NF-κB signaling plays irreplaceable roles in cisplatin-induced bladder cancer chemoresistance and tumor progression

YI SUN*, ZHENFENG GUAN*, LIANG LIANG, YONGYI CHENG, JIANCHENG ZHOU, JING LI and YONGGANG XU

Department of Urology, Shaanxi Provincial People's Hospital, Xi'an, P.R. China

Received August 16, 2015; Accepted October 14, 2015

DOI: 10.3892/ijo.2015.3256

Abstract. Bladder cancer (BCa) is the most vital urogenital malignant disease worldwide, bringing huge economic and social burden every year. Clinically, BCa is subdivided into superficial type and invasive type according to clinic-pathology, accompanied with different strategy of therapy. Number of reports indicate that 10-30% of superficial BCa will inevitable progress into invasive type, manifesting enhanced malignant behavior compared to original invasive type. Regardless of the original being an original invasive type or invasive type that progressed from superficial type of BCa, chemotherapy (including adjuvant or neo-adjuvant chemotherapy) in line with radiotherapy is the final regimen for BCa patients. Previous reports pointed out the high efficiency of cisplatin-containing chemotherapeutic regimen for BCa patients, leading to wide use of this regimen in BCa therapeutics. However, cisplatin-resistance inevitably appear, resulting in the failure of the BCa chemotherapy, the mechanism of which is still unknown. In the present study, parental BCa cell lines T24/J82 were used to obtain stable-cisplatin-resistance cell lines $T24^R$/$J82^R$, which showed enhanced capacity of malignancy, tumorigenesis and drug resistance, accompanied by elevated expression of EMT markers. The further mechanism investigation suggested that prolonged time of cisplatin-treatment contributed to the activation of the NF-κB signal, resulting in the upregulation of EMT markers, the maintenance of stem cell marker and the elevated expression of ABCB1. Thus, our study provides us a new view of the role of NF-κB signaling in BCa therapeutics.

Correspondence to: Professor Yi Sun, Department of Urology, Shaanxi Provincial People's Hospital, 256 Youyi west Road, Xi'an 710068, P.R. China
E-mail: adoremidfall@163.com

*Contributed equally

Key words: bladder cancer, NF-κB signaling, cisplatin, chemoresistance, progression

Introduction

Bladder cancer (BCa) is the 7th and 17th most common cancer in men and women worldwide, respectively, bringing huge economic and social burden (1). Clinically, BCa is classified as non-muscle-invasive type and muscle-invasive type, which are followed by definitely different therapeutic guidelines, with differing prognosis (2,3). For non-muscle invasive BCa, the recommended strategy is transurethral resection of bladder tumor (TURBT) followed by instillation of adjuvant intravesical chemotherapy. For muscle-invasive BCa, radical cystectomy is recommended, accompanied by neo-adjuvant chemotherapy, in addition, radical chemo- and radio-therapies are an options for the patients who are not fit for surgery (2,3). Non-muscle invasive BCa (10-30%) will unavoidably progress into muscle invasive type (2,4), in line with original muscle invasive BCa, bringing the irreplaceable roles of chemotherapy to BCa management, regardless whether adjuvant or neoadjuvant chemotherapy.

Cisplatin-based chemotherapeutic regimen is regarded as the most effective regimen for BCa (5,6), leading to the recommended use of cisplatin-containing regimen, such as MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) (7). However, chemoresistance unavoidably appears, resulting in the failure of chemotherapy. Besides some BCa patients present primary chemoresistance, although acquired chemoresistance attracts more attention, the mechanism of which is still unclear (8).

ATP-binding cassette (ABC) family, the vital ATP-dependent transporter located in cell membrane, is gaining interest in the process of mechanical investigation of chemoresistance in BCa therapy (8,9). ABC transporters 'pump' drugs, such as anti-neoplastic drugs, out of the cells against a concentration gradient, this is the basic mechanism of chemoresistance (10). To date, 48 members of the ABC transporter family have been isolated and identified (11,12). However, not all ABC family members are reported to participate in chemoresistance, Dean and Annilo (13) indicated that only *ABCA2, ABCB1, ABCC1, ABCC3* and *ABCG2* were proven to play crucial roles in the development of drug resistance.

The vast majority of studies point out the important roles of NF-κB signal pathway in the process of life evolution (14-16), including embryonic differentiation, organ formation, inflammatory reaction, wound healing and, the most vital one

of tumorigenesis (17). Our previous study, in line with others, suggested that NF-κB signaling played irreplaceable roles in BCa progression. Thus, inhibition of this signal pathway potentially could be used as therapeutic regimen for BCa (18). In the present study, we established cisplatin-resistant BCa cell lines, and demonstrated that these cells exhibited enhanced capacity of migration/invasion, proliferation and tumorigenesis compared with the parental cells. Mechanistically, NF-κB signaling was activated in the prolonged treatment of cisplatin for BCa cells, and played key roles in cisplatin-induced chemoresistance and malignant behavior in BCa. These data indicate that NF-κB signaling is critical in the development of resistance to cisplatin and potentially of interest in clinical work against BCa.

Materials and methods

Cell culture. Human bladder cancer cell lines T24, and J82 were obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in DMEM supplemented by 10% FBS (Invitrogen, Carlsbad, CA, USA). Cells were cultured in an atmosphere with 5% CO_2 at 37˚C (incubators: Thermo Scientific, Germany).

To generate cisplatin-resistant cell lines, the cultured parental T24 and J82 cells were supplemented with 20 μM cisplatin, the medium was refreshed every two days to remove the dead cells and washed thrice using sterile PBS (pH 7.2). This treatment lasted for more than three months until achieving stably cisplatin-resistant T24/J82 cells (tagged with $T24^R$/$J82^R$).

In NF-κB signaling activity assays, TNF-α (10 ng/ml), a classical activator of NF-κB signaling (19), or pyrrolidine dithiocarbamate (PDTC, Sigma-Aldrich, USA, 10 μM), an inhibitor of NF-κB pathway (20), was added into the medium of cultured cells before protein extraction or other analysis.

Western blot analysis. Pretreated cells were harvested at 80% confluence, and washed with cold PBS three times. Total cellular protein lysates were prepared with 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% inhibitor cocktail and 1 mM PMSF, both from Sigma, (St Louis, MO, USA)]. Protein (30 μg) was separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h with 5% skim milk in Tris-buffered saline (pH 7.6, TBS). Polyclonal primary antibodies were applied at different dilutions (Table I) in 5% skim milk in TBS at 4˚C overnight, followed by TBST (with Tween-20) washes. Membranes were incubated with fluorescent secondary antibodies (Licor, Rockford, IL, USA) coupled to the first antibody at room temperature in the dark for 1 h, followed by TBST washes, dried with neutral absorbent paper and scanned by Odyssey Detection System (Licor). MG-132 (Sigma-Aldrich) was used to inhibit the proteasome-dependent degradation when necessary (10 μM, 4 h before the protein harvest). GAPDH (for total cell fraction) and histone H1 (for nuclear fraction) were used as loading control.

