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Epithelial cell adhesion molecule (EpCAM) is associated with prostate cancer metastasis and chemo/radioresistance via the PI3K/Akt/mTOR signaling pathway

Jie Ni[a,b], Paul Cozzi[b,c,**], Jingli Hao[a,b], Julia Beretov[a,b,d], Lei Chang[a,b], Wei Duan[e], Sarah Shigdar[e], Warick Delprado[f], Peter Graham[a,b], Joseph Bucci[a,b], John Kearsley[a,b], Yong Li[a,b,*]

[a] Cancer Care Centre and Prostate Cancer Institute, St George Hospital, Kogarah, NSW 2217, Australia
[b] St George Clinical School, University of New South Wales (UNSW), Kensington, NSW 2052, Australia
[c] Department of Surgery, St George Hospital, Kogarah, NSW 2217, Australia
[d] SEALS, Anatomical Pathology, St George Hospital, Gray St Kogarah, NSW 2217, Australia
[e] School of Medicine, Deakin University, Waurn Ponds, VIC 3217, Australia
[f] Douglass Hanly Moir Pathology, Macquarie Park, NSW 2113, Australia

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ABSTRACT

Prostate cancer (CaP) is the second leading malignancy in men. The role of epithelial cell adhesion molecule (EpCAM), also known as CD326, in CaP progression and therapeutic resistance is still uncertain. Here, we aimed to investigate the roles of EpCAM in CaP metastasis and chemo/radioresistance. Expression of EpCAM in CaP cell lines and human CaP tissues was assessed using immunofluorescence and immunohistochemistry, respectively. EpCAM was knocked down (KD) in PC-3, DU145 and LNCaP-C4-2B cells using small interfering RNA (siRNA), and KD results were confirmed by confocal microscope, Western blotting and quantitative real time polymerase chain reaction (qRT-PCR). Cell growth was evaluated by proliferation and colony formation assays. The invasive potential was assessed using a matrigel chamber assay. Tumorigenesis potential was measured by a sphere formation assay. Chemo-/radiosensitivity were measured using a colony formation assay. Over-expression of EpCAM was found in primary CaP tissues and lymph node metastases including cancer cells and surrounding stromal cells. KD of EpCAM suppressed CaP proliferation and invasive ability, reduced sphere formation, enhanced chemo-/radiosensitivity, and down-regulated E-cadherin, p-Akt, p-mTOR, p-4EBP1 and p-S6K expression in CaP cells. Our findings suggest that EpCAM plays an important role in CaP proliferation, invasion, metastasis and chemo-/radioresistance associated with the activation of the PI3K/Akt/mTOR signaling pathway and is a novel therapeutic target to sensitize CaP cells to chemo-/radiotherapy.

Abbreviations: BPH, benign prostate hyperplasia; BSA, bovine serum albumin; CaP, prostate cancer; CPTC, circulating prostate tumor cells; CRPC, castration-resistant prostate cancer; CSC, cancer stem cell; CTC, circulating tumor cell; DAB, 3,3′ diaminobenzidine; DOX, doxorubicin; DTX, docetaxel; ECL, enhanced chemiluminescence; EMT, epithelial–mesenchymal transition; EpCAM, epithelial cell adhesion molecule; FBS, fetal bovine serum; HGPIN, high-grade prostatic intraepithelial neoplasia; HRP, horseradish peroxide; i.c., intracardiac; KD, knock down; MAb, monoclonal antibody; MMRD, mismatch repair deficient; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; o/n, overnight; PI, propidium iodide; PSA, prostate specific antigen; PTX, paclitaxel; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative real time polymerase chain reaction; RP, radical prostatectomy; RT, room temperature; s.c., subcutaneous; scr, scrambled; SD, standard deviation; siRNA, small interfering RNA; TBS, Tris-buffered saline; TMA, tissue microarray.

* Corresponding author at: Cancer Care Centre, St George Hospital, Gray St Kogarah, Sydney, NSW 2217, Australia. Tel.: +61 2 9113 2514; fax: +61 2 9113 4048.
** Corresponding author at: Department of Surgery, St George Hospital, Australia.
E-mail addresses: pcozzi@unsw.edu.au (P. Cozzi), y.li@unsw.edu.au (Y. Li).

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1. Introduction

Prostate cancer (CaP) remains a major health problem in males of Western countries and accounts for an estimated 33,720 deaths in the USA in 2011 (Beltran et al., 2011). Localized CaP patients have a long-term survival due to great advances in surgical resection and radiotherapy. However, patients with advanced and metastatic disease are often associated with a poor prognosis; up to 30% of treated patients suffer a relapse within 18 months after surgical resection. While initially responsive to androgen deprivation therapy, almost all CaP patients will inevitably progress to recurrent castration-resistant prostate cancer (CRPC) and die from metastasis.

Radiotherapy continues to be one of the most popular treatment options for CaP patients detected at early-stage or advanced-stage disease. Up to 20% of localized CaP is considered high-risk cases, defined as T3-4 or Gleason score 8–10 or baseline prostate specific antigen (PSA) > 20 ng/mL. For these high-risk cases, surgery or radiotherapy alone yields to high rates of local and distant relapse (Thoms et al., 2011), which results in cancer progression to metastatic disease. One main reason for these failures following radiotherapy is due to the radioresistance of a subpopulation of CaP clones within the tumor. Radioresistance is a major challenge in current CaP radiation therapy. Thus, it is urgent to identify novel therapeutic targets to improve CaP radiosensitivity and overcome radioresistance.

Chemotherapy is customarily used for relieving the resultant symptoms of advanced CaP. Two Docetaxel (DTX)-based clinical trials have unraveled the potential benefits of chemotherapy to prolong the survival time and improve the quality of life in CaP patients for the first time (Berthold et al., 2008; Petrylak et al., 2004). Although DTX-based regimens extended survival time, the overall benefit was modest, with most patients subjected to progression within 7 months and, ultimately, dying (Higano and Crawford, 2011). Its effectiveness is enormously challenged by the drug-resistant nature of CaP. Chemoresistance is another challenge for current CaP chemotherapy. Hence, it is important to investigate the mechanisms and signaling pathways of CaP metastasis and progression and to identify novel and effective therapeutic targets to improve current chemotherapeutic modalities.

Epithelial cell adhesion molecule (EpCAM, also known as CD326), initially discovered as a predominant antigen in human colon carcinoma, is a transmembrane glycoprotein that is highly expressed in rapidly proliferating carcinomas (Ni et al., 2012). Recent data suggest a more multipotent role of EpCAM that is not only in cell-cell adhesion, but also in cell signaling, migration, proliferation and differentiation (Patriarca et al., 2012). While there is an agreement that CaP specimens express a high-level of EpCAM, there are conflicting reports regarding the relationship between EpCAM expression and clinical parameters in CaP patients. One study found that EpCAM expression did not correlate with tumor stage, nodal stage, Gleason score or overall survival in 553 CaPs in different tumor stages (Went et al., 2004). However, another study reported that the expression of EpCAM in 102 localized CaPs treated with radical prostatectomy (RP) correlated with unfavorable clinical features such as higher preoperative and postoperative Gleason score, shorter biochemical recurrence-free survival and advanced tumor stage (Benko et al., 2011). The role of EpCAM in CaP metastasis is still unclear. To date, little attention has been paid to the regulation of EpCAM in CaP chemo/radioresistance. Because of the importance of EpCAM in cancer metastasis and progression, investigation of the roles of EpCAM in CaP metastasis and chemo-/radioresistance may provide novel insights to facilitate the development of novel treatment strategies for CaP.

In the current study, we hypothesized that overexpression of EpCAM is associated with the metastatic and progressive capacity of CaP; CaP chemo-/radioresistance is closely linked with EpCAM expression; the regulation of EpCAM in CaP metastasis and chemo-/radioresistance is correlated with the PI3K/Akt/mTOR signaling pathway. So far, little data exist on EpCAM signaling pathway in CaP metastasis. The impact of EpCAM expression on CaP cells was investigated in loss-of-function studies by silencing EpCAM expression in $EpCAM^+$ CaP cell lines, which resulted in a decrease in cell proliferation, migration, invasiveness and sphere formation, with a concurrent increase of the sensitivity to chemo-/radiotherapy and reduced PI3K/Akt/mTOR signaling proteins.

Here, we demonstrate for the first time that EpCAM has tumor initiation properties and is involved in proliferation, invasion, metastasis and chemo-/radiosensitivity via the activation of PI3K/Akt/mTOR signaling pathway in CaP cells, and is a useful therapeutic target to prevent CaP metastasis and increase the sensitivity to chemo-/radiotherapy.

2. Materials and methods

2.1. CaP and control tissues

Ethical approval was obtained from the South East Health Human Research Ethics Committee, South Section, Sydney, Australia. Primary CaP tissues ($n = 10$) from patients with localized CaP undergoing RP, lymph node metastases ($n = 10$) and benign prostate hyperplasia (BPH) tissues ($n = 10$) and normal prostate biopsies ($n = 10$) were obtained at Urology Sydney, St George Private Hospital, Sydney, Australia from 2000 to 2005.

2.2. Antibodies and reagents

Antibodies were obtained from different sources. The detailed information and conditions are listed in Table 1. Docetaxel (DTX), Paclitaxel (PTX) and Doxorubicin (DOX) were purchased from Sigma–Aldrich, Pty Ltd. (Castle Hills, NSW, Australia). The DTX and PTX were first diluted in 100% ethanol while DOX was diluted in DMSO, and the three drugs were added to the growth medium. The final concentrations of 100% ethanol or DMSO were 0.01%. siRNA targeting EpCAM and a scrambled (scr) sequence control for off-target effects were obtained from Applied Biosystems Pty Ltd Australia (Melbourne, VIC, Australia). EpCAM expression plasmid and empty vector were purchased from OriGene Technologies Inc (Rockville, MD, USA). Insulin, B27, EGF, bFGF and G418 were purchased from Invitrogen Australia Pty Ltd (Melbourne, VIC, Australia).

2.3. Cell culture

Androgen-non-responsive (PC-3, PC-3M, DU145, LNCaP-C4-2B) and androgen-responsive (LNCaP, LNCaP-LN3, DuCaP) CaP cell lines from different sources were studied (Table S1). All cell culture reagents were supplied by Invitrogen Australia Pty Ltd (Melbourne, VIC, Australia) unless otherwise stated. PC-3, PC-3M, DU145, C4-2B and LNCaP CaP cell lines were cultured in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 50 U/mL of penicillin and 50 μg/mL of streptomycin; LN3 cells in 1:1 RPMI-1640:F12-K; and DuCaP cells in DMEM. All cell lines were maintained in a humidified incubator at 37 °C and 5% CO_2.

PC-3, DU145 and LNCaP CaP cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). The PC-3M cell line was kindly provided by Dr. C. Power (Prince of Wales Hospital, UNSW, Australia); LN3 and C4-2B cell lines were kindly provided by Dr. C. Pettaway (M.D. Anderson Hospital, Austin, TX, USA). DuCaP cell line was provided by Dr. K. Pienta (University of Michigan Comprehensive Cancer Center, Ann Arbor, MI, USA).

