were added to the upper chamber. A total of 800 µl serum-free DMEM was added to the lower chamber. Following a 2 h incubation at 5% CO_2 and 37˚C, the chambers are washed with PBS (pH 7.4) three times to remove the cells in the upper chamber. Cells were fixed with 4% formalin for 15 min, and then stained with crystal violet (0.01% in ethanol) for 25 min, followed by three washes. Cells were counted under an inverted microscope. A total of five randomly selected fields were captured at x200 magnification and the average number of cells are analyzed.

For invasion analysis, the upper chamber was filled with 200 µl serum-free DMEM and Matrigel (Sigma-Aldrich; Merck KGaA; DMEM:Matrigel ratio, 8:1) and 1×10^4 cells from different groups. Cells were incubated for 36 h. Subsequent steps were performed as described above.

HUVEC recruitment. Boyden chambers (8-µm pore diameter; EMD Millipore) were used to assess the abilities of HUVEC recruitment in tumor cell lines. T24/J82 cells cultured in CM or control media for 24 h were seeded in 24-well plates until cells adhered to the bottom of the chamber. Subsequently, 1×10^4 HUVECs were seeded onto the upper chamber. After 24 h, the number of HUVECs were counted as described above.

Demonstration of the roles of the NF-κB signaling pathway. Cells were treated with the NF-κB pathway inhibitor pyrrolidinedithiocarbamic acid ammonium salt (PDTC; Sigma-Aldrich; Merck KGaA) at a final concentration of 10 µM for 12 h prior to protein or RNA extraction. In addition, small interfering RNA targeting p65 was used as the parallel experiment for inhibition of the NF-κB pathway by PDTC.

Cells were also treated with the NF-κB pathway activator tumor necrosis factor-α (TNF-α; Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration is 10 ng/ml for 12 h prior to proteins or RNA be extraction.

Immunofluorescence staining for nuclear translocation of NF-κB. Prepared cells were

washed three times with cold PBS (pH 7.4). Subsequently, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.5% Triton X-100 for 10 min and incubated in 1% BSA blocking solution for 1 h. Fixed cells were incubated overnight at 4˚C with rabbit anti-human-P65 in 1% BSA. Cells were washed and incubated with mouse anti-rabbit TRITC (Red) immunoglobulin G antibody (Santa Cruz Biotechnology, Inc.) diluted 1:100 in blocking buffer for 1 h. Nuclei were stained with DAPI (10 µg/ml; Sigma-Aldrich; Merck KGaA) for 5 min. Cells were examined under a fluorescent microscope equipped with narrow band-pass excitation filters to individually select for red and blue fluorescence. Cells were observed using the Image Pro Plus System mounted on a fluorescent microscope (Olympus Corporation). Experiments were repeated thrice.

5-bromo-2-deoxyuridine (BrdU) incorporation assay. T24/J82 cells were grown on 24-well plates until 50–70% confluence. Subsequently, BrdU (3 µg/ml) was added to the media. Media was removed after 4 h, and cells were rinsed three times with PBS for > 10 min to remove free BrdU. Cells were then fixed in 4% paraformaldehyde for 45 min, followed by five washes with PBS for > 20 min. Triton X-100 (0.1%) was used to destroy the cell membrane (15 min) and 2N HCl (25 min) was used to separate DNA into single strands for the primary antibody to access the incorporated BrdU. Prior to blocking nonspecific epitopes with 10% BSA for 20 min, cells were rinsed three times with PBS for > 10 min. Subsequently, anti-BrdU antibody (1:200) in 10% BSA was added to the cells and incubated overnight at 4˚C. Following primary antibody incubation, cells were rinsed five times with PBS to remove the free antibody, followed by incubation with TRTIC-labeled secondary antibody for 1 h at room temperature. Cells were rinsed three times with PBS to remove the free antibody. The fluorescence intensity of TRITC was measured using a SuperMicro Orifice Plate Spectrophotometer (Omega Bio-Tek, Inc.) at a wavelength of 547 nm.

Statistical analysis. ANOVA was performed to compare differences between n > 3 groups. Student's t-test was performed to compare differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Distribution of EC in BCa tissues increases from grade 1 to grade 3, manifesting as a risk factor for prognosis. Angiogenesis, defined as the formation of new blood vessels from existing vasculature, plays an important role in tumor growth and metastasis by providing oxygen, nutrients and growth factors to cancer cells. CD31, an existing marker in ECs, is widely used for evaluating microvessels in numerous studies [26]. Anti-CD31 microvessel immunostaining has several advantages over anti-factor VIII, and is a more sensitive method for detecting small, immature microvessels or single EC. This could be of importance in revealing a possible correlation of tumor angiogenesis with metastatic behavior, prognosis,

or angiogenic factor overexpression [27]. In the present study, CD31 was used as an EC marker in the BCa sections. As shown in Fig. 1A and B, the number of $CD31^+$ cells gradually increased according to tumor grade (grade 3 vs. grade 1; $P = 0.014 < 0.05$), indicating that the number of ECs in grade 3 is significantly increased compared with grade 1 in BCa sections. To further understand the association between the distribution of $CD31^+$ cells and the time from tumor diagnosis to relapse, 49 patients were followed up until the tumor relapse period. As expected, the time from tumor diagnosis to relapse is negatively related to the number of $CD31^+$ cells (Fig. 1C). All 49 patients are divided into two groups by the average number of $CD31^+$ cells (average, 5.69). Kaplan-Meier analysis suggested that number of $CD31^+$ cells is an independent risk factor for patients with BCa (Fig. 1D).

Tumor cells cultured in CM manifests with enhanced malignant and proliferation abilities. The role of stromal cells and the tumor microenvironment in general in modulating tumor sensitivity is increasingly becoming a key consideration for the development of active anticancer therapeutics [4]. Tumor-stroma interactions affects tumor cell signaling, survival, proliferation and drug sensitivity via different mechanisms, and among all stromal cells, microvessel endothelial cells are irreplaceable [12]. Consistent with a previous study [28], the present results showed that the number of ECs increased according to tumor progression and is negatively correlated to BCa prognosis. It was hypothesized that ECs may exert direct or indirect effects on BCa cells, aside from metabolism-related roles, such as oxygen supply. To further investigate this hypothesis, CM was used to culture T24 and J82 cells (Fig. 2A). As shown in Fig. 2C, Boyden chamber assay indicated that culture in CM resulted in enhanced malignancy of T24/J82 cells. This also lead to the process of epithelial to mesenchymal transition (EMT) as indicated by western blotting and RT-qPCR results (Fig. 2B).

Additionally, the proliferation abilities were assessed by BrdU incorporation to demonstrate whether culturing T24/J82 cells in CM affected the proliferative ability of tumor cells. As shown in Fig. 2D, comparing with the control, the fluorescence intensity of T24/J82 cells cultured in CM increased by > 5-fold, indicating that CM contribute to tumor cell proliferation in some way.

Tumor cells cultured in CM manifest with enhanced recruitment abilities. Due to the different distribution of ECs in differing grade of BCa sections and the role of CM on educating tumor cell lines, we determined whether there is discrepancy in HUVECs recruited by parental tumor cell and CM-cultured tumor cells. As shown in Fig. 2E, T24/J82 cells cultured in CM manifested with enhanced ability of HUVEC recruitment compared with parental T24/J82 cells. However, the mechanism is still unknown.

CM culture induces elevated expression of CXCL1/5/8 and CXCR2/3 in T24/J82 cells compared with controls. Recent studies showed that the CXC-chemokine family, which are produced by tumor and stromal cells, play important roles in the process of tumor angiogenesis, recruitment and progression [17, 29]. Our previous results showed evidence that the BCa cells cultured in CM showed enhanced malignancy, proliferation and HUVEC recruitment abilities. It was hypothesized that this chemokine family may be involved in this process. To investigate this hypothesis, CXC-chemokines and their receptors were monitored in parental and CM-cultured T24/J82 cells. CM culture resulted in different

expression of CXC chemokines in T24 and J82 cells, especially CXCL1, CXCL5 and CXCL8 (Fig. 3A). The corresponding receptors of these chemokines were also monitored, whereby CXCR2 and CXCR3 levels significantly increased compared with parental cell lines (Fig. 3B). This indicated that the CM contributed to the upregulation of those CXC-chemokines /receptors via an unknown mechanism, which was further investigated.