Real-time PCR. Cellular total RNA was isolated using TRIzol reagent (Invitrogen) and quantitated by absorbance at 260 nm. RNA (2 μg) was reverse transcribed using Revert Aid™ First Strand cDNA Synthesis kit (MBI Fermentas, St Leon-Rot, Germany) strictly according to the manufacturer's protocol. For real-time PCR, we used the SYBR Premix Ex Taq™ II system (Takara Biotechnology Co., Ltd., Dalian, China) and the Bio-Rad CFX96™ Real-time system (Bio-Rad, CA, USA). Primers are listed in Table II. Briefly, 12.5 μl SYBR Premix Ex Taq™ II, 1 μl primer (F and R, respectively), 200 ng cDNA and 9.5 μl distilled and deionized water were mixed, followed by two stage, pre-degeneration for 95˚C, 30 sec, one repeat; and PCR reaction, 95˚C 5 sec followed by 60˚C, 30 sec, 30 repeats; and the third stage as dissociation, 95˚C, 15 sec followed by 60˚C, 30 sec, and another 95˚C, 15 sec. GAPDH was used as the loading control.

MTT assay. Cell viability was assessed using a tetrazolium-based assay. Pretreated cells were incubated in the absence or presence of cisplatin for the indicated times, and then washed once with PBS and incubated with 0.5 mg/ml of MTT at 37˚C for 1 h. The reagent was reduced by living cells to form aninsoluble blue formazan product. After incubation, cells were lysed with DMSO. Colorimetric analysis using a 96-well microplate reader was performed at a wavelength of 490 nm. The experiments were performed in triplicate.

Cell migration/invasion assay. Ability of migration/invasion was demonstrated by Boyden Chamber assay, the chambers with pore of 8 μm diameter were obtained from Millipore (Millipore, Switzerland). For migration assay, 0.2 ml FBS-free DMEM medium suspension with 10,000 cells was added to the upper chamber in 24-well plate, and 0.8 ml FBS-free DMEM was added to the lower chamber. After 12-h incubation, the chambers were washed with PBS (pH 7.4) three times to remove the cells in the upper chamber and were fixed with 4% formalin for 15 min, then stained with crystal violet (0.01% in the ethanol) for 25 min followed by washing three times with PBS. The cells were counted using an inverted microscope, and five visions were randomly taken in the x200 magnification, and the average number of cells was analyzed. For the invasion assay, the cell suspension (10,000 cells/well) in the upper chamber contained 0.2 ml mixture of FBS-free DMEM/Matrigel at 8/1 ratio (Matrigel, Sigma, USA). Cells were incubated for 36 h and the rest of the protocol conducted in a similar manner as the migration assay.

Cell proliferative capacity assay. 5-Bromo-2-deoxyuridine (BrdU) incorporation assay was used for analysis of tumor proliferative ability. Briefly, pretreated cells were placed on 8-well glass (Millipore) until 50-70% confluence, and BrdU was added into the medium (3 μg/ml), followed by 4-h incubation and then rinsed 3 times with PBS over 10 min to remove residual free BrdU. Cells were then fixed by 4% paraformaldehyde for 45 min, followed by rinsing 5 times with PBS over 20 min. Triton X-100 (0.1%) was used to permeabilize the cell membrane for 15 min and 2N HCl was added for 25 min to separate DNA into single strands to allow primary antibody access to the incorporated BrdU. Cells were then rinsed 3 times with PBS over 10 min and non-specific epitopes blocked by 10% BSA for 20 min. Anti-BrdU antibody (1:200) in 10% BSA was added and then incubated overnight in 4˚C. Cells

Table I. The antibodies used.

Gene	ID	Dilution	Tag	Company
ABCB1	NM_000927.4	1:300	sc-13131	Santa Cruz
ABCC1	NM_004996.3	1:300	sc-136447	Santa Cruz
E-cadherin	NM_004360.3	1:600	sc-21791	Santa Cruz
GAPDH	NM_002046.5	1:15,000	ab181602	Abcam
Histone H1	NM_005325.3	1:400	sc-247158	Santa Cruz
MMP2	NM_001127891.2	1:400	ab86607	Abcam
MMP9	NM_004994.2	1:400	ab38898	Abcam
Nanog	NM_001297698.1	1:300	sc-374001	Santa Cruz
N-cadherin	NM_001308176.1	1:500	sc-271386	Santa Cruz
Oct-4	NM_001173531.2	1:300	sc-5279	Santa Cruz
P65	NM_001145138.1	1:300	sc-372	Santa Cruz
Snail	NM_005985.3	1:400	sc-28199	Santa Cruz
Vimentin	NM_003380.3	1:400	sc-7558	Santa Cruz

Table II. The primers for real-time PCR.

Gene	ID	Sequence
ABCA2	NM_001606	F: *AAG CCT GTG GAG GAT GAT GT* R: *GGT CAA CGG CCA GGA TAC G*
ABCB1	NM_000927.4	F: *GTC CCA GGA GCC CAT CCT* R: *CCC GGC TGT TGT CTC CAT A*
ABCC1	NM_004996.3	F: *GAA GGC CAT CGG ACT CTT CA* R: *CAG CGC GGA CAC ATG GT*
ABCC3	NM_001144070.1	F: *CAC ACG GAT CTG ACA GAC AAT GA* R: *ACA GGG CAC TCA GCT GTC TCA*
ABCG2	NM_001257386.1	F: *CAG GTC TGT TGG TCA ATC TCA CA* R: *TCC ATA TCG TGG AAT GCT GAA G*
E-cadherin	NM_004360.3	F: *TGC CCA GAA AAT GAA AAA GG* R: *GTG TAT GTG GCA ATG CGT TC*
GAPDH	NM_001256799.2	F: *AAC AGC GAC ACC CAT CCT C* R: *CAT ACC AGG AAA TGA GCT TGA CAA*
MMP2	NM_001127891.2	F: *CTC ATC GCA GAT GCC TGG AA* R: *TTC AGG TAA TAG GCA CCC TTG AAG A*
MMP9	NM_004994.2	F: *TGA CAG CGA CAA GAA GTG* R: *CAG TGA AGC GGT ACA TAG G*
Nanog	NM_001297698.1	F: *CTA AGA GGT GGC AGA AAA ACA* R: *CTG GTG GTA GGA AGA GTA AAG G*
N-cadherin	NM_001308176.1	F: *ACA GTG GCC ACC TAC AAA GG* R: *CCG AGA TGG GGT TGA TAA TG*
Oct-4	NM_001173531.2	F: *TTG GGC TAG AGA AGG ATG TGG TT* R: *GGA AAA GGG ACT GAG TAG AGT GTG G*
P65	NM_001145138.1	F: *ACG AAT GAC AGA GGC GTG TAT AAG G* R: *CAG AGC TGC TTG GCG GAT TAG*
Snail	NM_005985.3	F: *ACC CCA ATC GGA AGC CTA ACT* R: *GGT CGT AGG GCT GCT GGA A*
Vimentin	NM_003380.3	F: *GAG AAC TTT GCC GTT GAA GC* R: *GCT TCC TGT AGG TGG CAA TC*