Table 1
Antibodies used for immunofluorescence (IF) staining and western blot (WB).

Antibody	Source	Type	Dilution	Incubation time (min)	Temperature (°C)	Application
Rabbit anti-human EpCAM	Epitomics, Inc.	PAb	1:1000 (WB) 1:100 (IHC,IF)	O/N	4	WB, IHC, IF
Rabbit anti-human E-cadherin	Epitomics, Inc.	MAb	1:2000 (WB)	O/N	4	WB
Rabbit anti-human Vimentin	Epitomics, Inc.	MAb	1:2000 (WB)	O/N	4	WB
Rabbit anti-human Akt	Abcam	PAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human p-Akt	Abcam	PAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human mTOR	Cell signaling	PAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human p-mTOR	Cell signaling	PAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human S6k	Abcam	MAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human p-S6k	Abcam	PAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human 4EBP1	Cell signaling	MAb	1:1000 (WB)	O/N	4	WB
Rabbit anti-human p-4EBP1	Cell signaling	MAb	1:1000 (WB)	O/N	4	WB
Rabbit polyclonal IgG Isotype Control	Abcam	PAb	1:100 (IHC,IF)	O/N	4	IHC, IF
Rabbit anti-human GAPDH	Millipore	PAb	1:600 (WB)	O/N	4	WB
Mouse anti-human β-tubulin	Sigma–Aldrich	MAb	1:5000 (WB)	O/N	4	WB
Goat anti-rabbit Alexa Fluor® 488 Dye Conjugate	Invitrogen	IgG	1:1000 (IF)	60	RT	IF
Goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology	PAb	1:2500 (WB)	60	RT	WB
Goat anti-mouse IgG-HRP	Santa Cruz Biotechnology	PAb	1:2500 (WB)	60	RT	WB
Swine anti-goat, mouse, rabbit IgG/Biotinylated	Dako Cytomation	PAb	1:450 (IHC)	45	RT	IHC

Notes: HRP: horseradish peroxide; IF: immunofluorescence; MAb: monoclonal antibody; O/N: overnight; PAb: polyclonal antibody; RT: room temperature; WB: Western blot; IHC: Immunohistochemistry.

The identity of all cell lines was confirmed by short tandem repeat profiling and was tested within a few passages of initial authentication. Each cell line was regularly tested to confirm the absence of mycoplasma contamination using the LookOut® Mycoplasma PCR Detection Kit (Sigma–Aldrich, Castle Hill, NSW, Australia).

2.4. Immunofluorescence confocal microscope analysis

Immunofluorescence staining was performed as previously described (Chen et al., 2009). Briefly, cells grown on glass coverslips (10^5 cells) were fixed by methanol, rinsed by Tris-buffered saline (TBS) (pH 7.5) and incubated with a rabbit anti-EpCAM primary antibody (1:100 dilution) at 4 °C overnight (o/n). After rinsing in TBS, cells were incubated in Alexa Fluro-488 goat anti-rabbit IgG (1:1000 dilution) for 45 min at room temperature (RT). Propidium iodide (PI) was used for nuclei staining. Negative controls were treated identically but incubated with a rabbit isotype control. Immunofluorscence was then visualized using an FV300/FV500 Olympus laser scanning confocal microscope (Olympus, Tokyo, Japan).

2.5. Immunohistochemistry

Standard immunoperoxidase procedure was used to visualize EpCAM expression as previously published (Wang et al., 2011b). Briefly, paraffin-embedded slides were deparaffinized in xylene and rehydrated with various concentrations of ethanol. For the antigen retrieval, slides were immersed in 0.01 M citrate buffer (Thermo Fisher Pty Ltd, VIC, Australia), pH6, and heated in water bath for 15 min. Slides were then incubated with a rabbit anti-EpCAM polyclonal antibody (1:100 dilution) for 1 h at RT. After washing with TBS, the slides were incubated with a biotinylated secondary antibody (1:300 dilution) for 45 min at RT, rinsed in TBS and then incubated in streptavidin/horseradish peroxide (HRP) (1:300 dilution) for 30 min at RT. After rinsing in TBS, immunoreactivity was developed with 3,3′ diaminobenzidine (DAB) substrate (Thermo Fisher Pty Ltd, VIC, Australia) containing 0.03% hydrogen peroxide (VWR International, QLD, Australia) and counterstained with Harris Hematoxylin (Thermo Fisher Scientific, VIC, Australia) for 1 min. Negative controls were treated identically but with the primary antibody omitted.

2.6. Western blot

Protein expression levels were evaluated by Western blot as described (Hao et al., 2010). Briefly, whole cell lysates were run on NuPAGE Novex 4–12% Bis–Tris gel and then transferred to polyvinylidene difluoride (PVDF) membrane. After blocking non-specific sites with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween20 (TBST), the membrane was incubated with the different primary antibodies (Table 1) at 4 °C o/n, followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were detected using enhanced chemiluminescence (ECL) substrate (Pierce Chemical Co, Rockford, USA), and visualized using the ImageQuant LAS4000 system (GE Healthcare, USA). To confirm the equality of protein lysates loading, membranes were stripped using Restore Western Blot Stripping Buffer (Pierce Chemical Co., Rockford, USA), and re-probed using a mouse anti-human β-tubulin monoclonal antibody (MAb), then processed as above.

2.7. Short interfering RNA (siRNA) transfection

CaP cells were knocked down (KD) by 50 μM EpCAM-siRNAs using LipofectAMINE 2000 (Invitrogen, VIC, Australia) following the manufacturer's protocol. Incubation time with siRNA or scr complex was 72 h with cell lines as a published method (Xiao et al., 2012), after which the following experiments and assays were performed.

2.8. cDNA transfection

EpCAM expression plasmid and empty vector plasmid (used as a negative control) were transfected into $EpCAM^{low}$ expression LN3 CaP cell line using TurboFectin (OriGene Technologies Inc., MD, USA) according to the manufacturer's instructions. After 48 h incubation with transfection plasmid complex, stable transfection were selected and maintained by adding 0.6 mg/mL of G418 (Invitrogen,

VIC, Australia). Western blot was used to confirm the successful transfection of EpCAM.

2.9. RNA extraction

Approximately 4×10^5 of each cells from EpCAM-KD, EpCAM-scr and untreated control CaP cell lines were trypsinized, rinsed and centrifuged. The total RNA was extracted and purified using the RNeasy Plus Mini Kit (Qiagen, VIC, Australia) according to the manufacturer's instructions. The concentrations of total RNA from each cell line were measured by a ND-2000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.10. Quantitative real-time PCR

Two microgram (μg) of total RNA from each cell lines was reverse transcribed to cDNA using the SuperScript III First-strand Synthesis System Kit (Invitrogen Pty Ltd, VIC, Australia), according to the manufacturers' protocol. Quantitative real-time PCR (qRT-PCR) was used for the assessment of mRNA expression of the EpCAM gene. qRT-PCR was carried out in a solution containing 12.5 μL SYBR® Select Master Mix (Applied Biosystems, VIC, Australia), 2.75 μL TaqMan® Gene Expression primers (EpCAM: Hs00901885_mL), 7.25 μL of nuclease-free water and 2.5 μL of cDNA sample in a final volume of 20 μL. GAPDH (Hs02758991_g1) was used as an endogenous control. A Rotor-Gene instrument (Corbett Life Science, Sydney, Australia) was used for automated qRT-PCR setup of the reactions. After three independent experiments, the REST 2009 V2.0.13 (Qiagen, VIC, Australia) software was used for calculation and analysis of EpCAM gene expression.

2.11. Proliferation assay

Proliferating capacity of EpCAM-KD, EpCAM-scr and control CaP cells was determined using a proliferation assay. Briefly, 2×10^4 cells in 3 mL complete medium were seeded in 6-well plates and incubated at 37 °C and humidified 5% CO_2, and then the cell numbers of each well were counted by a haemocytometer in the following consecutive 7 days. The average numbers of cells were plotted (mean ± SD, $n = 3$).

2.12. Colony formation assays

EpCAM-KD, EpCAM-scr and control CaP cells were used for colony forming assay as previously described with minor modifications (Wang et al., 2011a). Briefly, 1500 cells/dish were seeded in 10 cm dishes at 37 °C and humidified 5% CO_2. The media were replaced regularly and all cultures were incubated for 10 days until the colonies were large enough to be clearly discerned. The colonies, defined as groups of >50 cells, were scored manually with the aid of an Olympus INT2 inverted microscope (Olympus, Tokyo, Japan). The average numbers of colonies were plotted (mean ± SD, $n = 3$).

2.13. Matrigel invasion assay

Invasive ability of EpCAM-KD, EpCAM-scr and control CaP cells was assessed using commercial matrigel and control transwell chambers (BD Bioscience, NSW, Australia). Briefly, 2×10^4 cells in 500 μL serum-free RPMI-1640 medium were added to each transwell insert, respectively. 750 μL of complete medium was added to the outer wells to provide chemoattractant and prevent dehydration. Cells were incubated at 37 °C and humidified 5% CO_2 for 48 hours and then stained with a Diff-Quik staining kit (Allegiance Healthcare Corp, Illinois, USA). The number of stained cells that invaded through matrigel or control inserts was counted in five high power fields by light microscope (Leica microscope, Nussloch, Germany). The ability of invasion was calculated as follows: invasion ratio = [(mean cells invading through matrigel insert)/(mean cells migrating through control insert)] × 100%. Cell invasion ratios were plotted (mean ± SD, $n = 3$).

2.14. Sphere formation assay

Cells were trypsinized, dissociated into single cells and then plated into an ultra low attachment round-bottom 96 well plate (Sigma–Aldrich Pty Ltd, Australia). Final cell dilution ranging from 1 to 100 cells in 100 μL serum-free DMEM/F12K media supplemented with 4 μg/mL insulin, B27 and 20 ng/mL EGF and bFGF was added into each well. Spheres that arose in 1 week were counted. The diameters of each sphere were observed and measured by an inverted phase microscope (CK-2, Olympus, Tokyo, Japan) fitted with an ocular eyepiece after 5 days. Sphere formation capacity was assessed as the number of spheres with the diameter of >50 μm.

2.15. MTT assay for chemodrug response

MTT assay was performed as described previously (Hao et al., 2010). Briefly, 2000 cells were seeded in 96-well plates and treated with a range of concentrations of DTX, PTX and DOX, which are commonly used in CaP chemotherapy. After 72 h incubation, the media were replaced with fresh medium containing 0.5 mg/mL MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The absorbance (OD) was read at a wavelength of 562 on a BIO-TEK microplate reader (Bio-Rad, Hercules, CA, USA). The growth inhibition curve was generated using the GraphPad Prism 4 (GraphPad, San Diego, CA, USA). Absolute IC_{50} values for the three drugs were calculated using the intersection of the 50% normalized drug response and the growth inhibition curves for each cell line, to find the x axis of IC_{50} for DTX, PTX and DOX, respectively.