NF-κB signaling in T24/J82 cells is activated by CM culture. Numerous studies showed that the NF-κB pathway played important roles in the progression of cancer, through which cancer progressed into more malignant types [30, 31]. This pathway in cancer cells could be activated by the components of the tumor niche or the interaction between tumor and stromal cells of the niche [32–34]. The present results demonstrate that in the BCa cell lines T24 and J82, CM culture lead to P65 nuclear translocation, which indicated the activation of the NF-κB signaling pathway (Column 2 vs. column 1; Fig. 4A).

Inhibition of the NF-κB signaling pathway in T24/J82 cells attenuates CM culture-induced malignancy, HUVEC recruitment and proliferation, accompanied by EMT reversal. In bladder transitional cancer, EMT is regarded to be one of the important processes in maintaining malignancy and promoting tumor progression [35]. Activation of the NF-κB signaling pathway was reported to be positively related to BCa progression but negatively related to prognosis. Based on this observation, siRNA for knocking down NF-κB, or PDTC, an inhibitor of NF-κB pathway, was used to inhibit this signaling. Both siRNA and PDTC resulted in the attenuation of CM culture-induced NF-κB signaling (Fig. 4A; column 3 vs. column 2; Fig. 4B-D), accompanied by attenuated malignancy (Fig. 5A). In addition, CM culture-induced HUVEC recruitment by T24/J82 cells was also attenuated (Fig. 5B). Inhibition of this signaling significantly inhibited the CM culture-induced proliferation of T24/J82 cells (Fig. 5C). This indicated that the NF-κB signaling pathway is a regulator during CM culture. Additionally, inhibition of NF-κB signaling also lead to the reversal of EMT, including downregulation of matrix metalloproteinase MMP 2, MMP9, N-Cadherin, Vimentin but upregulation of E-Cadherin (Fig. 4B), consistent with reports showing that the NF-κB pathway is a key regulator of EMT.

Inhibition of the NF-κB signaling pathway results in the decreased expression of CXCL1/8 and CXCR2 in CM-cultured tumor cell lines.

Previous results suggested that tumor cells cultured in CM resulted in different expression of CXC-chemokines/receptors, accompanied by the enhanced tumor cell malignancy, proliferation and ability of HUVEC recruitment, and inhibition of the NF-κB pathway attenuated these CM culture-induced phenomena. This result lead us to postulate the roles of the NF-κB signaling pathway on the expression of these CXC-chemokines/receptors. T24/J82 cells were cultured in CM in the presence of PDTC or si-p65, and RT-qPCR was performed to assess the expression of CXC-chemokines/receptors in tumor cells. As shown in Fig. 4A, the nuclear translocation of P65 was reversed by PDTC. RT-qPCR results also indicated that si-p65 induced the downregulation of *P65* expression (Fig. 4C) CM culture-induced elevation of *CXCL1/8* and its receptor *CXCR2* was significantly attenuated in the absence of NF-κB signaling (Fig. 5D); however, *CXCL5* and *CXCR3* expression was not significantly altered (Fig. 5D).

Activation of the NF-κB signaling pathway in T24/J82 cells by TNF-α mimics the effects induced by CM culture. To determine whether CM culture-induced effects involved the NF-κB signaling pathway, TNF-α, a classical activator of NF-κB signaling [30], was used to activate this pathway. This was followed by monitoring tumor cell malignancy, proliferation and HUVEC recruitment. TNF-α-treated T24/J82 manifested as P65 nuclear translocation, as indicated by immunofluorescence (Fig. 6A), indicating the activation of the NF-κB signaling pathway. As expected, this activation resulted in enhanced malignancy (Fig. 6D), proliferation (Fig. 6F) and ability of HUVEC recruitment (Fig. 6E). In addition, TNF-α induced the elevation of *CXCL1/8* and *CXCR2* levels (Fig. 6B) and contributed to EMT (Fig. 6C).

Functional inhibition of CXCR2 attenuates CM culture-induced HUVEC recruitment.

It was reported that CXC chemokines played important roles in inflammatory cell recruitment, and the present results also showed that CM culture-induced upregulation of CXCL1/8 in tumor cells was accompanied by enhanced HUVEC recruitment. As aforementioned, binding with the corresponding receptor is the first step for CXCL chemokine to play its role. In addition, CXCR2 is stably expressed on the surface of HUVECs, as shown by our results (data not shown). Therefore, it was hypothesized that enhanced HUVEC recruitment by CM-cultured T24/J82 cells might be mediated by the CXCL1/8-CXCR2 axis. CXCR2 neutralizing antibody (1 μg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) was used to treat tumor cells in the presence of CM. As shown in Fig. 7A, enhanced HUVEC recruitment induced by CM-cultured T24/J82 cells were attenuated. However, this functional inhibition showed no visible effects on tumor cell proliferation (Fig. 7B) and malignancy (Fig. 7C).

CM culture of T24/J82 cells resulted in IκBα degradation and activation of the NF-κB signaling pathway. IκBα is regarded as the key upstream inhibitor of NF-κB signaling, whereby the degradation of IκBα is necessary for NF-κB signaling activation [36]. It was hypothesized that CM-cultured T24/J82 cells might be involved in the degradation or decreased expression of IκBα. Therefore, the expression of this protein in CM-cultured T24/J82 cells was monitored using RT-qPCR and western blotting. Prior to protein extraction, cells were treated with MG-132, an inhibitor of the 26S-dependent protein degradation pathway, for 4 h to prevent spontaneous IκBα degradation. As shown in Fig. 7D (top panel), CM culture induced the attenuation of IκBα, however, this attenuation did not occur at the mRNA level (Fig. 7D; bottom panel). This indicated that CM culture-induced attenuation of IκBα may have been due to degradation instead of decreased expression.

Discussion

The main issue arising from BCa therapy is the high relapse ratio and inevitable tumor progression, the mechanism of which is still not clear [37]. Vasculogenesis, which is the main role of EC, is regarded to be play irreplaceable roles in tumor progression and resistance to chemotherapy [38]. However, the role of EC in the tumor microenvironment is not limited to this point, and increasing numbers of research have focused on the direct or indirect interaction of EC with cancer cells [12]. These interactions are involved different types of mechanisms and result in tumor progression. The present study provided evidence that,

in part, the interaction of ECs with BCa cells resulted in the upregulation of CXCL1/8 and CXCR2 via activation of the NF-κB signaling pathway. This was accompanied by enhanced tumor malignancy, proliferation and EC recruitment ability, providing further understanding of the role of EC in BCa progression.

An increasing number of studies have focused on the role of the tumor niche in tumor progression by excluding the malignant behavior of the tumor cell itself [39, 40]. The interaction between tumor and stromal cells, either direct or indirect, resulted in the activation of various pathways including Erk1/2, AKT/PKB, MAPK, Notch, Wnt [41] and NF-κB [42] signaling pathways, to promote cancer progression. These activated signaling pathways lead to the elevation of related factors including chemokines, cytokines and MMPs, which play roles not only in tumor progression, such as metastasis and proliferation, but also activating these signaling pathways as a positive feedback loop. These positive feedback loops modulate the activated-signal-induced tumor progression more precisely and efficiently. Among all involved stromal cells, EC has been less studied and investigated.