were rinsed 5 times with PBS, followed by incubation with TRTIC-labeled secondary antibody for 1 h, and finally rinsed 3 times with PBS to remove the free antibody. The fluorescence intensity of TRITC was monitored by SuperMicro Orifice Plate Spectrophotometer (BioTek, USA) at 547 nm.

Animal experiment. In order to demonstrate the ability of tumorigenesis, the parental BCa cells T24/J82, and chemoresistant BCa cells, $T24^R/J82^R$, were implanted subcutaneously into the flanks of the mice. In brief, 10^6 BCa cells mixed with matrigel (V/V=1:2) were injected subcutaneously into the flanks of the mice, 5 weeks later, tumor mass was harvested, weighed, fixed with 4% formalin, and prepared for pathological analysis.

Immunofluorescence staining. After designated treatment, the pretreated cells were washed three times with cold PBS (pH 7.4) and followed by fixing 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) IgG antibody (Santa Cruz, USA) diluted 1:100 in blocking buffer for 1 h. Nuclei were stained with DAPI for 5 min. Cells were examined with a fluorescent microscope equipped with narrow band-pass excitation filters to individually select for red, and blue fluorescence. Cells were observed through the Image Pro Plus System™ mounted on the fluorescent microscope (Olympus, Japan). The experiment was repeated three times.

Statistical analysis. ANOVA test was used to analyze the statistical discrepancy in >3 groups. Student's t-test was used to detect any statistically significant difference between 2 groups. P-values <0.05 were considered significant.

Results

Stable-chemoresistant cell lines induced by cisplatin manifest enhanced malignancy, proliferative capacity and tumorigenic ability. Chemoresistance is regarded as a vital obstacle of BCa chemotherapeutics (8). Cisplatin, one of the major drugs in chemotherapeutics for BCa, is recommended in first-line of clinical work (5). Therefore, cisplatin-resistance is ubiquitous in BCa patients, dissecting the mechanism of which potentially brings benefits to BCa patients. In order to obtain cisplatin-resistant BCa cell lines, T24 and J82, two BCa cell lines were used to generate stably cisplatin-resistant cell lines (tagged by $T24^R$ and $J82^R$, respectively) as indicated in Materials and methods. Comparing with parental T24/J82, $T24^R/J82^R$ showing cisplatin-resistance demonstrated by MTT assay (Fig. 1A). The following *in vivo* analysis suggested the enhanced tumorigenic capacity (Fig. 1B); in addition, the *in vitro* analysis suggested that these cisplatin-resistant BCa cells, $T24^R/J82^R$ possessed enhanced ability of proliferation (Fig. 1C) and migration/invasion (Fig. 1D and E).

$T24^R/J82^R$ shows enhanced expression of EMT markers and ABCB1/ABCC1. The enhanced capacity of migration/invasion and proliferation of $T24^R/J82^R$ drove us to monitor the expression of related genes, including EMT markers. As expected, both the western blot (Fig. 2A) and real-time PCR (Fig. 2B) analyses suggested elevated expression of EMT related makers. In another aspect, ABC transporter family, was regarded to play irreplaceable roles in chemoresistance. The expression of drug-resistant related members of ABC family were examined by real-time PCR, and the results indicated elevated expression of *ABCB1* and *ABCC1* in the two cell lines (Fig. 2C). In addition to real-time PCR, western blot analyses also suggested elevated expression of the two members (Fig. 2D).

Expression of stem cell markers are enhanced in $T24^R/J82^R$. Previous *in vivo* results (Fig. 1B) indicated the enhanced tumorigenic capacity of $T24^R/J82^R$ cell lines, evoking us to dissect the stem cell related characteristics of the two stable cell lines. The western blot and real-time PCR analyses both indicated elevated expression of Nanog and Oct-4 in $T24^R$ and $J82^R$ cell lines (Fig. 2A and B), providing evidence to support our postulation that cisplatin-induced drug-resistant cell lines acquired the characteristics of stem cells.

NF-κB signaling is activated in $T24^R/J82^R$. Plenty of studies have pointed out the important role of NF-κB signaling in tumorigenesis and cancer metastasis (14,17), yet maintenance of stem cell characteristics was reported (21-23), leading us to link the cisplatin-induced phenomenon to this signal pathway. As indicated in Fig. 3A, $T24^R/J82^R$ cells had significant P65 nuclear translocation, suggesting the activation of this signaling pathway. In parallel experiment, nuclear lysates from $T24^R/J82^R$ cells were demonstrated by western blot analysis, indicating the accumulation of P65 in the nucleus (Fig. 3B). Further investigation indicated that in $T24^R/J82^R$ cells, expression of IκB was attenuated, which was the inhibitor of NF-κB signaling (Fig. 3C). Thus, we concluded that NF-κB signaling was activated in $T24^R/J82^R$ cells due to decreased expression of IκB.

Inhibition of NF-κB signaling ablates the capacity of migration/invasion, proliferation and chemoresistance in $T24^R/J82^R$ cells. Previous results suggested that the enhanced malignancy, proliferative capacity and chemoresistance were accompanied by activation of NF-κB signaling, thus we speculated that these phenomena were induced by the activation of NF-κB signaling. In order to further clarify this point, PDTC, a specific inhibitor of NF-κB signaling, was used to inhibit this pathway, followed by Boyden Chamber assay, MTT assay and BrdU incorporation assay. Our results revealed that PDTC efficiently inhibited the nuclear translocation of P65 (Fig. 4A), accompanied by attenuated capacity of migration/invasion (Fig. 4B), chemoresistance (Fig. 4C) and proliferation (Fig. 4D).