2.16. Chemosensitivity assay

Briefly, 1500 cells/dish were seeded in 10 cm dishes for 48 h at 37 °C and 5% CO_2 and then treated with a fixed dose (1/2 dose of the IC_{50}) of DTX, PTX and DOX respectively, from the MTT assay in EpCAM-KD, EpCAM-scr and wild type control CaP cells, or the same volume of vehicle control (100% ethanol for DTX and PTX groups, and DMSO for DOX groups). After 3 days treatment, the drug-containing media was replaced with fresh media and all cultures were incubated for an additional 7 days until colonies were large enough to be clearly distinguished. The scoring and statistical process was carried out as previously described (Hao et al., 2012). The treatment details are shown in Fig. S1.

2.17. Radiosensitivity assay

Briefly, 1000 cells/flask were seeded in 25 cm^2 flasks. EpCAM-KD, EpCAM-scr and wild type control CaP cells were exposed to a single dose (2 Gy) irradiation using a linear accelerator (Elekta, Stockholm, Sweden) at a dose rate of 2.7 Gy/min with 6 MV photons (Cancer Care Center, St George Hospital, Sydney, Australia). The colony formation assay was then carried out in radiation-treated and untreated cells in a published method (Xiao et al., 2012). The media were replaced regularly and all cultures were incubated for 10 days until the colonies were large enough to be clearly distinguished. The colonies, defined as groups of >50 cells, were scored manually with the aid of an Olympus INT2 inverted microscope (Olympus, Tokyo, Japan). The average numbers of colonies were plotted (mean ± SD, $n = 3$).

2.18. Assessment of immunostaining

Staining intensity (0–3) in CaP cell lines and in CaP tissues was assessed using a confocal microscope (Olympus, Tokyo, Japan) and a light microscopy (Leica, Germany), respectively. The criteria used for assessment of CaP tissues were as previously reported (Cozzi et al., 2005), where: 0 (negative, <25%); 1+ (weak, 25–50%); 2+ (moderate, 50–70%); 3+ (strong, >75%) of the tumor cells stained. Evaluation of cell and tissue staining was performed independently by two experienced observers (JN and YL). All specimens were scored blind and an average of grades was taken finally.

2.19. Statistical analysis

All numerical data were expressed as the average of the values (mean), and the standard deviation (SD) was calculated. Data from different groups were compared using the two-tailed *t*-test. All *P* values were 2-sided. $P < 0.05$ was considered significant. All numerical statistical analyses were performed using the GraphPad Prism 4.00 package (GraphPad, San Diego CA, USA).

3. Results

3.1. Expression of EpCAM in metastatic CaP cell lines

Immunofluorescence labeling of CaP cells with anti-EpCAM antibody showed positive and heterogeneous expression in PC-3, PC-3M, DU145, C4-2B, LNCaP, LN3 and DuCaP cells, with variation between cell lines (Fig. 1A). Strong (Grade 3) expression of EpCAM was seen in PC-3, DU145, C4-2B and DuCaP cell lines. Medium (Grade 2) expression of EpCAM was seen in PC-3M and LNCaP cell lines. Low expression of EpCAM was found in LN3 cell lines. Expression of both membrane and cytoplasm was found in all positive CaP cell lines. The immunostaining grades are summarized in Table S1. The immunofluorescence results for the expression of EpCAM in CaP cell lines were further confirmed by western blotting (Fig. 1B).

3.2. Expression of EpCAM in primary CaP tissues, lymph node metastases, BPH and normal prostates

Immunoreactivity identified in primary CaP tissues, lymph node metastases, BPH and normal prostates using paraffin sections stained with EpCAM antibody is summarized in Table 2. Typical staining results are shown in Fig. 1C. In primary CaP tissues, strong EpCAM expression was found in 9 out of 10 (90%) and moderate EpCAM expression in 1 out of 10 (10%). In lymph node metastases, strong EpCAM expression was found in 10 out of 10 (100%). The staining of EpCAM in primary CaP tissues and lymph node metastases is both membrane and cytoplasm with more homogeneous patterns. The EpCAM expression is also strongly positive in stromal cells in both primary CaP tissues and lymph node metastases (Fig. 1C). In the BPH samples, 1 out of 10 (10%) BPH tissues was found negative, 8 out of 10 (80%) were weakly positive and 1 out of 10 (10%) was found moderately positive to EpCAM. The positive staining in BPH was found mostly in epithelial cells but not in stromal cells and was heterogeneous. The positive staining was prominently associated with the cell membrane and the cytoplasm. Normal prostate tissues from the control group were very weakly positive (2 out of 10) for EpCAM expression. The staining pattern is very similar to that in BPH samples.

3.3. Expression of EpCAM in CaP-KD and CaP-control cells

To further investigate the roles of EpCAM in CaP metastasis and progression, we selected three CaP cell lines (PC-3, DU145 and C4-2B) with high level expression of EpCAM for knocking down study. After knocking down EpCAM, the reduction of EpCAM expression was significant in all three CaP cell lines compared to the off target scr control and wild type control (Fig. 1D). No detectable staining was seen in the cells incubated with isotype control (data not shown), indicating the siRNA knockdown was successful. The immunofluorescence staining intensity in different CaP cell lines is summarized in Table S2. The immunofluorescence staining results in different CaP cell lines after KD were further confirmed by Western blot (Fig. 1E) and qRT-PCR (Fig. 1F).

3.4. Knock down of EpCAM reduces proliferative and clonogenic ability in CaP cells

To investigate the proliferative ability after knocking down EpCAM, the cell numbers were observed for 7 days. Compared to scr and wild type control groups, the numbers of PC-3-EpCAM-KD, DU145-EpCAM-KD and C4-2B-EpCAM-KD cells were significantly decreased ($P < 0.05$) (Fig. 2A) whereas, no significant difference was found between scr group and wild type group ($P > 0.05$). The numbers of cells on different time points were plotted from day 1 to day 7 (Fig. 2A)

To investigate whether KD of EpCAM affects the clonogenicity of cell lines, we assessed EpCAM-KD, EpCAM-scr and wild type-control CaP cells in culture. The number of colonies in EpCAM-KD cells was significantly decreased compared with that in EpCAM-scr and wild type control cells in all three CaP cell lines ($P < 0.005$) while there was no significant difference between the colonies generated from EpCAM-scr control and wild type group cells ($P > 0.05$) (Fig. 2B). Representative images for colony formation in different groups are shown in Fig. 2C.

3.5. Knock down of EpCAM reduces CaP cell invasion and tumor sphere forming ability

After knocking down EpCAM, cell invasion was significantly reduced for EpCAM-KD cells, compared with EpCAM-scr and wild type control cells in all three cell lines ($P < 0.05$), while there was no significant difference between scr and wild type control cells (Fig. 3A). The average percentage of invasion for each subline is summarized in Table S2. Representative images for each subline are shown in Fig. 3B.

To assess the relative presence of sphere-forming cells in a cell population, different densities of CaP cells from the sublines were calculated using a linear regression of wells without spheres by a published method (Singh et al., 2003). The mean *x*-intercept value of the graph indicates the number of cells needed to form one sphere per well, which was much higher in the EpCAM-KD cells, when compared to the EpCAM-scr and control groups ($P < 0.01$), while there is no significant difference between EpCAM-scr and control groups ($P > 0.05$), suggesting that knocking down of EpCAM reduces the relative number of cells capable of forming spheres in the population of three CaP cell lines (Fig. 3C).

3.6. Cytotoxicity of chemodrugs in CaP cells in vitro

MTT assay was used to determine the effect of DTX, PTX and DOX treatment on single cell cytotoxicity. Our results indicated a dose-independent cell proliferation inhibition in PC-3-scr, DU145-scr, C4-2B-scr CaP cell lines. Each cell line displayed a variable response to different drugs and no cytotoxic effect was found for vehicle control in all cell lines tested (data not shown). The PC-3-scr cell line is the most sensitive to DTX (IC_{50}: 5.2 nM); the DU145-scr cell line is the most sensitive to DOX (IC_{50}: 14.5 nM); the C4-2B-scr cell line is the most sensitive to PTX (IC_{50}: 2.8 nM). The IC_{50} values of each drug on each cell lines are summarized in