Vasculogenesis is the process of new blood vessel formation by recruited EC cells. Vasculogenesis is a critical biological process under both physiological and pathological conditions [43], which is regarded as the main role of EC in tumor progression in the past few years; however, new challenges on this view have recently appeared. It was reported that during the inflammatory process, EC plays vital roles in the recruitment of neutrophils [44] and macrophages to the target organ. Additionally, the matrix secreted by activated EC, such as all types of chemokines, enzymes [45], matrix products, growth factors and inflammatory mediators [46], are regarded to regulate inflammation more precisely. Activated ECs in the inflammatory microenvironment are involved in many signaling pathways and chemokines/factors, most of which also can be found in the tumor microenvironment [6]. Among all chemokines, CXC chemokine is the most important.

The expression of ELR^+CXC chemokines is found in the context of aberrant vasculogenesis, which suggested that specific stimuli within the microenvironment or epigenetic cellular events contributed to the expression of these angiogenic CXC chemokines [43]. It was reported that all ELR^+CXC chemokine promoters contain a putative *cis*-element that recognizes the NF-κB family of transcriptional factors. Therefore, NF-κB plays an important role as a ‘master switch’ in the transactivation of those angiogenic CXC chemokines [20, 22, 47–49]. Several studies found that the NF-κB controlled expression of angiogenic CXC chemokines is critical to angiogenesis relevant to tumor growth and potential metastases [20]. In the present study, activating or inhibiting the NF-κB signaling pathway resulted in increased or decreased expression of *CXCL1/8* and *CXCR2*, which indicated that in BCa cell lines, *CXCL1/8* and *CXCR2*, as ELR^+ chemokines/receptors, are at least partly mediated by NF-κB signaling. *CXCL5* and *CXCR3* were elevated in CM-cultured T24/J82 cells, but inhibition or activation of NF-κB signaling had no significant effect on their expression (Fig. 5D and Fig. 6B), which suggested that CM culture induced the upregulation of *CXCL5* and *CXCR3* via mechanisms different from the NF-κB signaling pathway.

NF-κB signaling is activated during the inflammatory process, targeting the recruitment of inflammatory cells. The present results indicated that T24/J82 cells cultured in CM manifested with elevated *CXCL1/8* and *CXCR2*, accompanied by enhanced tumor cell malignancy and HUVEC recruitment ability. Meanwhile, functional inhibition of CXCR2 has no significant effect on tumor malignancy when compared with controls, but lead to the attenuation of HUVEC recruitment. In conclusion, the present data indicated that tumor cell malignancy was induced by activation of the NF-κB signaling pathway, while HUVEC recruitment was induced by activation of the CXCL5/CXCR2 axis. This result contradicted with a published report showing that CXCL1/8 promoted cancer cell malignancy by directly binding to their receptors on tumor cells [50]. It seems that tumor cell malignancy and HUVEC recruitment are two independent of each other in these two cell lines.

The mechanism of CM-induced activation of NF-κB signaling is still unclear. However, as indicated in Fig. 7D, CM resulted in decreased expression of IκBα at the protein level but had no significant effect at the mRNA level, indicating that CM culture contributed to the degradation of IκBα in an unknown manner, leading to the release of NF-κB to translocate into the nucleus. As reported, degradation of IκBα involved mechanisms which include ubiquitination and phosphorylation at different sites, which led to the hypothesis that an unknown mechanism is involved, which will be investigated in further studies.

To summarize the present study (Fig. 7E), we found that in the BCa niche, the interaction between tumor cells and ECs lead to the degradation of IκBα to activate NF-κB signaling, resulting in BCa cell malignancy and EC recruitment. Recruited ECs interacted with tumor cells, either directly or indirectly, to promote tumor progression and EC recruitment as a positive feedback loop. Although the exact mechanism is still unclear, the present study discovered that the positive feedback loop between EC recruitment and aberrant NF-κB signaling activation contributed to the tumor cell malignancy and proliferation, providing information regarding the role of ECs in BCa tissue.

Conclussion

In the present investigation, we proved evdience to surport another role of endothelial cell in BCa progression—not only by angogensis but also by interacting with tumor cell directly. Althoug the mechanism is still unclear, we belive this point will be very intresting in the further stduy.

Declarations

Ethics approval and consent to participate : The human cancer tissue in this paper has been approved by the ethics committee of Xi'an university;

Consent for publication:Not applicable;

Availability of data and materials: Data sharing is not applicable to this article as no datasets were generated or analysed during the current study;

Competing interests:The authors declare that they have no competing interests;

Funding:The present study is supported by National Natural Science Foundation of China grants (81572520) and Shaanxi Provincial People's Hospital incubation Fund Project (2018YXQ-03);

Authors' contributions: GZ,Design and coordinate experiments, data analysis and article writing; SY, Case follow-up and survival analysisn; JY, Immunohistochemical staining and laser confocal analysis; WX, The tumor tissue paraffin embedded and sectioned plus cell culture ,FJ,WB and Qt-PCR analysis, data analysis and discussion

Acknowledgements: Not applicable;

References

1. Sanli O, Dobruch J, Knowles MA, Burger M, Alemozaffar M, Nielsen ME, Lotan Y. Bladder cancer. NAT REV DIS PRIMERS. 2017;3:17022.
2. Smith ZL, Guzzo TJ. Urinary markers for bladder cancer. F1000Prime Rep. 2013;5:21.
3. Aldebasi YH, Rahmani AH, Khan AA, Aly SM. The effect of vascular endothelial growth factor in the progression of bladder cancer and diabetic retinopathy. INT J CLIN EXP MED. 2013;6(4):239–51.
4. McMillin DW, Negri JM, Mitsiades CS. The role of tumour-stromal interactions in modifying drug response: challenges and opportunities. NAT REV DRUG DISCOV. 2013;12(3):217–28.
5. Hanahan D, Weinberg RA. The hallmarks of cancer. CELL. 2000;100(1):57–70.
6. Coussens LM, Werb Z. Inflammation and cancer. NATURE. 2002;420(6917):860–7.
7. Bhowmick NA, Neilson EG, Moses HL. Stromal fibroblasts in cancer initiation and progression. NATURE. 2004;432(7015):332–7.
8. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. CELL. 2006;124(2):263–6.
9. Weinberg RA. Coevolution in the tumor microenvironment. NAT GENET. 2008;40(5):494–5.
10. Polyak K, Haviv I, Campbell IG. Co-evolution of tumor cells and their microenvironment. TRENDS GENET. 2009;25(1):30–8.
11. van Beijnum JR, Rousch M, Castermans K, van der Linden E, Griffioen AW. Isolation of endothelial cells from fresh tissues. NAT PROTOC. 2008;3(6):1085–91.
12. Franses JW, Baker AB, Chitalia VC, Edelman ER. Stromal endothelial cells directly influence cancer progression. SCI TRANSL MED. 2011;3(66):65r–66r.
13. Orr FW, Wang HH, Lafrenie RM, Scherbarth S, Nance DM. Interactions between cancer cells and the endothelium in metastasis. J PATHOL. 2000;190(3):310–29.
14. Kramer RH, Nicolson GL. Interactions of tumor cells with vascular endothelial cell monolayers: a model for metastatic invasion. Proc Natl Acad Sci U S A. 1979;76(11):5704–8.

