

## Original Article

# Enrichment of prostate cancer stem cells from primary prostate cancer cultures of biopsy samples

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**Abstract:** This study was to enrich prostate cancer stem cells (PrCSC) from primary prostate cancer cultures (PPrCC). Primary prostate cancer cells were amplified in keratinocyte serum-free medium with epidermal growth factor (EGF) and bovine pituitary extract (BPE), supplemented with leukemia inhibitory factor (LIF), stem cell factor (SCF) and cholera toxin. After amplification, cells were transferred into ultra-low attachment dishes with serum-free DMEM/F12 medium, supplemented with EGF, basic fibroblast growth factor (bFGF), bovine serum albumin (BSA), insulin, and N2 nutrition. Expression of cell-type-specific markers was determined by RT-qPCR and immunostaining. Tumorigenicity of enriched PrCSC was determined by soft agar assay and xenograft assay in NOD/SCID mice. Biopsy samples from 19 confirmed prostate cancer patients were used for establishing PPrCC, and 18 cases (95%) succeeded. Both basal marker (CK5) and luminal markers (androgen receptor and CK8) strongly co-expressed in most of PPrCC, indicating their basal epithelial origin. After amplification under adherent culture condition *in vitro*, transient amplifying cells were the dominant cells. Sphere formation efficiency (SFE) of passaged PPrCC was about 0.5%, which was 27 times lower than SFE of LNCaP (13.67%) in the same condition. Compared with adherent cells from PPrCC, prostasphere from PPrCC showed up regulated stem cell markers and increased tumorigenic potential in soft-agar assay. However, spheroid cells from PPrCC prostasphere failed to initiate tumor in xenograft assay in 6 months. Thus, PPrCC can be established and amplified from prostate cancer biopsy samples. Our modified sphere culture system can enrich PrCSC from PPrCC.

**Keywords:** Prostate cancer (PrCa), primary prostate cancer cultures (PPrCC), prostate cancer stem cells (PrCSC), sphere culture

## Introduction

Prostate cancers are the most frequently diagnosed cancers and the second most cause of cancer-related death in American men, accounting for 11% of all cancer related deaths [1]. In recent years, there has been a rapid increase in the incidence of prostate cancer in Chinese male [2], with ageing male population increasing briskly. Although there is a great progress in the diagnosis of localized prostate cancer and the disease can be treated early with surgery or radiation therapy, it recurs in approximately 20% to 30% of patients, which eventually progresses in most patients who receive further treatment [3]. Because most

prostate cancers are hormone dependent and respond to androgen deprivation therapy, androgen ablation remains the main treatment of the metastatic disease [4]. While initially effective, this treatment is followed by tumor recurrence in a few years, because the tumors eventually become androgen refractory and metastases, which give the strongest indicators of poor outcome [4, 5].

Cancer stem cells are a defined subset of cells within a cancer, which have marked capacity for proliferation, self-renewal and differentiation [6, 7]. Since putative leukemic stem cell were first identified [6], following studies have identified cancer stem cells in many cancers such as

neurological cancer, cervical cancer, breast cancer, colon cancer, liver cancer, lung cancer, melanoma, ovarian cancer, testicular cancer and prostate cancer. Many prostate cancers relapse and metastasis in part due to the presence of prostate cancer stem cells (PrCSC) [8], which do not express androgen receptor and do not directly respond to androgen deprivation therapy [9]. PrCSC may provide insight into the origin of prostate cancer and new therapeutics for prostate cancer. Cell-surface markers (also termed cancer stem cell markers) utilized in PrCSC research include CD133, CD44, integrin  $\alpha 2\beta 1^{\text{hi}}$  in prostate cancer tissue [10], CD44, CD133, integrin  $\alpha 2\beta 1^{\text{hi}}$ , CD24<sup>+</sup>, ALDH1 in human prostate cancer cell lines [11-16] and Lin<sup>-</sup>Sca-1<sup>+</sup>, CD49f<sup>hi</sup>, Trop2 in mouse model [17-19]. Single or different combinations of these cancer stem cell markers are utilized to enrich PrCSC population.

Many human prostate cancer cell lines are used to isolate PrCSC, such as LNCaP [14, 16], PC3 [12, 13, 16, 20], DU145 [11-13, 21]. Few labs isolate PrCSC and do advanced research in PrCSC directly from prostate cancer tissues [10, 22], and definitive evidence for the existence of PrCSC in prostate cancer is still lacking [23].