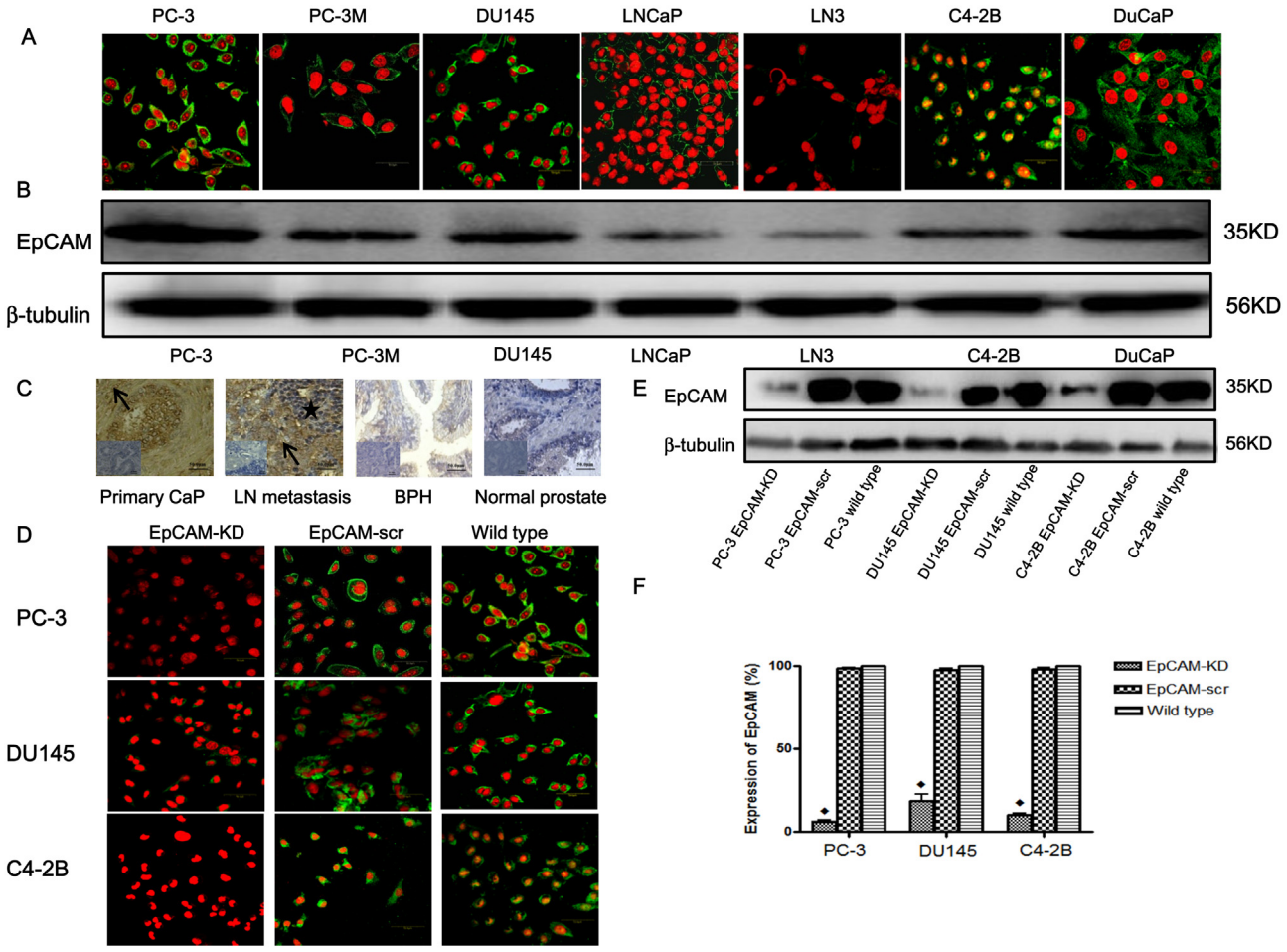

Fig. 1. Expression of EpCAM in metastatic CaP cell lines, primary CaP tissues and lymph node metastases and down-regulation of EpCAM by EpCAM-siRNA in metastatic CaP cells. (A) Representative immunofluorescence images of EpCAM (green) in CaP cell lines. Nuclei are stained with PI (red). Magnification: all images 400×. (B) Western blot is shown to confirm the immunofluorescence staining in all CaP cell lines. β-tubulin was chosen as a loading control. (C) Representative images of EpCAM (brown) in primary CaP tissues and matched lymph node metastases (the lymph node in the image from the same patient), BPH tissues and normal prostate tissues by immunohistochemistry. Nuclei are stained with Harris hematoxylin (blue). *Arrows* indicated positive staining for surrounding stromal cells. *Asterisk* denotes lymphocytes. Magnification: all images (including insets) 400×. (D) Representative immunofluorescence images of EpCAM (green) in the selected PC-3, DU145 and C4-2B cell lines for KD study. Nuclei are stained with PI (red). Magnification: all images 400×. EpCAM expression in KD groups is obviously reduced compared to scr and wild type CaP control groups. (E) and (F) Knock-down effects are further confirmed by Western blot and qRT-PCR ($P < 0.01$). All results were from three independent experiments (mean ± SD, $n = 3$). BPH: benign prostatic hyperplasia; CaP: prostate cancer; KD: knock-down; LN: lymph node; scr: scrambled siRNA control. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Immunostaining results for normal prostates, BPH tissues, different human prostate tissues and LN metastases for EpCAM expression.

Tissue type	*N*	Tissue staining intensity			
		+++	++	+	–
Normal	10	0	0	2	8
BPH	10	0	1	8	1
CaP (G > 7)	10	9	1	0	0
LN metastasis	10	10	0	0	0

Notes: BPH: benign prostatic hyperplasia; CaP: primary prostate cancer; G: Gleason score; LN: lymph node; *N*: number; –: negative; + weak; ++: moderate; +++: strong.

Table S3. The ½ dose of IC_{50} values from MTT assay were chosen for the following chemosensitivity study.

3.7. Knock down of EpCAM increases CaP cells chemosensitivity and radiosensitivity

After treatment with ½ dose of IC_{50} values of each drugs, three EpCAM-KD CaP cells demonstrated obviously reduced colony formative percentage compared to EpCAM-scr groups, normalized by untreated groups ($P < 0.05$) (Fig. 4**A**). The most sensitive chemodrug in PC-3-KD, DU145-KD and C4-2B-KD cells is DTX, and the colony formation is 7.2%, 3.2%, 9.4%, respectively. The average percentage of colony formation in different CaP sublines after chemodrug treatment is summarized in Table S4. These results suggest that knocking down EpCAM increases the chemosensitivity to DTX, PTX and DOX in PC-3, DU145 and C4-2B CaP cells, and that the DTX is the most sensitive chemodrug among them. Typical images of colony formation to different chemodrugs in three CaP cell lines are shown in Fig. 4B.

After treatment by a single dose of 2 Gy irradiation, three EpCAM-KD CaP cells showed significant reduction in colony formation, compared to EpCAM-scr and wild type controls as well as untreated control ($P < 0.05$) (Fig. 5A). Although 2 Gy radiation treatment caused a reduction in colony formation in EpCAM-scr and wild type controls, no significant difference was found between these two controls (EpCAM-scr and wild type treated with radiation) with the untreated CaP control ($P > 0.05$) (Fig. 5A). The most radiosensitive CaP-KD cell line is PC-3 cells and the colony formation is 5.6% after radiation treatment. The average percentage of

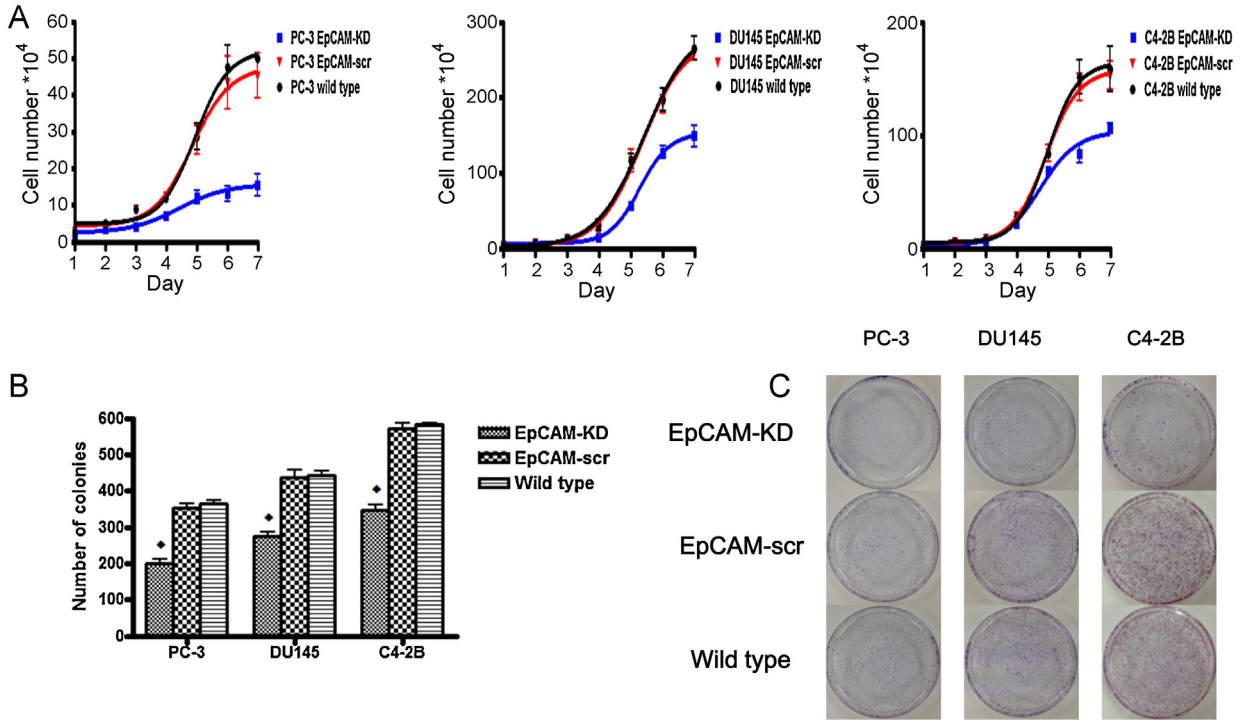

Fig. 2. Proliferation, colony formation after knocking down EpCAM in CaP cells. (A) Proliferation rate in PC-3, DU145 and C4-2B cells was significantly reduced in KD cells compared to scr and wild type control cells ($P < 0.05$) while there is no significant difference between scr cells and wild type cells in three cell lines, respectively. (B) Nine CaP cell lines (KD and controls) were seeded in 100 mm dishes and cultured in growth medium for 10 days. Results are presented as the number of colonies formed. (C) Typical images for colony formation are shown. Significant decreases in colony formation were observed in KD group of PC-3, DU145 and C4-2B cells respectively, compared to the scr and wild type control groups ((♦) indicates that $P < 0.05$). All results were obtained from three independent experiments (mean ± SD, $n = 3$). KD: knock-down; scr: scrambled siRNA control.

colony formation in different CaP sublines after radiation treatment is summarized in Table S5. These findings indicate that the knocking down of EpCAM increases the radiosensitivity of PC-3, DU145 and C4-2B CaP cells. Typical images of colony formation to RT in three CaP cell lines are shown in Fig. 5B.

3.8. Epithelial–mesenchymal transition (EMT) and PI3K/Akt/mTOR signaling pathway are related to the expression of EpCAM in CaP cells

After knocking down EpCAM, the level of E-cadherin (EMT marker) was increased, while the expression of Vimentin (EMT marker) p-mTOR, p-Akt, p-4EBP1, t-S6K, p-S6K were downregulated compared to EpCAM-scr cells, whereas no obvious changes were seen in t-mTOR, t-Akt, and t-4EBP1 expression in all CaP cell lines (Fig. 6A). On the other hand, after successful transfection of EpCAM for overexpression in LN3 cells (Fig. 6B), the expression of p-mTOR, p-Akt, p-4EBP1, t-S6K, p-S6K were upregulated compared to control vector group and wild type group, respectively, whereas no obvious changes were observed in t-mTOR, t-Akt, and t-4EBP1expression (Fig. 6C). These data indicate that KD of EpCAM results in reduced EMT and inactivation of PI3K/Akt/mTOR signaling pathway in CaP cells while overexpression of EpCAM leads to activation of PI3K/Akt/mTOR signaling pathway.

4. Discussion

In this study, we examined the expression of EpCAM in metastatic CaP cell lines, primary CaP, lymph node metastase, BPH and normal prostate tissues using a tissue archive to further investigate the roles of EpCAM in CaP metastasis and progression, chemo-/radiosensitivity, and the association with PI3K/Akt/mTOR signaling pathway. High levels of EpCAM were observed in most metastatic CaP cell lines and in specimens of primary CaP and lymph node metastases, but not in BPH and normal prostate tissues. EpCAM is highly involved in CaP proliferation, invasion, metastasis and chemo-/radioresistance. To the best of our knowledge, this is the first study to investigate expression of EpCAM in a large number of CaP metastatic cell lines, lymph node metastases and the roles of EpCAM in CaP metastasis and progression, chemoresistance and radioresistance, as well as associated signaling pathway.

One interesting finding in this study is that over-expression of EpCAM was seen in both androgen-nonresponsive (PC-3, DU145, C4-2B) and androgen-responsive (LNCaP and DuCaP) metastatic CaP cells from different sites tested (Table S1). This observation indicates that EpCAM may be involved in CaP progression in both early and advanced events. Went et al. also reported that strong EpCAM expression was found in luminal cells of high-grade prostatic intraepithelial neoplasias (HGPIN) as well as in hormone-refractory CaPs rather than in untreated primary CaPs (Went et al., 2004), suggesting that increasing levels of EpCAM expression represent an early and late events in the development of CaP. Another interesting finding is that the over-expression of EpCAM was seen not only in CaP cells, but also in the surrounding stroma in both primary CaP and lymph node metastases, in high-grade tumors (Gleason score >7). Our observation in the pattern of expression in CaP tissues was similar to that seen in a study conducted by Mukherjee et al., where the stromal cells were reported to predominantly express EpCAM (Mukherjee et al., 2009). However, they did not observe lymph node metastases.