15. Mukaida N, Baba T. Chemokines in tumor development and progression. EXP CELL RES. 2012;318(2):95–102.
16. Keeley EC, Mehrad B, Strieter RM. Chemokines as mediators of tumor angiogenesis and neovascularization. EXP CELL RES. 2011;317(5):685–90.
17. Vandercappellen J, Van Damme J, Struyf S. The role of CXC chemokines and their receptors in cancer. CANCER LETT. 2008;267(2):226–44.
18. Pappa CA, Tsirakis G, Kanellou P, Kaparou M, Stratinaki M, Xekalou A, Alegakis A, Boula A, Stathopoulos EN, Alexandrakis MG: **Monitoring serum levels ELR + CXC chemokines and the relationship between microvessel density and angiogenic growth factors in multiple myeloma**. *CYTOKINE* 2011, **56**(3):616–620.
19. Lazennec G, Richmond A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. TRENDS MOL MED. 2010;16(3):133–44.
20. Strieter RM, Burdick MD, Mestas J, Gomperts B, Keane MP, Belperio JA. Cancer CXC chemokine networks and tumour angiogenesis. EUR J CANCER. 2006;42(6):768–78.
21. Mantovani A. The chemokine system: redundancy for robust outputs. Immunol Today. 1999;20(6):254–7.
22. Richmond A. Nf-kappa B, chemokine gene transcription and tumour growth. NAT REV IMMUNOL. 2002;2(9):664–74.
23. Maxwell PJ, Gallagher R, Seaton A, Wilson C, Scullin P, Pettigrew J, Stratford IJ, Williams KJ, Johnston PG, Waugh DJ. HIF-1 and NF-kappaB-mediated upregulation of CXCR1 and CXCR2 expression promotes cell survival in hypoxic prostate cancer cells. ONCOGENE. 2007;26(52):7333–45.
24. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. CLIN CANCER RES. 2008;14(21):6735–41.
25. Levidou G, Saetta AA, Korkolopoulou P, Papanastasiou P, Gioti K, Pavlopoulos P, Diamantopoulou K, Thomas-Tsagli E, Xiromeritis K, Patsouris E. Clinical significance of nuclear factor (NF)-kappaB levels in urothelial carcinoma of the urinary bladder. VIRCHOWS ARCH. 2008;452(3):295–304.
26. Sharma S, Sharma MC, Sarkar C. Morphology of angiogenesis in human cancer: a conceptual overview, histoprognostic perspective and significance of neoangiogenesis. HISTOPATHOLOGY. 2005;46(5):481–9.
27. Giatromanolaki A, Koukourakis MI, Theodossiou D, Barbatis K, O'Byrne K, Harris AL, Gatter KC. Comparative evaluation of angiogenesis assessment with anti-factor-VIII and anti-CD31 immunostaining in non-small cell lung cancer. CLIN CANCER RES. 1997;3(12 Pt 1):2485–92.
28. Huang YJ, Qi WX, He AN, Sun YJ, Shen Z, Yao Y. Prognostic Value of Tissue Vascular Endothelial Growth Factor Expression in Bladder Cancer: a Meta-analysis. Asian Pac J Cancer Prev. 2013;14(2):645–9.
29. Slettenaar VI, Wilson JL. The chemokine network: a target in cancer biology? Adv Drug Deliv Rev. 2006;58(8):962–74.

30. Li CW, Xia W, Huo L, Lim SO, Wu Y, Hsu JL, Chao CH, Yamaguchi H, Yang NK, Ding Q, et al: **Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappaB-mediated transcriptional upregulation of Twist1**. *CANCER RES* 2012, **72**(5):1290–1300.
31. Cheng ZX, Sun B, Wang SJ, Gao Y, Zhang YM, Zhou HX, Jia G, Wang YW, Kong R, Pan SH, et al. Nuclear factor-kappaB-dependent epithelial to mesenchymal transition induced by HIF-1alpha activation in pancreatic cancer cells under hypoxic conditions. PLOS ONE. 2011;6(8):e23752.
32. Sun SC. The noncanonical NF-kappaB pathway. IMMUNOL REV. 2012;246(1):125–40.
33. Shih VF, Tsui R, Caldwell A, Hoffmann A. A single NFkappaB system for both canonical and non-canonical signaling. CELL RES. 2011;21(1):86–102.
34. Liu M, Sakamaki T, Casimiro MC, Willmarth NE, Quong AA, Ju X, Ojeifo J, Jiao X, Yeow WS, Katiyar S, et al. The canonical NF-kappaB pathway governs mammary tumorigenesis in transgenic mice and tumor stem cell expansion. CANCER RES. 2010;70(24):10464–73.
35. McConkey DJ, Choi W, Marquis L, Martin F, Williams MB, Shah J, Svatek R, Das A, Adam L, Kamat A, et al. Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer. CANCER METAST REV. 2009;28(3–4):335–44.
36. Harhaj EW, Dixit VM. Regulation of NF-kappaB by deubiquitinases. IMMUNOL REV. 2012;246(1):107–24.
37. Witjes JA, Hendricksen K. Intravesical pharmacotherapy for non-muscle-invasive bladder cancer: a critical analysis of currently available drugs, treatment schedules, and long-term results. EUR UROL. 2008;53(1):45–52.
38. El GK, Al-Kikhia L, Mansuri N, Syrjanen K, Al-Fituri O, Elzagheid A. **Angiogenesis in urinary bladder carcinoma as defined by microvessel density (MVD) after immunohistochemical staining for Factor VIII and CD31**. *LIBYAN J MED* 2011, 6.
39. Farrow B, Albo D, Berger DH. The role of the tumor microenvironment in the progression of pancreatic cancer. J SURG RES. 2008;149(2):319–28.
40. Lowe SW, Cepero E, Evan G. Intrinsic tumour suppression. NATURE. 2004;432(7015):307–15.
41. Wend P, Holland JD, Ziebold U, Birchmeier W. Wnt signaling in stem and cancer stem cells. SEMIN CELL DEV BIOL. 2010;21(8):855–63.
42. Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H, Wirth T. NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. CANCER LETT. 2010;295(2):214–28.
43. Strieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane MP. CXC chemokines in angiogenesis. CYTOKINE GROWTH F R. 2005;16(6):593–609.
44. Rot A. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. Immunol Today. 1992;13(8):291–4.
45. Wang L, Zhang ZG, Zhang RL, Gregg SR, Hozeska-Solgot A, LeTourneau Y, Wang Y, Chopp M. Matrix metalloproteinase 2 (MMP2) and MMP9 secreted by erythropoietin-activated endothelial cells

promote neural progenitor cell migration. J NEUROSCI. 2006;26(22):5996–6003.

46. Sumpio BE, Riley JT, Dardik A. Cells in focus: endothelial cell. Int J Biochem Cell Biol. 2002;34(12):1508–12.
47. Wang D, Richmond A. Nuclear factor-kappa B activation by the CXC chemokine melanoma growth-stimulatory activity/growth-regulated protein involves the MEKK1/p38 mitogen-activated protein kinase pathway. J BIOL CHEM. 2001;276(5):3650–9.
48. Chow MT, Luster AD. Chemokines in Cancer. CANCER IMMUNOL RES. 2014;2(12):1125–31.
49. Karashima T, Sweeney P, Kamat A, Huang S, Kim SJ, Bar-Eli M, McConkey DJ, Dinney CP: **Nuclear factor-kappaB mediates angiogenesis and metastasis of human bladder cancer through the regulation of interleukin-8**. *CLIN CANCER RES* 2003, **9**(7):2786–2797.
50. Takamori H, Oades ZG, Hoch OC, Burger M, Schraufstatter IU: **Autocrine growth effect of IL-8 and GROalpha on a human pancreatic cancer cell line, Capan-1**. *PANCREAS* 2000, **21**(1):52–56.