Inhibition of NF-κB signaling in $T24^R/J82^R$ results in decreased expression of EMT markers, stem cell makers and ABCB1. Malignancy and proliferative ability was attenuated in the presence of NF-κB signal inhibition, evoking the hypothesis that the inhibition of NF-κB signaling pathway affected tumor cell phenotype and the expression of ABCB1/ABCC1. Therefore, NF-κB signal was inhibited by PDTC in $T24^R/J82^R$ cells followed by monitoring the expression of EMT/stem cell

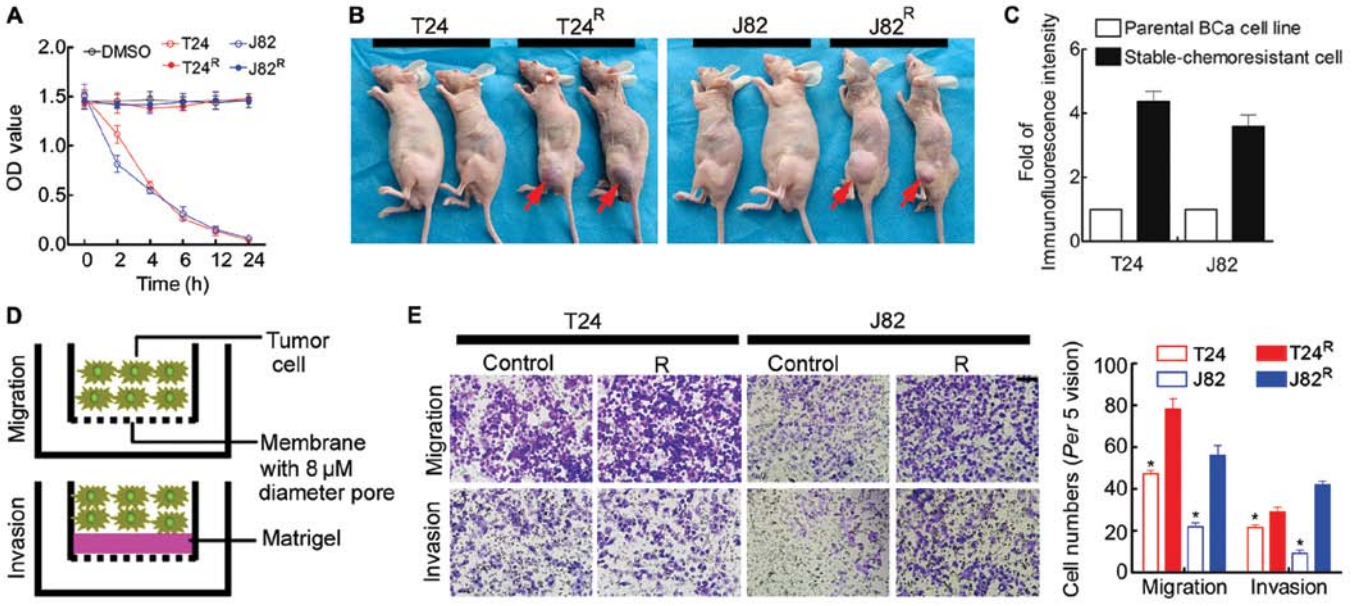

Figure 1. Chemoresistant BCa cell lines manifest enhanced capacity of malignancy, proliferation and tumorigenesis. (A) MTT suggests that chemoresistant BCa cell lines T24^R and J82^R obtained as indicated in Materials and methods show cisplatin resistance; (B) Subcutaneous xenograft indicates the enhanced capacity of tumorigenesis by T24^R/J82^R vs T24/J82, tumors are highlighted with a red arrow; (C) BrdU incorporation shows >3-fold of tumor cell proliferative ability, T24^R/J82^R vs T24/J82, as indicated by immunofluoresence intensity; (D) The schematic of Boyden Chamber assay; (E) Boyden Chamber assay reveals the enhanced capacity of tumor cell migration and invasion, T24^R/J82^R vs T24/J82. Left, the representative figures; right, quantification of Boyden Chamber assay; bar, 100 μm, *P<0.05.

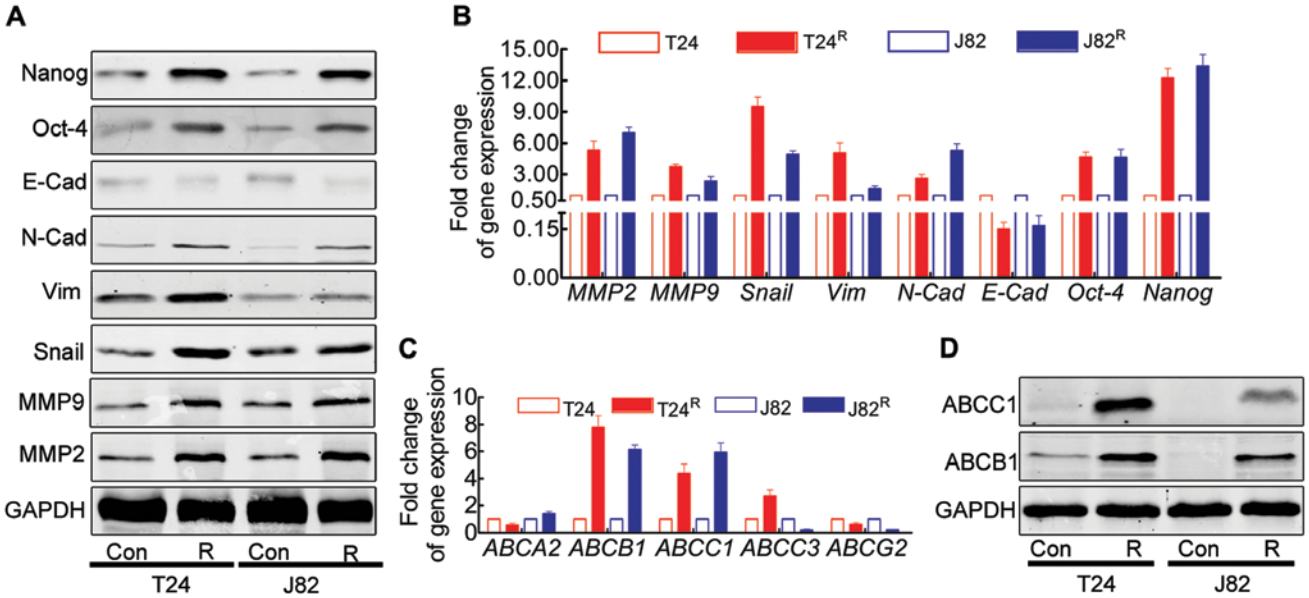

Figure 2. T24^R/J82^R show elevated expression of EMT markers, stem cell markers and multiple drug-resistant genes. (A) Western blot analysis indicates elevated expression of MMP2, MMP9, Snail, Vim, N-Cad, Oct-4 and Nanog, accompanied by decreased expression of E-Cad in T24^R/J82^R vs T24/J82; (B) Real-time PCR indicates that compared with parental T24/J82 cells, the expression of *MMP2, MMP9, Snail, Vim, N-Cad, Oct-4* and *Nanog* are elevated >3-fold, still accompanied by decreased expression of *E-Cad*; (C) Real-time PCR reveals that chemoresistant-related genes are also increased in T24^R/J82^R cells especially *ABCB1* (MDR1) and *ABCC1* (MRP1); (D) Western blot analysis suggests the enhanced expression of ABCB1 and ABCC1 in T24^R/J82^R vs T24/J82.