The role of EpCAM in the stromal cells of CaP remains unclear. In a separate study, we also found that EpCAM was strongly positive in DU145 and LNCaP spheroids as well as the tumor xenografts from subcutaneous (s.c.) models and tibial metastases from intracardiac (i.c.) injection bone metastasis models with a PC-3M-luc metastatic CaP cell line (Ni et al., 2012). The over-expression of

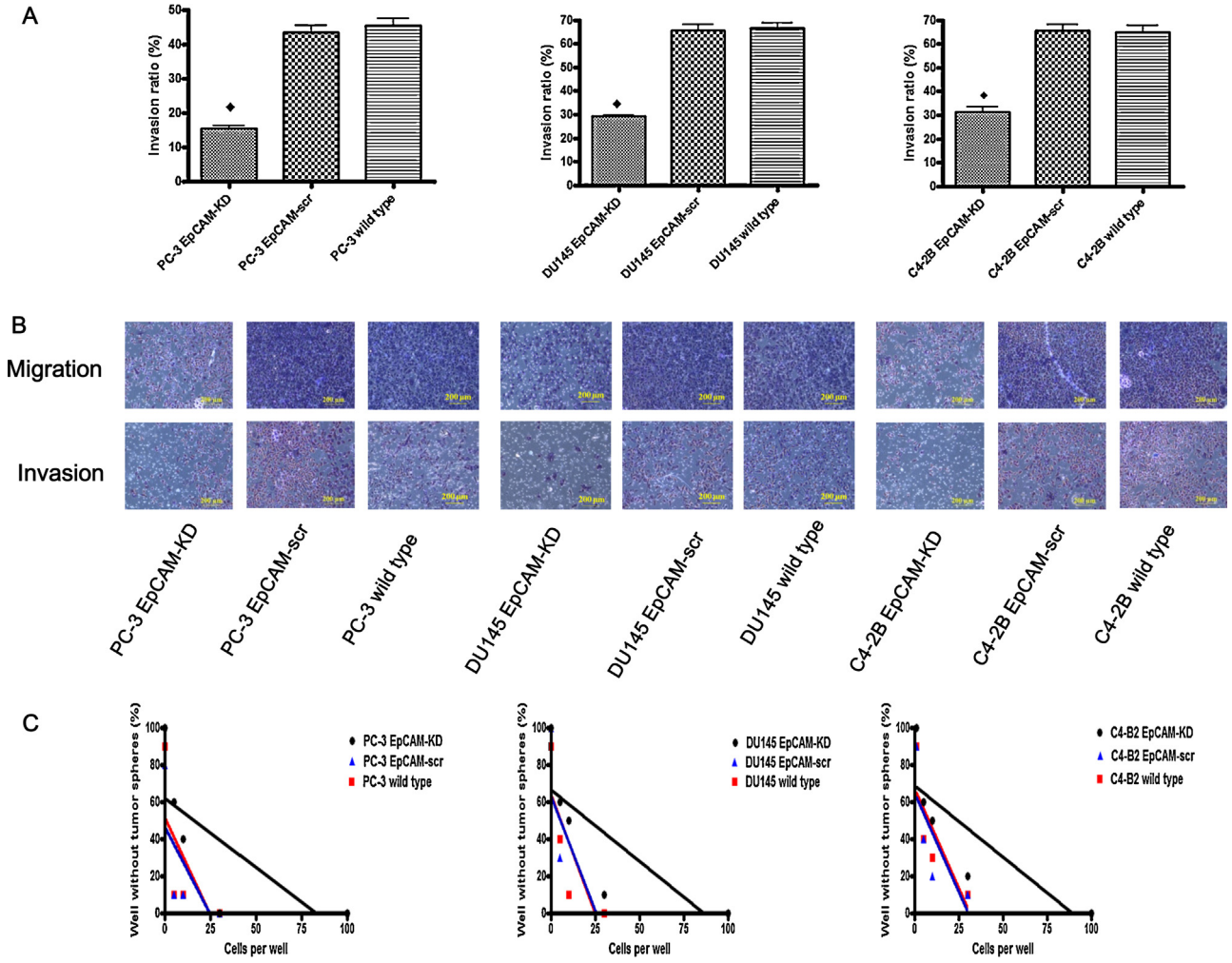

Fig. 3. Matrigel invasion and sphere formation after EpCAM knock down in CaP cells. Matrigel invasion assay was used to study the change in invasion potential of cells. (A) After knocking down, the invasive potential was significantly reduced by 60.8%, 55.2% and 48.8% in PC-3-KD, DU145-KD and C4-2B-KD cell lines compared to control cells, respectively ((♦) indicates that $P < 0.05$). (B) Representative images for CaP cell invasion and migration in KD and control cells by light microscope are shown. Magnification: 200×. (C) The mean *x*-intercept values calculated from limiting dilution analysis for each cell subtype reveal that the number of cells required to form at least 1 tumor sphere/well was much higher in KD groups, compared to the scr and wild type groups ($P < 0.05$). All results were obtained from three independent experiments (mean ± SD, $n = 3$). KD: knock-down; scr: scrambled siRNA control.

EpCAM found in both primary CaP tumors and lymph node metastases indicates that cancer clones that escape from primary tumors do not lose their EpCAM antigens via lymphatics. It was reported that the majority (>80%) of circulating CaP cells in patients with metastatic CRPC express EpCAM (Armstrong et al., 2011), suggesting that EpCAM$^+$ metastatic CaP cells can also spread via blood. EpCAM is found to be expressed at a low level on the basolateral membrane in most normal human epithelial tissues, but at a high level on the apical membrane in cancer cells and progenitor cells (Munz et al., 2009; Trzpis et al., 2007). It was reported that the over-expression of EpCAM was found in CaP tissues, compared with that in benign prostate tissues (Benko et al., 2011; Zellweger et al., 2005) as well as in normal prostate tissues from healthy individuals (Poczatek et al., 1999). In this study, we only found low level of EpCAM expression in normal prostates and BPH epithelial cells, which is consistent with the previous reports. The difference in distribution of EpCAM makes it much less accessible to antibodies on normal epithelia than the over-expressed EpCAM in CaP tissues, where it is homogenously localized on the cancer cell surface. This feature makes EpCAM a very suitable cancer target with lower toxicity. All these data indicate that EpCAM may provide a basis for targeting primary CaP cells, metastatic CaP cells in transit via blood and lymphatic and microenvironment by systemic delivery of anti-EpCAM directed therapeutic agents to prevent CaP metastasis.

EpCAM is a transmembrane glycoprotein that is intensively over-expressed in many cancers including CaP (van der Gun et al., 2010; Went et al., 2006). It was reported that the expression of EpCAM in various cancers is inversely related to the prognosis of the patients (Baeuerle and Gires, 2007). EpCAM plays different roles in different cancers. Whether EpCAM acts as a tumor suppressive gene or as an oncogene might depend on the tumor microenvironment (van der Gun et al., 2010). Dependent on the tumor type, EpCAM over-expression is associated with either increased or decreased overall survival of patients (van der Gun et al., 2010). The role of EpCAM is not limited to cell-cell adhesion but includes stem cell signaling, cell migration, proliferation and differentiation (Trzpis et al., 2007). However, the functions of EpCAM still remain largely unknown.

In EpCAM and CaP clinical research, there are conflicting reports regarding the relationship between EpCAM expression and clinical parameters in CaP patients. Poczatek et al. found that there was no significant correlation between EpCAM expression in CaP and Gleason score, and progress after RP (Poczatek et al., 1999). Using tissue microarrays (TMA) technique, Zellweger et al. demonstrated that over-expression of EpCAM was significantly associated

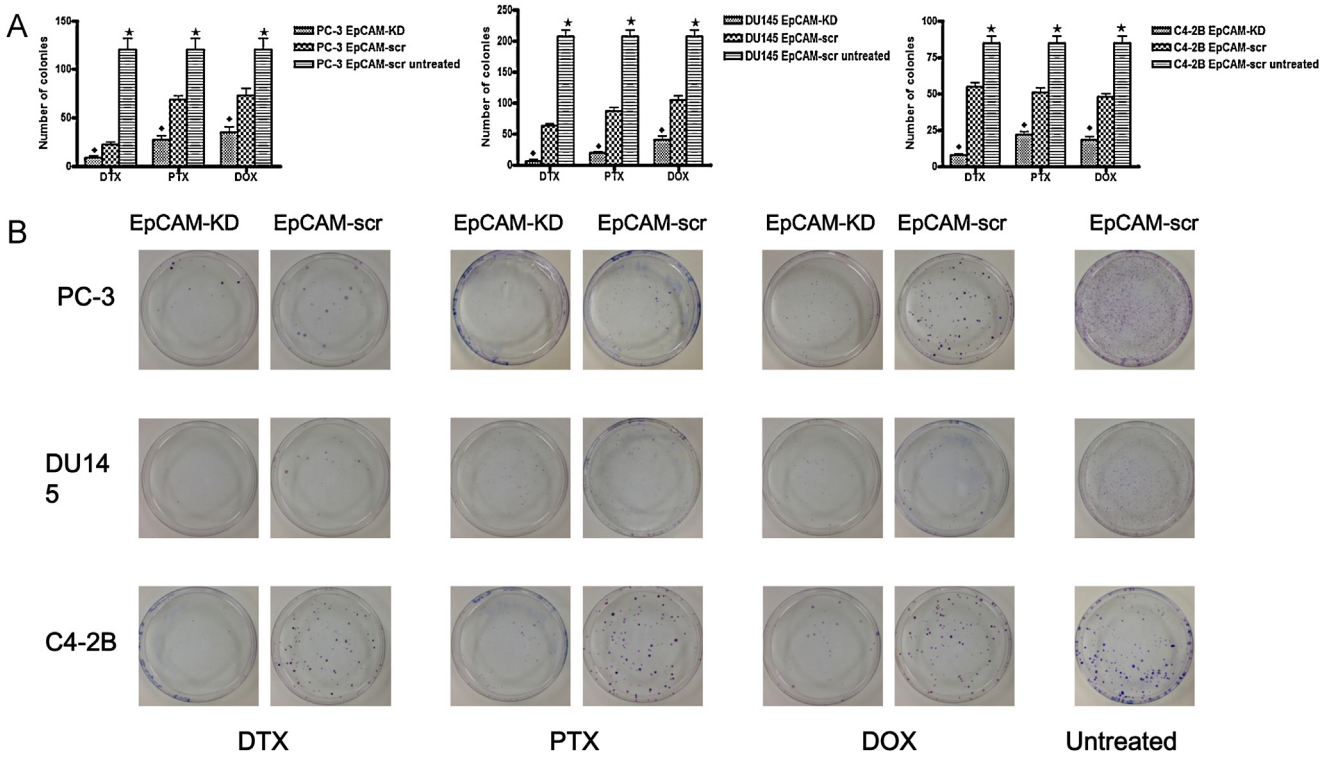

Fig. 4. Chemosensitivity after knocking down EpCAM in CaP cells. Cells were seeded in 100 mm dishes and treated with fixed DTX/PTX/DOX doses (1/2 IC_{50}) for 3 days. Following treatment, cells were cultured in fresh growth medium for 7 days. Results are presented as the number of colonies formed. (A) Typical results are shown for colony formation in CaP cell lines treated with DTX, PTX and DOX, "♦" indicates that the significant decreases in the average number of colonies were seen in EpCAM-KD cells compared to the EpCAM-scr and untreated cells in response to all three drugs tested ($P < 0.05$). "★" indicates that the significant decreases in the average number of colonies were seen in EpCAM KD cells and EpCAM-scr cells compared to the untreated cells in response to all three drugs tested ($P < 0.05$). (B) Typical images are shown for obvious reduction of colony formation found in EpCAM-KD CaP cells after different chemodrug treatments compared with the EpCAM-scr cells. The images were taken by a Sony camera (Tokyo, Japan). All results were obtained from three independent experiments (mean ± SD, $n = 3$). DOX: doxorubicin; DTX: docetaxel; KD: knock-down; p-4EBP1: phosphorylated-4EBP1; p-Akt: phosphorylated-Akt; p-mTOR: phosphorylated-mTOR; p-S6K: phosphorylated-S6K; PTX: paclitaxel; scr: scrambled siRNA control.