Tables

Table 1
information of the antibodies for Western Blot

Gene ID	Antibody	Dilutions	Species	Supplied by
NM_004360.3	E-Cadherin	1:600	Homo	Santa Cruz
NM_001792.3	N-Cadherin	1:300	Homo	Santa Cruz
NM_003380.3	Vimentin	1:300	Homo	Santa Cruz
NM_001145138.1	NF-κB (P65)	1:300	Homo	Santa Cruz
NM_004530.4	MMP2	1:400	Homo	Santa Cruz
NM_004994.2	MMP9	1:400	Homo	Santa Cruz
NM_002046.4	GAPDH	1:15000	Homo	Santa Cruz
NM_005325.3	Histon H1	1:300	Homo	Santa Cruz
NM_020529.2	IκBα	1:300	Homo	Santa Cruz

Table 2
Primers for real time PCR and siRNA for P65 knocking down

Gene ID	Gene	Primers	Seqence
NM_001145138.1	*P65*	F	*ACG AAT GAC AGA GGC GTG TAT AAG G*
		R	*CAG AGC TGC TTG GCG GAT TAG*
NM_002046.4	*GAPDH*	F	*AAC AGC GAC ACC CAT CCT C*
		R	*CAT ACC AGG AAA TGA GCT TGA CAA*
NM_001127891.1	*MMP2*	F	*CTC ATC GCA GAT GCC TGG AA*
		R	*TTC AGG TAA TAG GCA CCC TTG AAG A*
NM_004994.2	*MMP9*	F	*TGA CAG CGA CAA GAA GTG*
		R	*CAG TGA AGC GGT ACA TAG G*
NM_004360.3	*E-Cadherin*	F	*TGC CCA GAA AAT GAA AAA GG*
		R	*GTG TAT GTG GCA ATG CGT TC*
NM_001792.3	*N-Cadherin*	F	*ACA GTG GCC ACC TAC AAA GG*
		R	*CCG AGA TGG GGT TGA TAA TG*
NM_003380.3	*Vimentin*	F	*GAG AAC TTT GCC GTT GAA GC*
		R	*GCT TCC TGT AGG TGG CAA TC*
NM_001511.3	*CXCL1*	F	*AAC CGA AGT ATA GCC ACA C*
		R	*GTT GGA TTT GTC ACT GTT CAG C*
NM_002089.3	*CXCL2*	F	*TGC AGG GAA TTC ACC TCA AG*
		R	*TGA GAC AAG CTT TCT GCC CA*
NM_002090.2	*CXCL3*	F	*CTG CAG GGA ATT CAC CTC AAG AA*
		R	*ACT TCT CTC CTG TCA GTT GGT GCT C*
NM_002619.3	*CXCL4*	F	*CTG AAG AAG ATG GGG ACC TG*
		R	*TTC AGC GTG GCT ATC AGT TG*
NM_002994.4	*CXCL5*	F	*TCA CAG AGT AGA ACC TGG GTT AGA G*
		R	*TGT GTC CCA CCA GGA CTA GAA*
NM_002993.3	*CXCL6*	F	*AAT TTT GGA CAG TGG AAA CAA GAA A*
		R	*AGA AAA CTG CTC CGC TGAAGA CT*
NM_002704.3	*CXCL7*	F	*ACC ATG AGC CTC AGA CTT GAT ACC*

Gene ID	Gene	Primers	Seqence
		R	*TTA ATC AGC AGA TTC ATC ACC TGC C*
NM_000584.3	*CXCL8*	F	*GTG CAG TTT TGC CAA GGA GT*
		R	*CTC TGC ACC CAG TTT TCC TT*
NM_002416.1	*CXCL9*	F	*AGG GTC GCT GTT CCT GCA TC*
		R	*TTC ACA TCT GCT GAA TCT GGG TTT A*
NM_001565.3	*CXCL10*	F	*GAC ATA TTC TGA GCC TAC AGC AGA G*
		R	*GTT GAT TAC TAA TGC TGA TGC AGG T*
NM_005409.4	*CXCL11*	F	*CCT TGG CTG TGA TAT TGT GTG CTA*
		R	*ACT TGG GTA CAT TAT GGA GGC TTT C*
NM_000609.6	*CXCL12*	F	*AAG CCC GTC AGC CTG AGC TA*
		R	*TTA GCT TCG GGT CAA TGC ACA C*
NM_006419.2	*CXCL13*	F	*GAG GCA GAT GGA ACT TGA GC*
		R	*CTG GGG ATC TTC GAA TGC TA*
NM_004887.4	*CXCL14*	F	*AAG CTG GAA ATG AAG CCA AA*
		R	*TTC CAG GCG TTG TAC CAC TT*
NM_000634.2	*CXCR1*	F	*ACA CGC ACA CTG ACC CAG GAA*
		R	*CAT CCG CCA TTT TGC TGT GT*
NM_001168298.1	*CXCR2*	F	*TCA CATT CCA AGC CTC ATG TCC*
		R	*GCA GAG CTC CAG CAA ATG ACA TA*
NM_001142797.1	*CXCR3*	F	*TAC AAG GCA TGG CGT AGA GG*
		R	*GGT CCT GAC GAT CTT GTT TAT TGA*
NM_001008540.1	*CXCR4*	F	*CCT GCC TGG TAT TGT CAT CCT G*
		R	*ACT GTG GTC TTG AGG GCC TTG*
NM_001716.4	*CXCR5*	F	*TCA GAC TGG TTG AGT TCA GGT AGC T*
		R	*ACC CAG GAT CCG GTG ACA T*
NM_001145138.1	P65 siRNA	*F*	AAG AGC ATC ATG AAG AAG AGT CCT GTC TC
		R	AAA CTC TTC TTC ATG ATG CTC CCT GTC TC