markers and ABCB1/ABCC1. In line with our expectation, inhibition of NF-κB signal significantly induced the decreased expression of MMP2, MMP9, Snail, vimentin and N-cadherin, accompanied by increasing of E-cadherin (Fig. 4E and F). In addition, this inhibition of NF-κB pathway also led to the attenuation of Oct-4 and Nanog, both of which were important stem cell markers. According to MTT assay (Fig. 4C), PDTC rendered decreased chemoresistance of T24^R/J82^R, thus possibly accompanied by decreased expression of ABCB1 and ABCC1. The inhibition of NF-κB signal, however, led to the

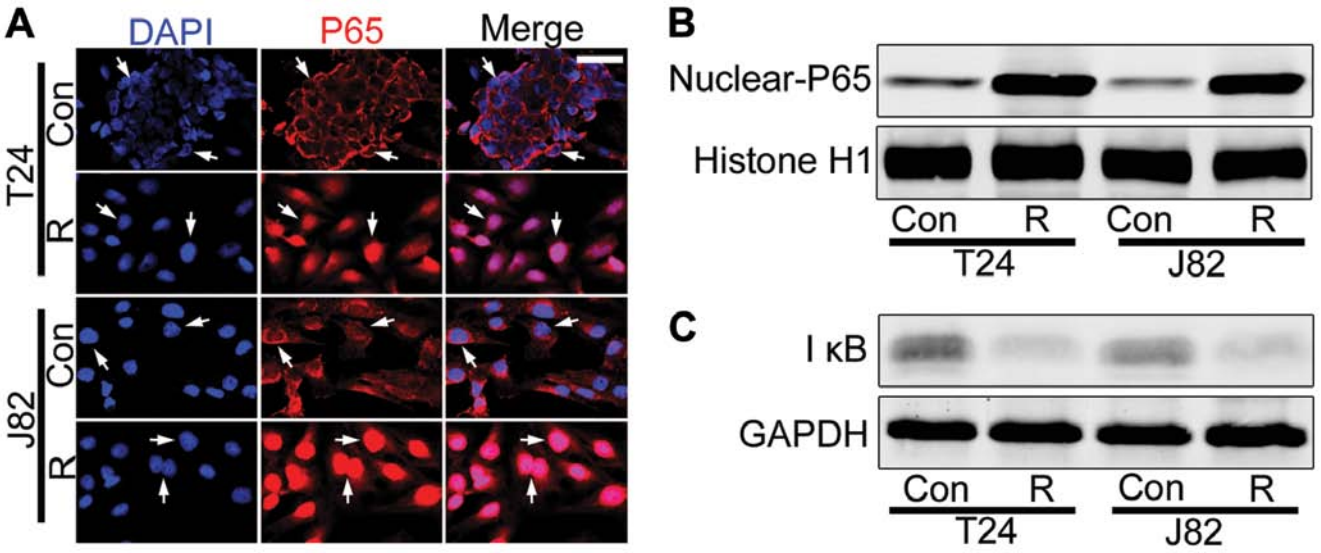

Figure 3. NF-κB signaling is activated in $T24^R/J82^R$ vs T24/J82; (A) Immunofluorescence staining reveals that the functional subunit of NF-κB signals P65 translocation into the nucleus, indicating the activation of this signaling pathway as tagged by the white arrows; bar, 100 μM; (B) Western blot analysis shows that the nuclear lysates from $T24^R/J82^R$ vs T24/J82 manifested higher expression of P65; (C) Western blot analysis suggests that, compared with T24/J82, the expression of IκB, the inhibitor of NF-κB signaling, is decreased in $T24^R/J82^R$, indicating the activation of the NF-κB pathway.