with high Gleason grade in 181 clinically localized CaPs from transurethral resection, RP specimens, transurethral resection from hormone-refractory local recurrences of CaPs and metastases of CaP (Zellweger et al., 2005). Using the same TMA technique, Went et al. found that EpCAM expression did not correlate with tumor stage, nodal stage, Gleason score or overall survival in 553 CaPs in different tumor stages (Went et al., 2004). In the circulating prostate tumor cells (CPTC), which largely account for haematogenous metastasis, EpCAM has been one of the most commonly used markers for cancer cell isolation and detection (Jost et al., 2010). It was reported that the majority (more than 80%) of CPTCs in patients with metastatic CRPC express EpCAM (Armstrong et al., 2011), supporting its tumor promoting role in CRPC. On the contrary, a recent study showed that none of the circulating tumor cell (CTC) fractions from metastatic CRPC patients that express EpCAM could form tumors, suggesting that CTCs have relatively low tumor-forming potential (Carvalho et al., 2013). A possible explanation of these conflicting findings lies with the discrepancies in methodology such as experimental design, the type of antibodies used, processing of tissue samples, patient population and the complexity of CaP itself. Due to the dual roles of EpCAM in different cancers, to date, limited data have been reported to demonstrate its roles in CaP progression as well as the signaling pathways involved. Therefore, understanding the roles of EpCAM in CaP metastasis and its related signaling pathway is very important in developing novel targeted therapy.

In the current study, we knocked down EpCAM using siRNA and found a reduced proliferation rate in all three $EpCAM^+$ CaP cell lines, suggesting that over-expression of EpCAM is related to CaP growth and progression. Colony formation assay provides a more appropriate measure of the long-term effects of potential therapeutic agents, assessing the ability of cells to retain proliferative after treatment, a characteristic that clinically facilitates tumor recurrences in patients (Hao et al., 2012). The results from our clonogenic assay showed that the down-regulation of EpCAM suppressed the survival potential of CaP cells in all three cell lines, further confirming that EpCAM plays an important role in CaP growth, proliferation and recurrence. Similar results have been reported in hepatocellular carcinoma cells (Kimura et al., 2007).

The matrigel invasion assay mimics the extracellular matrix microenvironment by providing growth factors and creating a matrix scaffold for tumor cells to invade through. The dual roles of EpCAM are paralleled in inconsistency of the cell invasive ability in different cancer types. Over-expression of EpCAM was reported to be associated with increased invasion in breast cancer (Osta et al., 2004), but with decreased invasion in colorectal and lung cancers (Tai et al., 2007; Went et al., 2006). In this study, we found the reduced invasion in all three CaP-KD cell lines, indicating that EpCAM plays an important role in CaP cell invasion. One possible underlying mechanism for the reduced invasion in our study could be the down-regulation of EMT (see Fig. 6) as EMT is important in CaP metastasis (Chen et al., 2013; Sethi et al., 2010). Another possibility is the reduced activity of the PI3K/Akt/mTOR signaling pathway which will be discussed in the following section.

Over the last decade, EpCAM has been 'rediscovered' as a cancer stem cell (CSC) marker in breast, colon, hepatocellular, gastric, ovarian and pancreatic cancers (Al-Hajj et al., 2003; Dalerba et al., 2007; Han et al., 2011; Li et al., 2007; Meirelles et al., 2012; O'Brien et al., 2007; Terris et al., 2010; Yamashita et al., 2009). However, the progress in elucidating relationship between EpCAM and CSC in CaP is very slow. The sphere culture assay has been proposed as

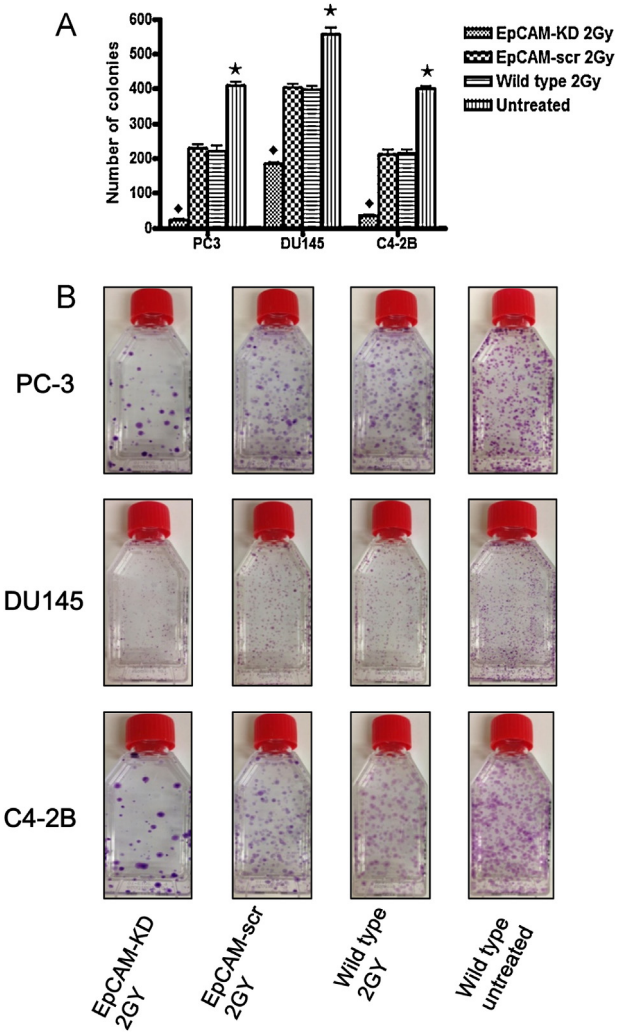

Fig. 5. Radiosensitivity after knocking down EpCAM in CaP cells. CaP cell line subtypes were seeded in 25 cm^2 flasks and treated with a single dose (2 Gy) irradiation. Following treatment, cells were cultured for 10 days. Results are presented as the number of colonies formed. (A) Typical results of radiosensitivity in colonies of CaP cell lines are shown. "♦" indicates a significant difference in the average number of colonies between EpCAM-KD cells and EpCAM-scr, wild type after 2 Gy treatment as well as untreated cells ($P < 0.05$). "⋆" indicates that the significant decreases in the average number of colonies were seen in EpCAM KD cells after 2 Gy treatment and EpCAM-scr cells compared to the untreated cells ($P < 0.05$). (B) Typical images are shown for obvious reduction of colony formation found in EpCAM-KD CaP cells after radiation treatment compared with the controls. The images were taken by a Sony camera (Tokyo, Japan). All results were obtained from three independent experiments (mean ± SD, $n = 3$). KD: knock-down; scr: scrambled siRNA control.

a valuable method for isolating cancer cells with conserved stemness determinants that are able to propagate in defined media (Jung et al., 2011; Lobo et al., 2007; Vermeulen et al., 2008). Several studies have suggested that CSCs can be enriched in spheres when these are cultured in serum-free medium supplemented with adequate mitogens, such as the bFGF and EGF (Lee et al., 2006; Zhong et al., 2010). Sphere formation assay best mimics the process of enriching and proliferating of CSCs. It is generally agreed that, like all stem cells, the tumor sphere-forming cells are capable of proliferation, self-renewal and possess higher tumorigenicity. Recent studies revealed that the sphere formation is essential for cancer-initiating ability of CSCs (Cicalese et al., 2009; Dontu et al., 2003; Mani et al., 2008). The sphere forming cells in human hepatoma were reported to be associated with the expression of stemness markers such as EpCAM (Yamashita et al., 2009) and CD44 (Zhu et al., 2010). In addition, the sphere-forming cells from primary tumors, such as breast cancer and ovarian cancer, showed stem-like properties and expressed their CSC markers (Ponti et al., 2005; Zhang et al., 2008). The anchorage-independent sphere culture of stem cells was instrumental in the study of adult stem cells including the CaP (Shi et al., 2007). Guo et al. have recently shown that EpCAM, CD44 and CD49 from primary human prostate had sphere forming capabilities and showed difference in tubule initiation capabilities and differentiation after sorting by FACS (Guo et al., 2012), suggesting that EpCAM is associated with prostate stemness. In this study, we found that all three $EpCAM^+$ CaP cell lines can form spheres in an appropriate cell number, and that knocking down of EpCAM significantly reduces sphere formative ability, suggesting that EpCAM has stem cell-like properties and is closely associated with CaP stemness. This finding may provide a novel insight to facilitate the development of novel treatment strategies for CaP.

As CSCs plays an important role in various chemotherapies and radiotherapy, and are responsible for tumor recurrence, based on our above observation, it will be very interesting to know weather EpCAM is involved in CaP chemo-/radioresistance. Investigation of the link between overexpression of EpCAM and chemo-/radioresitance in CaP has clinical significance in developing novel treatment approaches. Currently, there are only very limited data demonstrating the link between chemo-/radiosensitivity with EpCAM expression in other cancers. Gostner et al. transfected EpCAM to Hs578T and MDA-MB-231 breast cancer cells to produce $Hs578T^{EpCAM}$ and $MDA\text{-}MB\text{-}231^{EpCAM}$ cell lines, and found $Hs578T^{EpCAM}$ but not $MDA\text{-}MB\text{-}231^{EpCAM}$ cells showed enhanced chemosensitivity to DTX treatment compared to their empty vector counterparts (Gostner et al., 2011). The reason for this difference is that Wnt signaling was only activated significantly in $MDA\text{-}MB\text{-}231^{EpCAM}$ but not in $Hs578T^{EpCAM}$ (Gostner et al., 2011). However, over-expression of EpCAM was reported to be associated with increased chemoresistance in ovarian cancer cell lines (Richter et al., 2010) and tissues (Bellone et al., 2009). Maletzki et al. recently developed three patient-derived mismatch repair deficient (MMR-D) colorectal cancer cell lines (HROC24, HROC87, and HROC113) along with their corresponding xenografts with EpCAM expression and found MMR-D cell lines exhibited variable responsiveness toward chemotherapeutics *in vitro* and *in vivo* (Maletzki et al., 2012). The chemosensitivity results from our current study show that reducing EpCAM expression can greatly improve chemosensitivity in all three CaP cell lines with the treatment of DTX, PTX and DOX, suggesting that over-expression of EpCAM is highly correlated with CaP chemoresistance and that combination of targeting EpCAM with traditional chemotherapy may reduce side-effects and improve current approaches for late stage metastatic CaP. The exact mechanisms for the link between EpCAM expression and chemosensitivity are still unclear. As EpCAM is a putative CSC marker, also known as tumor-initiating cell marker which embodies the refractory nature including chemoresistance observed in many cancers, it is possible that knocking down EpCAM in CaP cells can reduce such resistance to chemodrug treatment.