Figures

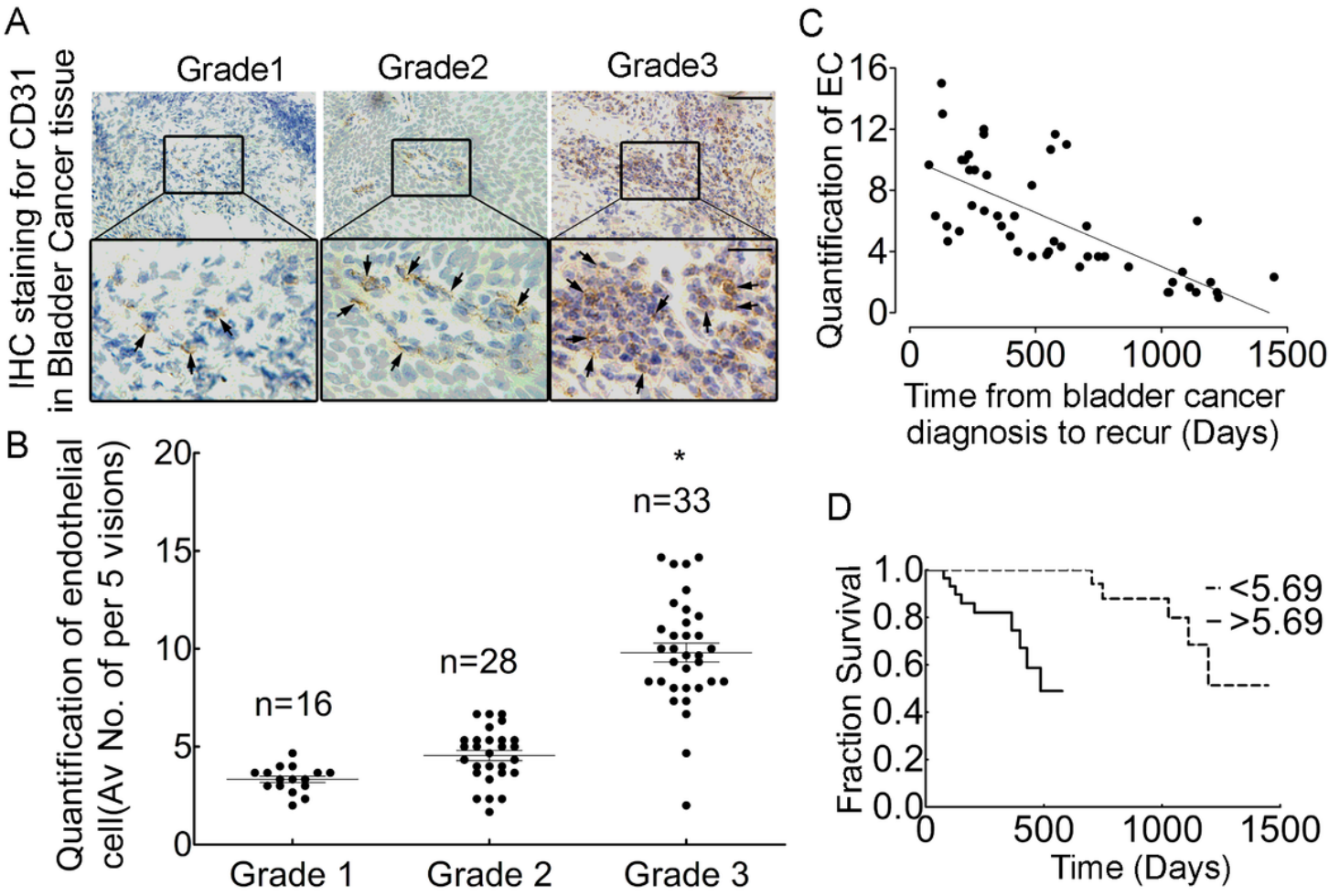

Figure 1

Figure 1

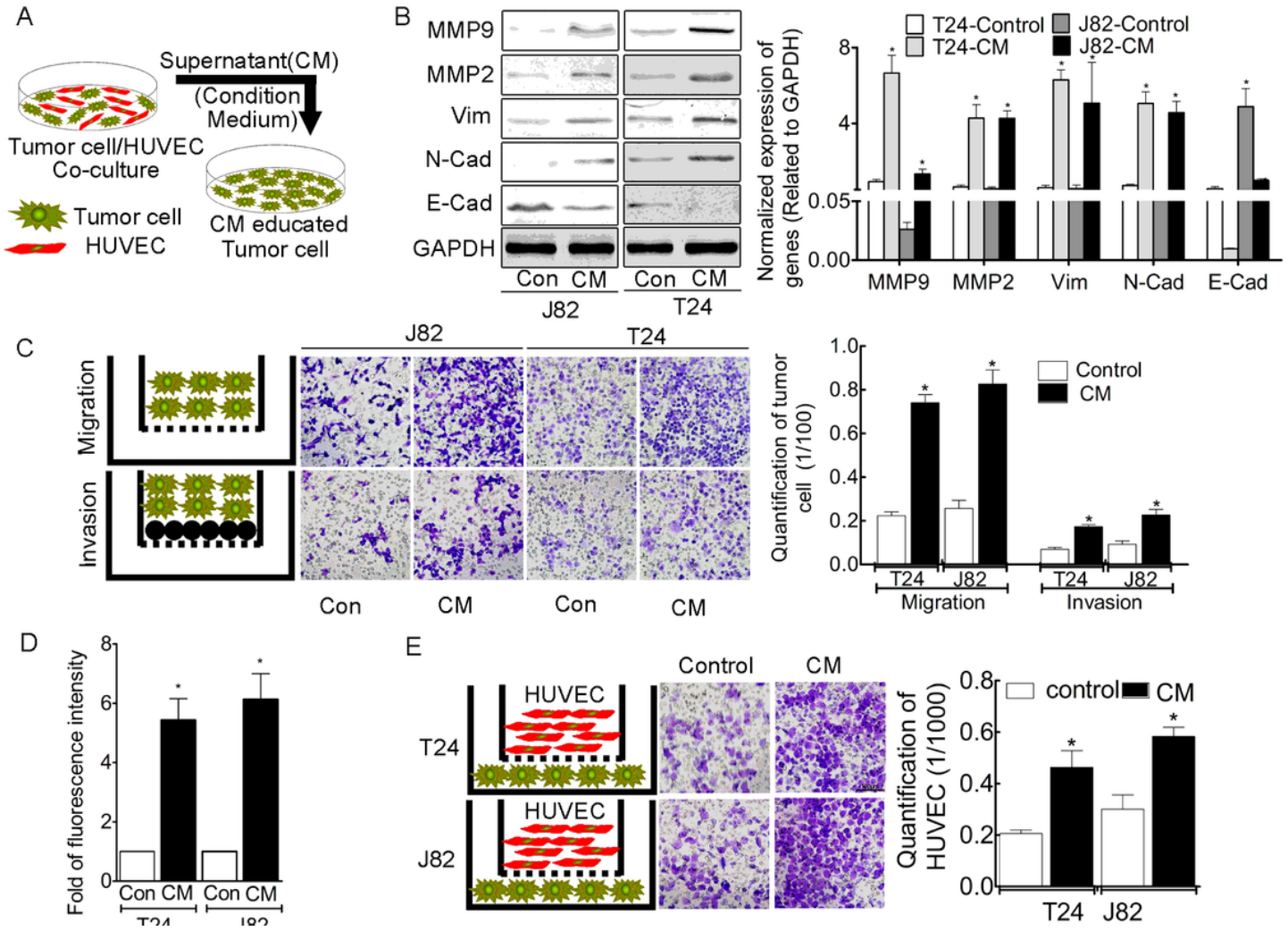

Figure 2

Figure 2