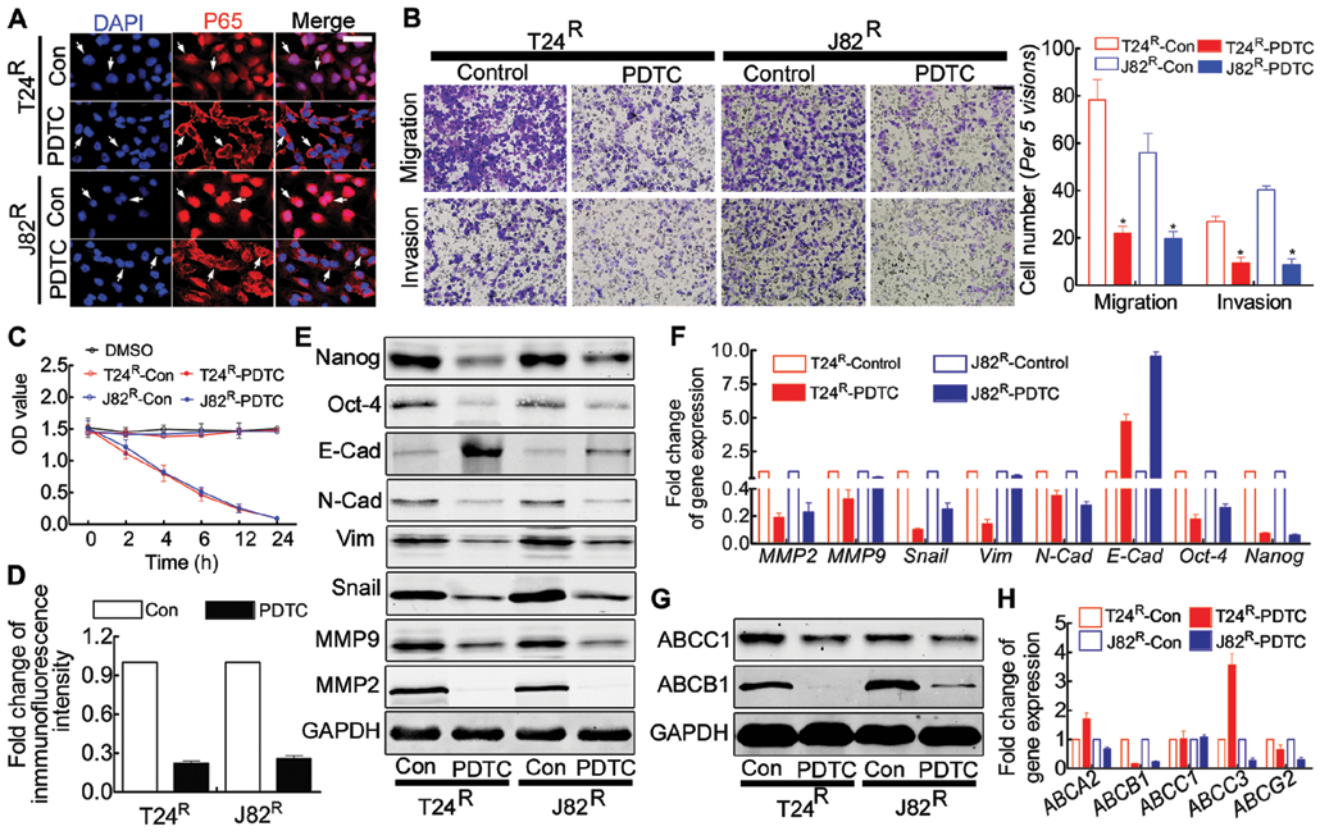

Figure 4. The malignancy, chemoresistance and proliferative ability of $T24^R/J82^R$ cells are attenuated by inhibiting NF-κB signaling, accompanied by switching of MET and decreased expression of chemoresistant related genes. (A) Immunofluorescence staining suggests that in the presence of PDTC, a specific inhibitor of NF-κB signaling, nuclear translocation of P65 is inhibited in $T24^R$ and $J82^R$ as tagged by the white arrows, indicating high efficiency of inhibiting this pathway; bar, 100 μm; (B) Boyden Chamber assay reveals that the malignant behavior is significantly inhibited by PDTC compared with control. Right, representative figures of Boyden Chamber assay; left, quantification of Boyden Chamber assay; bar, 100 μm, $^*P<0.05$; (C) MTT assay suggests attenuated chemoresistance in the presence of PDTC; (D) BrdU incorporation suggests attenuated proliferative ability of $T24^R/J82^R$ vs T24/J82; (E) Western blot analysis elucidates that EMT is reversed, manifesting as decreased expression of MMP2, MMP9, Snail, Vim and N-Cad, accompanied by elevated expression of E-Cad. In addition, stem cell makers, Oct-4 and Nanog are also attenuated in the presence of PDTC in $T24^R/J82^R$; (F) Real-time PCR shows that inhibition of NF-κB signaling by PDTC results in decreased expression of EMT and stem cell markers; (G) Western blot analysis suggests that inhibition of NF-κB signaling leads to the decreased expression of ABCB1 without significant change on *ABCC1* expression; (H) Real-time PCR shows that the expression of *ABCB1* is inhibited by PDTC.

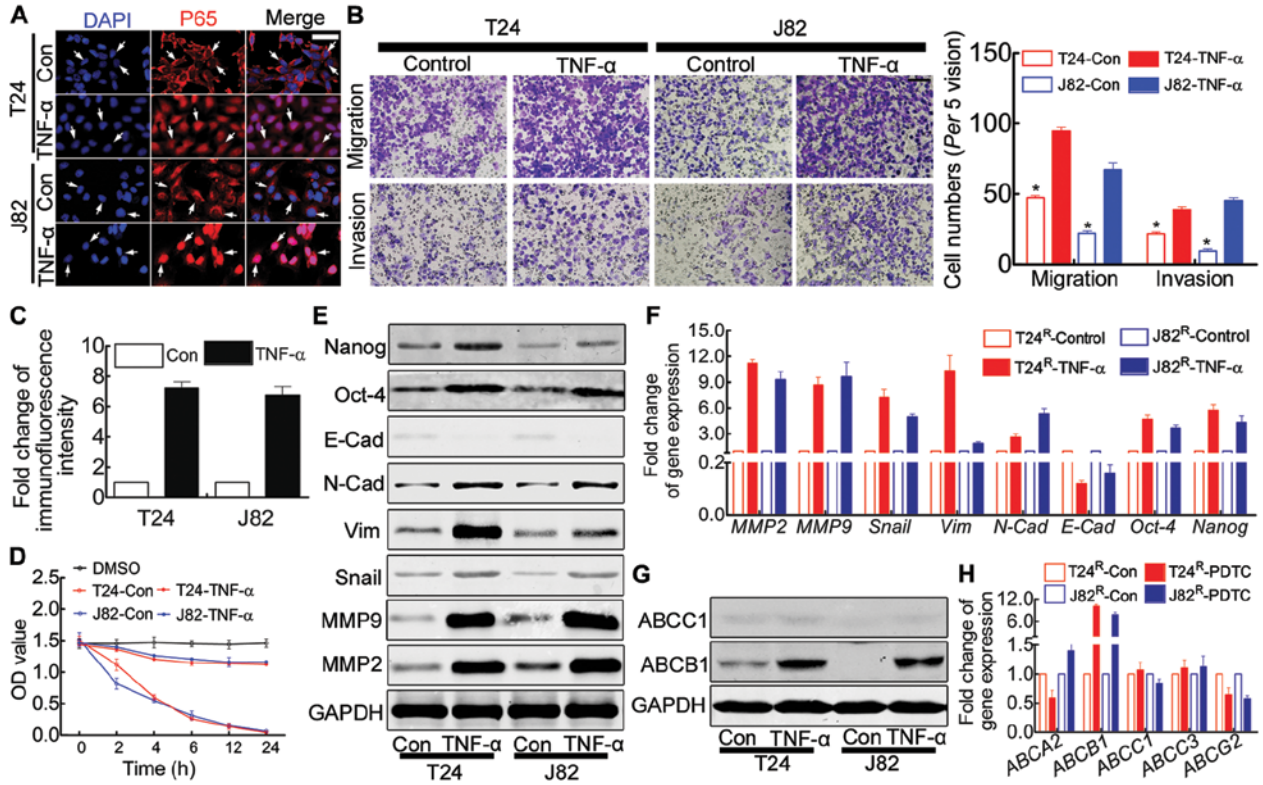

Figure 5. Forced activation of NF-κB signaling in parental T24/J82 cells results in enhanced malignancy, chemoresistance and proliferative ability, accompanied by processing of EMT and elevated expression of stem cell makers. (A) Immunofluorescence staining shows that, in the presence of TNF-α, a classical NF-κB signaling activator, P65 manifests nuclear translocation in parental T24/J82 cells indicated by the white arrows; bar, 100 μm; (B) Boyden Chamber assay suggests that malignancy of T24/J82 is enhanced by TNF-α. Right, representative figures of Boyden Chamber assay; left, quantification of Boyden Chamber assay; bar, 100 μm *P<0.05; (C) MTT assay shows that TNF-α renders enhanced chemoresistance to T24/J82; (D) BrdU incorporation indicates that activation of NF-κB signaling by TNF-α results in enhanced tumor cell proliferative capacity; (E) Western blot analysis reveals that in T24/J82 cells, TNF-α promotes the process of EMT and maintenance of stem cell markers; (F) Real-time PCR suggests that TNF-α contributes to the process of EMT and the maintenance of stem cell makers; (G) Western blot analysis shows that activation of NF-κB signaling by TNF-α induces the elevation of ABCB1, but has no effects on ABCC1; (H) Real-time PCR suggests that in the present of TNF-α, *ABCB1* is extremely elevated in T24/J82 cells.

attenuation of ABCB1, but without effects on ABCC1 (Fig. 4G and H).