Over-expression of EpCAM can also affect cancer radiosensitivity. Kaori et al. reported that down-regulation of EpCAM by siRNA increased the radiosentivity in ME-180 cervical adenosquamous carcinoma cells (Imadome et al., 2010). Our results from the EpCAM knock down study are consistent with Kaorie's report and indicate that reducing EpCAM expression increased radiosensitivity to radiation treatment in all three CaP cell lines, implying its important role in CaP growth and radiosensitivity, which may help to understand the mechanism that is associated with CaP progression as well as recurrence after radiotherapy. This finding suggests that targeting EpCAM can improve CaP radiotherapy. Given that chemo-/radioresistance is one of the main characteristics in CSCs,

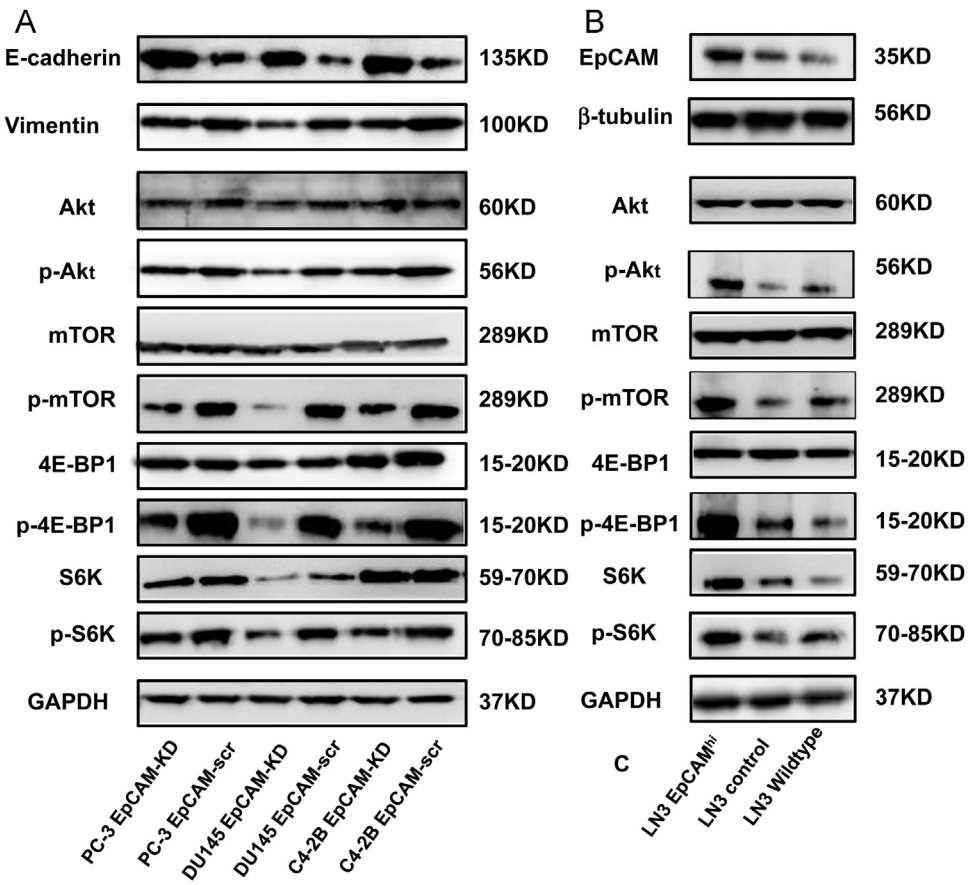

Fig. 6. The changes of EMT markers and PI3K/Akt/mTOR signaling pathway proteins after knocking down or knocking in EpCAM in CaP cells. Two markers (E-cadherin and Vimentin) involved in EMT and eight signal transduction molecules (mTOR, p-mTOR, Akt, p-Akt, 4EBP1, p-4EBP1, S6K, p-S6K) involved in PI3K/Akt/mTOR signaling pathway were assessed to investigate the relationship between EpCAM and cell signaling pathways. (A) The level of E-cadherin was increased in KD cell lines, while the levels of Vimentin, p-mTOR, p-Akt, p-4EBP1, p-S6K, S6K were reduced in KD cell lines, compared to scr and wild-type controls. (B) Overexpression of EpCAM after cDNA transfection on LN3 cell line was confirmed. (C) Levels of p-mTOR, p-Akt, p-4EBP1, p-S6K, S6K were increased in EpCAM-overexpression LN3 cell line, compared to control and wild-type groups. β-tubulin and GAPDH were used as loading controls. All results were obtained from three independent experiments (mean ± SD, $n = 3$). KD: knock-down; p-4EBP1: phosphorylated-4EBP1; p-Akt: phosphorylated-Akt; p-mTOR: phosphorylated-mTOR; p-S6K: phosphorylated-S6K; scr: scrambled siRNA control.

our findings further support that EpCAM is associated with CaP stemness.

CaP cells utilize multiple signaling pathways to proliferate and invade during the course of tumor progression and metastasis (Martin, 2003). Among several independent cell survival signaling pathways, the PI3K/Akt/mTOR pathway is a key pathway that has been linked to both tumorigenesis and resistance to therapy in CaP and other solid tumors (Bitting and Armstrong, 2013). Activation of the PI3K/Akt/mTOR pathway has been strongly implicated in CaP progression (Pourmand et al., 2007; Taylor et al., 2010). Alterations of components of the PI3K/Akt/mTOR pathway, including mutation, altered expression, and copy number alterations, have been reported in 42% of primary prostate tumors and 100% of metastatic tumors (Taylor et al., 2010). Several lines of evidence indicate that this signaling system plays a key role in CSC biology (Martelli et al., 2011). Preclinical studies suggest that the PI3K/Akt/mTOR pathway is important in maintaining a CSC population (Dubrovska et al., 2009) and is involved in EMT in CaP cells (Lim et al., 2011; Mulholland et al., 2012). However, the correlation of EpCAM expression and PI3K/Akt/mTOR pathway in CaP metastasis and progression has not been yet clarified. In this study, we have shown that silencing EpCAM concomitantly downregulated PI3K/Akt/mTOR signaling pathway proteins and EMT, suggesting that the activation of this pathway is associated with EpCAM expression and enhanced EMT. In addition, we have also demonstrated that transfection of EpCAM into $LN3^{low\ EpCAM}$ cells could activate the PI3K/Akt/mTOR signaling pathway, further confirming association between EpCAM and PI3K/Akt/mTOR signaling pathway. These findings are also consistent with the reduced proliferation, colony formation and cell invasion ability, and increased chemo-/radiosensitivity after reducing EpCAM expression and suggest that targeting this signaling pathway using PI3K/Akt/mTOR inhibitors is promising for future CaP treatment.

As EpCAM is over-expressed in primary CaP tumors and lymph node metastases and is associated with CaP proliferation, invasion, metastasis, chemo-/radioresistance, targeting EpCAM using a targeted therapy or combination of targeting EpCAM with traditional therapies will have clinical significance in the treatment of metastatic and refractory CRPC which is the major challenge in current CaP therapy. We have recently reviewed the different approaches using EpCAM-guided targeting therapies in preclinical studies and clinical trials (Ni et al., 2012). Possible targeted therapies include antibodies to inactivate EpCAM proteins, vaccination against tumor-specific EpCAM, radiolabelled or toxin-conjugated anti-EpCAM antibodies, molecules that block the certain EpCAM-related signaling pathways or proteins as well as gene therapies that restore the function of defective tumor-suppressor genes. Our research team has recently isolated an RNA aptamer that interacts specifically with a number of live human cancer cells derived from breast, colorectal, and gastric cancers that express EpCAM (Shigdar et al., 2011). This EpCAM RNA aptamer is very promising for future CaP therapy.

In conclusion, we have demonstrated that over-expression of EpCAM was found in metastatic CaP cell lines, primary CaP tissues and lymph node metastases including cancer cells and stromal cells; that EpCAM$^+$ cancer cells possess stem cell-like properties and EpCAM is involved in CaP cell proliferation, invasion and chemo-/radiosensitivity via activation of PI3k/Akt/mTOR signaling pathway *in vitro*. Our findings shed light on a potential role of EpCAM in CaP spread and metastasis, and support the use of EpCAM-guided targeting therapy for metastatic and refractory CaP to increase sensitivity to chemo-/radiotherapy. Investigation into anti-EpCAM therapies represents an ongoing effort to improve the clinical outcome for treating CRPC.

Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.09.008.