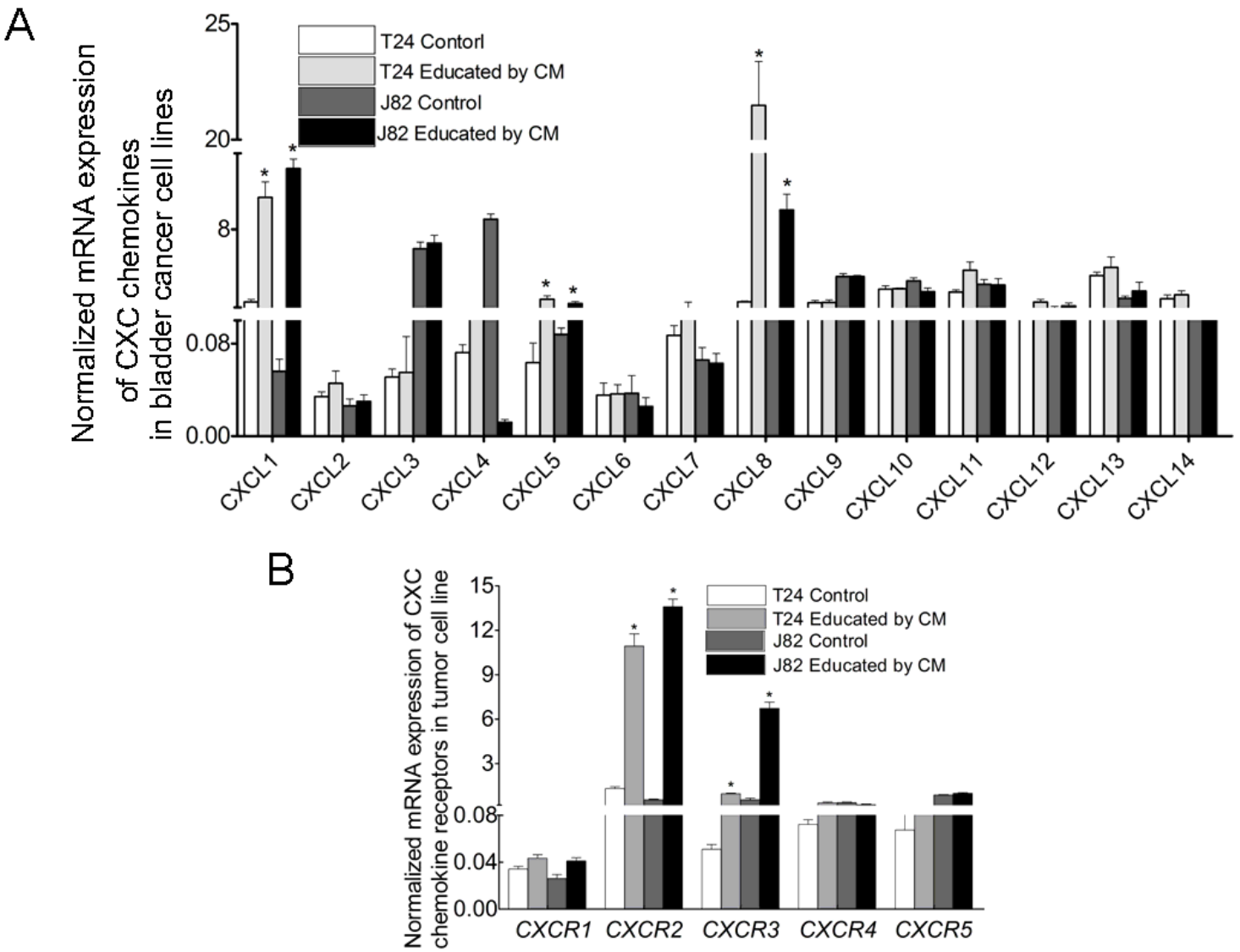

Figure 3

Figure 3

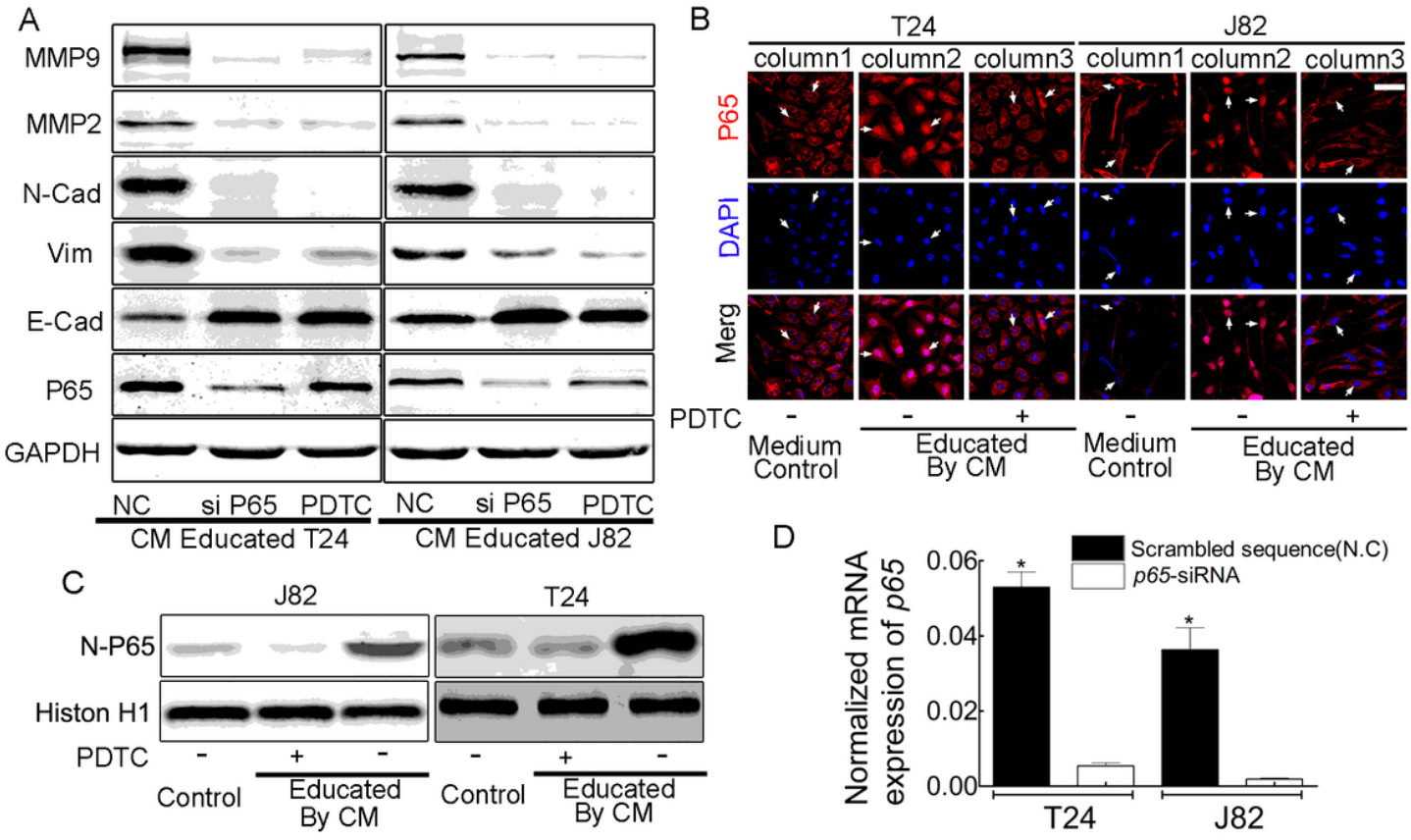

Figure 4

Figure 4

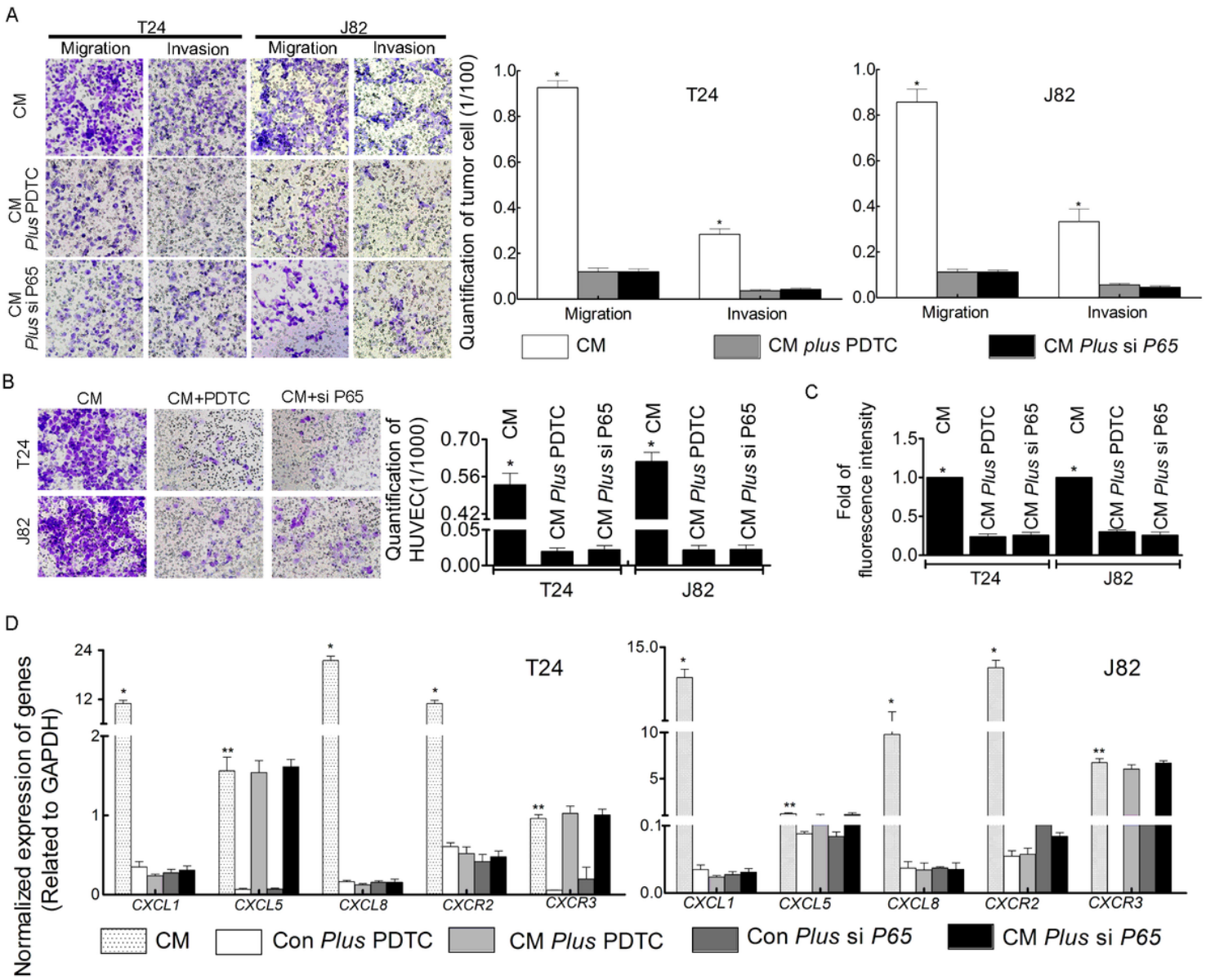