Forced activation of NF-κB signal in parental T24/J82 cells potentiates the capacity of migration/invasion, chemoresistance and proliferation of the tumor cell. Inhibition of NF-κB signal in T24^{R}/J82^{R} resulted in reversal of the EMT and other accompanied phenomena, leading us to further speculate that the phenomenon was induced by forced activation of NF-κB signal in the parental T24/J82 cells. TNF-α, a classical activator of the NF-κB signal, was used according to the protocol. Immunofluorescence staining suggested that this activator significantly induced the nuclear translocation of P65, indicating the activation of this pathway (Fig. 5A). The Boyden Chamber assay (Fig. 5B) and BrdU incorporation (Fig. 5C) revealed that activation of NF-κB signal in parental T24/J82 cells contributed to the capacity of migration/invasion and proliferation, accompanied by enhanced chemoresistance demonstrated by the MTT assay (Fig. 5D).

Forced activation of NF-κB signal in parental T24/J82 cells results in the elevated expression of EMT markers, stem cell markers and ABCB1. Our previous results suggested that suppression of NF-κB signal led to the reversal of EMT, decreased expression of stem cell markers and ABCB1. Therefore, expression of EMT markers, stem cell markers, ABCB1 and ABCC1 were monitored in T24/J82 cells stimulated by TNF-α. Our results revealed that the forced activation of NF-κB signal in parental T24/J82 induced the increased expression of MMP2, MMP9, vimentin, Snail and N-cadherin, accompanied by decreased expression of E-cadherin (Fig. 5E and F). In line with T24^{R}/J82^{R} cells the parental T24/J82 with forced activation of NF-κB signal possessed higher expression of the stem cell markers, Oct-4 and Nanog (Fig. 5E and F), compared with the control. In addition, consistent with our previous results, forced activation of NF-κB signal in parental T24/J82 cells led to the elevated expression of ABCB1, but still had no effects on ABCC1 (Fig. 5G and H).

The expression of EMT/stem cell markers and ABCB1 were higher in human chemoresistant-BCa tissue vs chemosensitive BCa tissue. Previous mechanistic investigation indicated that prolonged time of cisplatin treatment of BCa cells caused the enhanced expression of EMT/stem cell markers and ABCB1. Therefore, to verify this conclusion *in vivo*, human BCa tissues from the same tumor grade were used, including chemoresistant and chemonsensitive BCa, to monitor the expression of EMT/stem cell markers and

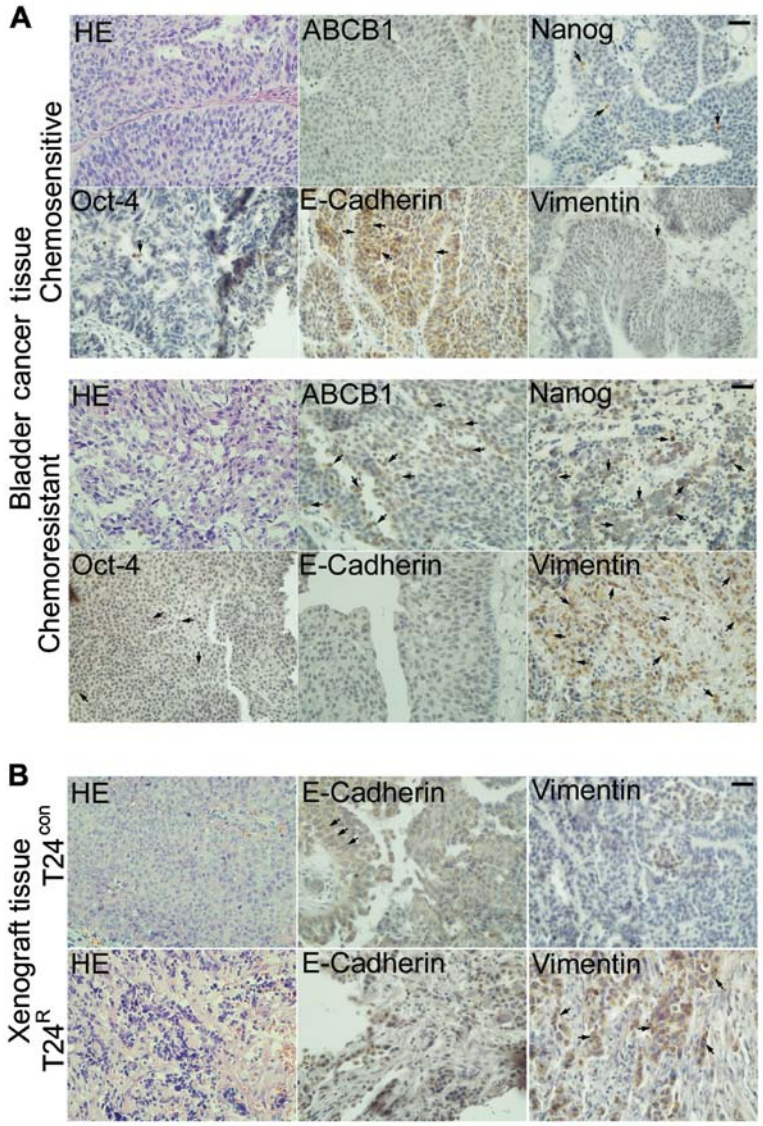

Figure 6. Mechanistic validation *in vivo*. (A) Representative figures of H&E and IHC staining for EMT/stem cell markers and ABCB1 in chemoresistant human BCa tissue vs chemosensitive BCa tissue, indicating that comparing with chemosensitive BCa tissue, chemoresistant BCa tissue manifests as higher expression of ABCB1, vimentin, Nanog and Oct-4, but lower expression of E-cadherin, which is consistent with our conclusion of the mechanistic investigation. Bar, 100 μm; black arrows, the positive cells. (B) Representative figures of H&E and IHC staining for EMT markers in xenograft tumor mass from T24/T24^{R} cells, indicating that comparing with parental T24, the expression of vimentin and E-cadherin is increased and decreased, respectively, in tumor mass from T24^{R} vs parental T24 (T24^{con}); bar, 100 μm; black arrows, the positive cells.