References

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proceedings of the National Academy of Sciences of the United States of America 2003;100:3983–8.
Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bitting RL, Turnbull JD, et al. Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. Molecular Cancer Research 2011;9:997–1007.
Baeuerle PA, Gires O. EpCAM (CD326) finding its role in cancer. British Journal of Cancer 2007;96:417–23.
Bellone S, Siegel ER, Cocco E, Cargnelutti M, Silasi DA, Azodi M, et al. Overexpression of epithelial cell adhesion molecule in primary, metastatic, and recurrent/chemotherapy-resistant epithelial ovarian cancer: implications for epithelial cell adhesion molecule-specific immunotherapy. International Journal of Gynecological Cancer 2009;19:860–6.
Beltran H, Beer TM, Carducci MA, de Bono J, Gleave M, Hussain M, et al. New therapies for castration-resistant prostate cancer: efficacy and safety. European Urology 2011;60:279–90.
Benko G, Spajic B, Kruslin B, Tomas D. Impact of the EpCAM expression on biochemical recurrence-free survival in clinically localized prostate cancer. Urologic Oncology 2011;31:468–74.
Berthold DR, Pond GR, Soban F, de Wit R, Eisenberger M, Tannock IF. Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer: updated survival in the TAX 327 study. Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology 2008;26:242–5.
Bitting RL, Armstrong AJ. Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. Endocrine-related Cancer 2013;20:R83–99.
Carvalho FL, Simons BW, Antonarakis ES, Rasheed Z, Douglas N, Villegas D, et al. Tumorigenic potential of circulating prostate tumor cells. Oncotarget 2013;4:413–21.
Chen CL, Mahalingam D, Osmulski P, Jadhav RR, Wang CM, Leach RJ, et al. Single-cell analysis of circulating tumor cells identifies cumulative expression patterns of EMT-related genes in metastatic prostate cancer. The Prostate 2013;73:813–26.
Chen H, Hao J, Wang L, Li Y. Coexpression of invasive markers (uPA, CD44) and multiple drug-resistance proteins (MDR1, MRP2) is correlated with epithelial ovarian cancer progression. British Journal of Cancer 2009;101:432–40.
Cicalese A, Bonizzi G, Pasi CE, Faretta M, Ronzoni S, Giulini B, et al. The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell 2009;138:1083–95.
Cozzi PJ, Wang J, Delprado W, Perkins AC, Allen BJ, Russell PJ, et al. MUC1, MUC2, MUC4, MUC5AC and MUC6 expression in the progression of prostate cancer. Clinical & Experimental Metastasis 2005;22:565–73.
Dalerba P, Cho RW, Clarke MF. Cancer stem cells: models and concepts. Annual Review of Medicine 2007;58:267–84.
Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes & Development 2003;17:1253–70.
Dubrovska A, Kim S, Salamone RJ, Walker JR, Maira SM, Garcia-Echeverria C, et al. The role of PTEN/Akt/PI3K signaling in the maintenance and viability of prostate cancer stem-like cell populations. Proceedings of the National Academy of Sciences of the United States of America 2009;106:268–73.
Gostner JM, Fong D, Wrulich OA, Lehne F, Zitt M, Hermann M, et al. Effects of EpCAM overexpression on human breast cancer cell lines. BMC Cancer 2011;11:45.
Guo C, Liu H, Zhang BH, Cadaneanu RM, Mayle AM, Garraway IP. Epcam, CD44, and CD49f distinguish sphere-forming human prostate basal cells from a subpopulation with predominant tubule initiation capability. PLoS ONE 2012;7:e34219.
Han ME, Jeon TY, Hwang SH, Lee YS, Kim HJ, Shim HE, et al. Cancer spheres from gastric cancer patients provide an ideal model system for cancer stem cell research. Cellular and Molecular Life Sciences 2011;68:3589–605.
Hao J, Chen H, Madigan MC, Cozzi PJ, Beretov J, Xiao W, et al. Co-expression of CD147 (EMMPRIN), CD44v3-10, MDR1 and monocarboxylate transporters is associated with prostate cancer drug resistance and progression. British Journal of Cancer 2010;103:1008–18.
Hao J, Madigan MC, Khatri A, Power CA, Hung TT, Beretov J, et al. In vitro and in vivo prostate cancer metastasis and chemoresistance can be modulated by expression of either CD44 or CD147. PLoS ONE 2012;7:e40716.
Higano CS, Crawford ED. New and emerging agents for the treatment of castration-resistant prostate cancer. Urologic Oncology 2011;29:S1–8.
Imadome K, Iwakawa M, Nakawatari M, Fujita H, Kato S, Ohno T, et al. Subtypes of cervical adenosquamous carcinomas classified by EpCAM expression related to radiosensitivity. Cancer Biology & Therapy 2010;10:1019–26.
Jost M, Day JR, Slaughter R, Koreckij TD, Gonzales D, Kinnunen M, et al. Molecular assays for the detection of prostate tumor derived nucleic acids in peripheral blood. Molecular Cancer 2010;9:174.
Jung P, Sato T, Merlos-Suarez A, Barriga FM, Iglesias M, Rossell D, et al. Isolation and in vitro expansion of human colonic stem cells. Nature Medicine 2011;17:1225–7.
Kimura H, Kato H, Faried A, Sohda M, Nakajima M, Fukai Y, et al. Prognostic significance of EpCAM expression in human esophageal cancer. International Journal of Oncology 2007;30:171–9.
Lee J, Kotliarova S, Kotliarov Y, Li A, Su Q, Donin NM, et al. Tumor stem cells derived from glioblastomas cultured in bFGF and EGF more closely mirror the phenotype and genotype of primary tumors than do serum-cultured cell lines. Cancer Cell 2006;9:391–403.
Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Research 2007;67:1030–7.
Lim M, Chuong CM, Roy-Burman P. PI3K, Erk signaling in BMP7-induced epithelial–mesenchymal transition (EMT) of PC-3 prostate cancer cells in 2- and 3-dimensional cultures. Hormones & Cancer 2011;2:298–309.
Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. Annual Review of Cell and Developmental Biology 2007;23:675–99.
Maletzki C, Stier S, Gruenert U, Gock M, Ostwald C, Prall F, et al. Establishment, characterization and chemosensitivity of three mismatch repair deficient cell lines from sporadic and inherited colorectal carcinomas. PLoS ONE 2012;7:e52485.
Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial–mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–15.
Martelli AM, Evangelisti C, Follo MY, Ramazzotti G, Fini M, Giardino R, et al. Targeting the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin signaling network in cancer stem cells. Current Medicinal Chemistry 2011;18:2715–26.
Martin GS. Cell signaling and cancer. Cancer Cell 2003;4:167–74.
Meirelles K, Benedict LA, Dombkowski D, Pepin D, Preffer FI, Teixeira J, et al. Human ovarian cancer stem/progenitor cells are stimulated by doxorubicin but inhibited by Mullerian inhibiting substance. Proceedings of the National Academy of Sciences of the United States of America 2012;109:2358–63.
Mukherjee S, Richardson AM, Rodriguez-Canales J, Ylaya K, Erickson HS, Player A, et al. Identification of EpCAM as a molecular target of prostate cancer stroma. The American Journal of Pathology 2009;175:2277–87.
Mulholland DJ, Kobayashi N, Ruscetti M, Zhi A, Tran LM, Huang J, et al. Pten loss and RAS/MAPK activation cooperate to promote EMT and metastasis initiated from prostate cancer stem/progenitor cells. Cancer Research 2012;72:1878–89.
Munz M, Baeuerle PA, Gires O. The emerging role of EpCAM in cancer and stem cell signaling. Cancer Research 2009;69:5627–9.
Ni J, Cozzi PJ, Duan W, Shigdar S, Graham PH, John KH, et al. Role of the EpCAM (CD326) in prostate cancer metastasis and progression. Cancer Metastasis Reviews 2012;31:779–91.
O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature 2007;445:106–10.
Osta WA, Chen Y, Mikhitarian K, Mitas M, Salem M, Hannun YA, et al. EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. Cancer Research 2004;64:5818–24.

Patriarca C, Macchi RM, Marschner AK, Mellstedt H. Epithelial cell adhesion molecule expression (CD326) in cancer: a short review. Cancer Treatment Reviews 2012;38:68–75.
Petrylak DP, Tangen CM, Hussain MH, Lara PN Jr, Jones JA, Taplin ME, et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. New England Journal of Medicine: Research 2004;351:1513–20.
Poczatek RB, Myers RB, Manne U, Oelschlager DK, Weiss HL, Bostwick DG, et al. Ep-CAM levels in prostatic adenocarcinoma and prostatic intraepithelial neoplasia. Journal of Urology 1999;162:1462–6.
Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Research 2005;65:5506–11.
Pourmand G, Ziaee AA, Abedi AR, Mehrsai A, Alavi HA, Ahmadi A, et al. Role of PTEN gene in progression of prostate cancer. Urology Journal 2007;4:95–100.
Richter CE, Cocco E, Bellone S, Bellone M, Casagrande F, Todeschini P, et al. Primary cervical carcinoma cell lines overexpress epithelial cell adhesion molecule (EpCAM) and are highly sensitive to immunotherapy with MT201, a fully human monoclonal anti-EpCAM antibody. International Journal of Gynecological Cancer: Official Journal of the International Gynecological Cancer Society 2010;20:1440–7.
Sethi S, Macoska J, Chen W, Sarkar FH. Molecular signature of epithelial–mesenchymal transition (EMT) in human prostate cancer bone metastasis. American Journal of Translational Research 2010;3:90–9.
Shi X, Gipp J, Bushman W. Anchorage-independent culture maintains prostate stem cells. Developmental Biology 2007;312:396–406.
Shigdar S, Lin J, Yu Y, Pastuovic M, Wei M, Duan W. RNA aptamer against a cancer stem cell marker epithelial cell adhesion molecule. Cancer Science 2011;102:991–8.
Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Research 2003;63:5821–8.
Tai KY, Shiah SG, Shieh YS, Kao YR, Chi CY, Huang E, et al. DNA methylation and histone modification regulate silencing of epithelial cell adhesion molecule for tumor invasion and progression. Oncogene 2007;26:3989–97.
Taylor BS, Schultz N, Hieronymus H, Gopalan A, Xiao Y, Carver BS, et al. Integrative genomic profiling of human prostate cancer. Cancer Cell 2010;18:11–22.
Terris B, Cavard C, Perret C. EpCAM, a new marker for cancer stem cells in hepatocellular carcinoma. Journal of Hepatology 2010;52:280–1.
Thoms J, Goda JS, Zlotta AR, Fleshner NE, van der Kwast TH, Supiot S, et al. Neoadjuvant radiotherapy for locally advanced and high-risk prostate cancer. Nature Reviews Clinical Oncology 2011;8:107–13.
Trzpis M, McLaughlin PM, de Leij LM, Harmsen MC. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. The American Journal of Pathology 2007;171:386–95.
van der Gun BT, Melchers LJ, Ruiters MH, de Leij LF, McLaughlin PM, Rots MG. EpCAM in carcinogenesis: the good, the bad or the ugly. Carcinogenesis 2010;31:1913–21.
Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, et al. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proceedings of the National Academy of Sciences of the United States of America 2008;105:13427–32.
Wang L, Chen H, Liu F, Madigan MC, Power CA, Hao J, et al. Monoclonal antibody targeting MUC1 and increasing sensitivity to docetaxel as a novel strategy in treating human epithelial ovarian cancer. Cancer Letters 2011a;300:122–33.
Wang L, Chen H, Pourgholami MH, Beretov J, Hao J, Chao H, et al. Anti-MUC1 monoclonal antibody (C595) and docetaxel markedly reduce tumor burden and ascites, and prolong survival in an in vivo ovarian cancer model. PLoS ONE 2011b;6:e24405.
Went P, Vasei M, Bubendorf L, Terracciano L, Tornillo L, Riede U, et al. Frequent high-level expression of the immunotherapeutic target Ep-CAM in colon, stomach, prostate and lung cancers. British Journal of Cancer 2006;94:128–35.
Went PT, Lugli A, Meier S, Bundi M, Mirlacher M, Sauter G, et al. Frequent EpCam protein expression in human carcinomas. Human Pathology 2004;35:122–8.
Xiao W, Graham PH, Power CA, Hao J, Kearsley JH, Li Y. CD44 is a biomarker associated with human prostate cancer radiation sensitivity. Clinical & Experimental Metastasis 2012;29:1–9.
Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, et al. EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. Gastroenterology 2009;136:1012–24.
Zellweger T, Ninck C, Bloch M, Mirlacher M, Koivisto PA, Helin HJ, et al. Expression patterns of potential therapeutic targets in prostate cancer. International Journal of Cancer 2005;113:619–28.
Zhang S, Balch C, Chan MW, Lai HC, Matei D, Schilder JM, et al. Identification and characterization of ovarian cancer-initiating cells from primary human tumors. Cancer Research 2008;68:4311–20.
Zhong Y, Guan K, Guo S, Zhou C, Wang D, Ma W, et al. Spheres derived from the human SK-RC-42 renal cell carcinoma cell line are enriched in cancer stem cells. Cancer Letters 2010;299:150–60.
Zhu Z, Hao X, Yan M, Yao M, Ge C, Gu J, et al. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. International Journal of Cancer 2010;126:2067–78.