Figure 5

Figure 5

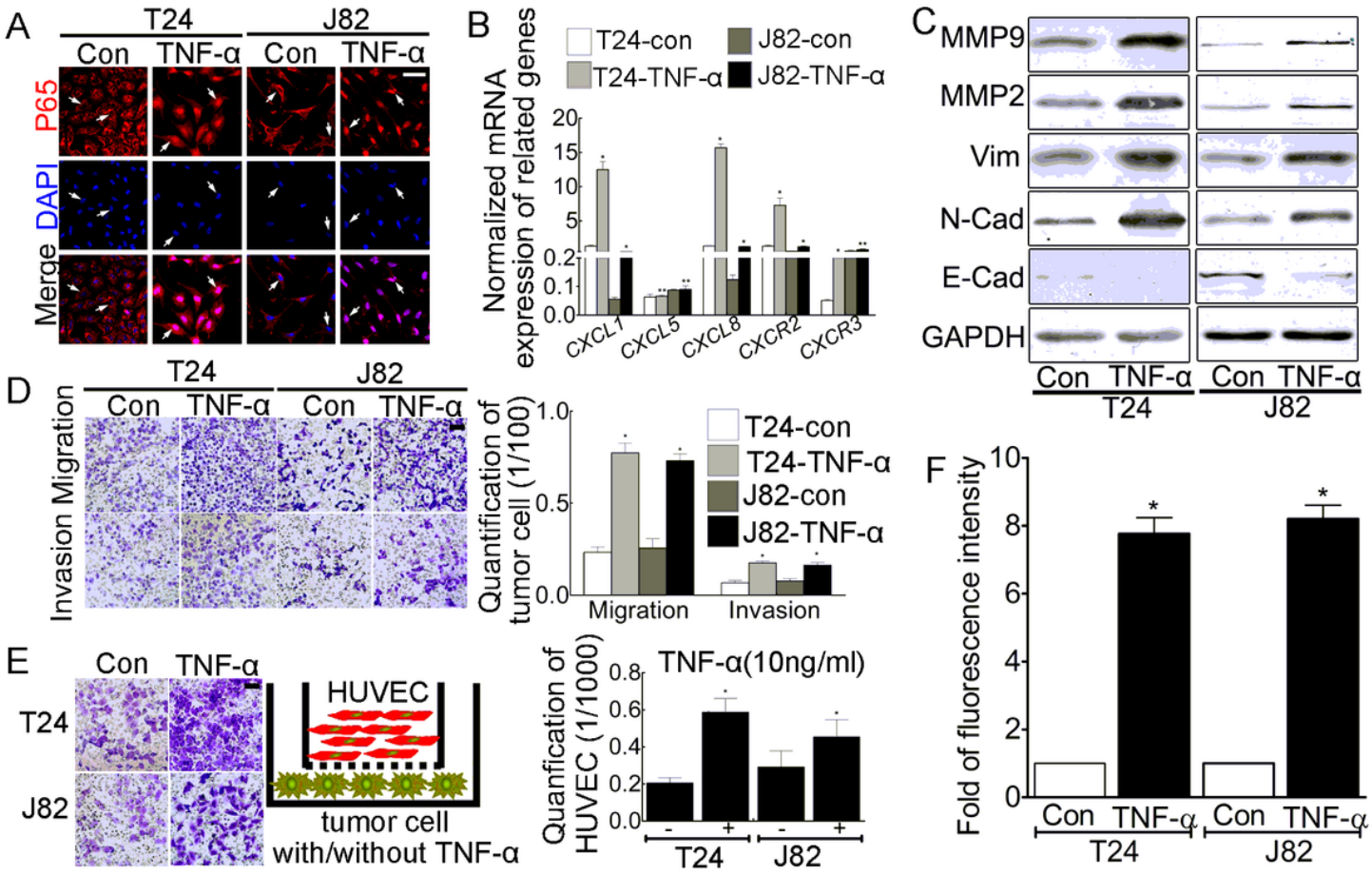

Figure 6

Figure 6

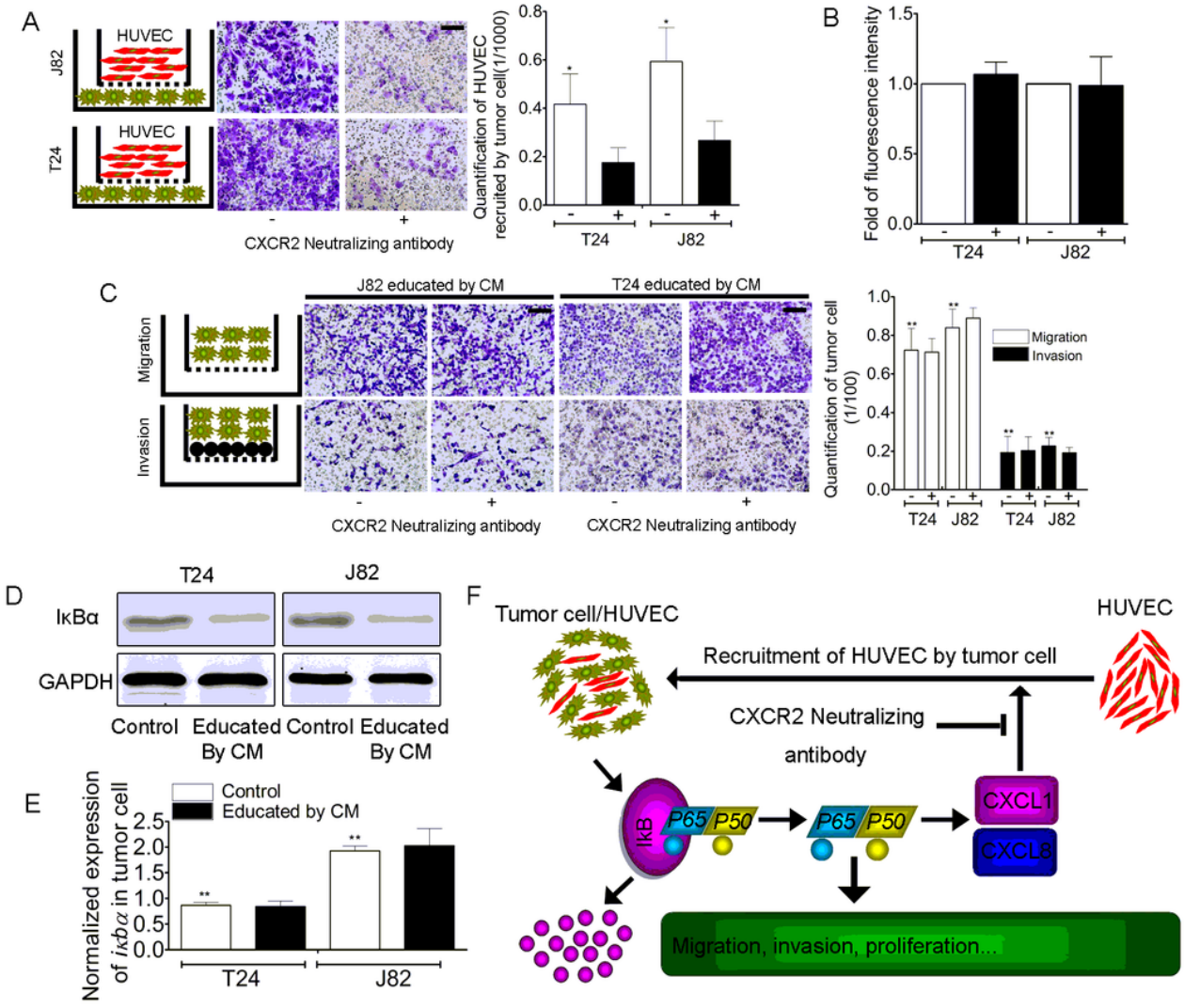

Figure 7

Figure 7