ABCB1. As indicated in Fig. 6A, consistent with our mechanistic investigation, chemoresistant BCa tissue manifested higher expression of ABCB1, Nanog, Oct-4, and vimentin, but lower expression of E-cadherin compared to chemosensitive BCa tissue. In addition, expression of EMT related markers were compared in our xenograft tumor tissue from T24^{R}/T24, indicating elevated expression of vimentin and decreased expression of E-cadherin in tumor tissue from T24^{R} vs T24, as indicated in Fig. 6B.

Discussion

One of the main obstacles of cancer chemotherapeutics is chemo-resistance, especially the acquired chemoresistance, leading to failure of the treatments against cancer (8,9), and BCa is not an exception. As the final regimen for BCa patients, efficiency of chemotherapy plays a decisive role in BCa prognosis. Therefore, mechanistic investigation of how chemo-resistance occurs in BCa has attracted great attention from clinical workers in the past decades; however, problems still exist (24,25). Cisplatin, as the basic reagent in BCa chemotherapeutic regimen, however, chemoresistance still widely exist. Moreover, the mechanism of cisplatin-induced chemoresistance unknown.

In order to further investigate this point, we used cisplatin-treatment to obtain chemoresistant cell lines as described in Materials and methods. We found chemosensitivity was decreased in T24^{R}/J82^{R} (Fig. 1A), as usually occur in BCa therapy. As per our expectation, T24^{R}/J82^{R} manifested enhanced ability of tumorigenesis (Figs. 1B and 6B: H&E staining), proliferation (Fig. 1C) and malignancy (Fig. 1E), followed by EMT (Fig. 2A and B), all the above results are consistent with clinical BCa behavior that chemoresistance renders BCa enhanced malignancy. In another aspect, we

speculated that capacity of chemoresistance was possibly mediated by ABC transporter family members; in addition, according to the reports by Dean and Annilo (13), we used real-time PCR to monitor the expression of drug-resistance related ABC family members, including *ABCA2, ABCB1, ABCC1, ABCC3* and *ABCG2*. We found that not all drug-resistance related ABC family members were elevated in $T24^R/J82^R$ cells, as showed in Fig. 2C. Only ABCB1 and ABCC1 consistently showed results in $T24^R/J82^R$ that the expression was significantly elevated compared with control, and the western blot analysis also confirmed this point (Fig. 2D). The elevated expression of ABCB1 and ABCC1 were unexpected. Firstly, there was scarce information to indicate that both the proteins, ABCB1 (MDR1) and ABCC1 (MRP1), could be induced by cisplatin-treatment. Secondly, many investigations pointed out that there was no expression of ABCC1 in BCa tissue.

Our unpublished data suggested that knocking down the expression of ABCB1 in $T24^R/J82^R$ led to enhanced chemo-sensitivity, whereas, forced expression of ABCB1 in parental T24/J82 cells confer chemoresistance, indicating the important roles of ABCB1 in chemoresistance. A previous study reported that both ABCB1 and ABCC1 were target genes of NF-κB signaling, furthermore, the elevated expression of ABCB1/ABCC1, enhanced the malignant phenotype and induced EMT in our $T24^R/J82^R$ cells making us speculate the activity of NF-κB signaling. The following immunofluorescence staining and western blot analysis proved our speculation that P65 was accumulated in the nucleus in $T24^R/J82^R$, which meant the activation of this signal (Fig. 3).

To further support this postulation, activity of NF-κB signaling was monitored by inhibition or forced activation. In the condition of NF-κB signal inhibition by PDTC, malignancy and proliferative ability of $T24^R/J82^R$ cells were attenuated compared with control (Fig. 4B and D), accompanied by enhanced chemo-sensitivity (Fig. 4C). Moreover, expression of EMT markers was also reversed by PDTC in $T24^R/J82^R$, indicating the irreplaceable roles of NF-κB signaling in the process of EMT. However, PDTC exhibited no significant effects on the expression of ABCC1 but could significantly decrease the expression of ABCB1 in $T24^R/J82^R$ demonstrated by western blot analysis (Fig. 4G) and real-time PCR analyses (Fig. 4H). The followed experiment suggested that forced activation of NF-κB signaling in parental T24/J82 led to the reversal of all the phenomena induced by PDTC, yet still with no effects on ABCC1 (Fig. 5G and H), giving us a clue that the elevated expression of ABCC1 in $T24^R/J82^R$ was possibly not induced by NF-κB signaling.

The enhanced proliferative capacity of $T24^R/J82^R$ drove us to monitor the tumorigenic ability. Subcutaneous xenografts in nude mice suggested the enhanced tumorigenic ability for $T24^R/J82^R$ vs T24/J82 (Figs. 1B and 6: H&E staining), leading us to link the stem cell characteristics of these chemoresistant cells. Our previous study (ref. ?) had indicated that in T24/J82 cells, activated NF-κB signaling contributed to the expression of Oct-4 and Nanog, both of which are well-defined stem cell markers. As expected, elevated expression of the stem cell markers in $T24^R/J82^R$ were definitely mediated by NF-κB signaling in the present study. In the IHC staining of the xenograft tissue, our conclusion was proved to be correct as that tumor mass from $T24^R$ cells manifested increased expression of vimentin and ABCB1, but decreased expression of E-cadherin (Fig. 6B, $J82^R$ data not showed). This point offered an explanation for the accelerated tumorigenesis when the chemoresistance occurred, though this was not the only reason. Finally, human BCa tissue, including chemoresistant and chemosensitive tumors, were used to monitor the expression of EMT/stem cell markers and ABCB1, as indicated in Fig. 6A, which suggested similar results with our mechanistic study that vimentin, Nanog, Oct-4 and ABCB1 were elevated with decreased E-cadherin in chemoresistant BCa tissue vs chemosensitive BCa tissue.

Taken together, in the present study, we provided evidence that in BCa cell line T24/J82, cisplatin-treatment contributed to the activation of NF-κB signaling, resulting in EMT, elevated expression of the chemoresistant gene ABCB1 and maintaining of the stem cell characteristics. We thus provided a new view for the role of NF-κB signaling in BCa chemotherapeutics.

Acknowledgements

This study was partly supported by the National Natural Science Foundation of China (NSFC no. 81172436 to Sun Yi) and Overall Innovation Projects of Scientific and Technological Resources of Shaanxi Province (no. 2013KTCL03-04 to Yonggang Xu).

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