We adopted a sphere culture method to isolate and enrich PrCSC from primary prostate cancer cultures (PPrCC). Sphere culture has been used to isolate cancer stem cells from many types of cancers, and tumorsphere culture in serum-free growth factor defined medium was also used to isolate and propagate PrCSC from prostate cancer tissues [22], but has not been optimized to enrich PrCSC from PPrCC originating from prostate biopsy tissues. In this study, we amplify the PPrCC by optimized adherent culture condition and enrich PrCSC by optimized suspension culture conditions. Our study demonstrates that this modified sphere culture system can be used to isolate and enrich PrCSC from prostate cancer biopsy samples.

### Material and methods

#### *Tissue collection and isolation of prostatic cancer cells*

Human prostatic tissue was obtained, with patient consent, from 28 patients (age range 50 to 86) undergoing radical prostatectomy for prostate cancer and/or prostatic needle biopsy

samples for cancer diagnosis. Initial PSA levels of these patients were from 5  $\mu\text{g/l}$  to 370  $\mu\text{g/l}$ , and Gleason score from 2+2 to 4+5. The condition of prostatic cancer was confirmed by histological examination of representative fragments. After pathological examination, primary cells cultured from prostate cancer were prepared for next step.

Tissues dissected from radical prostatectomy specimens were minced into small pieces (about 1  $\text{mm}^3$ ), incubated with mixture of Trypsin/EDTA and collagenase I for 20 minutes at 37 °C, and passed through sterile 40-micron cell strainer (Falcon) to prepare single-cell suspension. The single cells were seeded into the 6-well plate coated by collagen I (10  $\mu\text{g}/\text{cm}^2$ ).

Needle biopsy samples were cut into small pieces (about 1  $\text{mm}^3$ ), and the pieces seeded in the 6-well plate coated by collagen I (10  $\mu\text{g}/\text{cm}^2$ ) with 0.5 ml of culture medium for the first 12 hours and add 2 ml of culture medium, which increase the adhesion between the pieces and the plate bottom.

#### *Cell culture medium and cell cultures*

The single cells from radical prostatectomy specimens or the small pieces from needle biopsy samples were maintained in complete keratinocyte growth medium (keratinocyte serum-free medium with epidermal growth factor and bovine pituitary extract; Invitrogen), which were also supplemented with 2 ng/ml of leukemia inhibitory factor (LIF, Sigma), 2 ng/ml of stem cell factor (SCF, Sigma), and 100 ng/ml of cholera toxin (Sigma), referring to the method described previously [10].

Cells were cultured in the plates or dishes coated with collagen I in a humidified incubator at 37 in an atmosphere of 95% air and 5% carbon dioxide. The medium was changed twice a week, and cells were passaged after detachment with TrypLE™ Express.

#### *Immunocytochemistry*

Prostate cancer cells were planted on glass coverslips in 6-well plate. After 72 hours, the plate was washed with PBS. Immunocytochemistry was performed according to standard procedures. Dilutions of antibody (see **Table 1**) used were: rabbit monoclonal anti-cytokeratin 5 Ab (Abcam Ab52635, 1:400), mouse mono-

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**Table 1.** Antibodies used for immunocytochemical Staining (ICCS)

Antibodies	Dilution	Clone number	Source and catalog number
ICCS primary antibodies			
Mouse monoclonal anti-CK8	1:200	M20	Abcam, ab9023
Rabbit monoclonal anti-CK5	1:400	EP1601Y	Abcam, ab52635
Rabbit polyclonal Anti-AR	1:200	H-280	Santa Cruz Biotechnology, sc-13062
DAPI	1:8000		Sigma, D-8417, 1 mg/ml
ICCS secondday antibodies			
Goat anti-Rabbit-Cy3	1:1000		Jackson, 111-165-045
Goat anti-Mouse-Alexa488	1:1000		Invitrogen, A-11029

CK, Cytokeratin; AR, Androgen Receptor.

**Table 2.** Primer sequences for real-time PCR experiments

Gene	Sequence
GAPDH	5'CGACCACTTTGTCAAGCTCA3' and 5'AGGGGAGATTCAAGTGTGGTG3'
ABC1	5'GGCAGGAAAGTGTCTGCTTG3' and 5'CACCCGGCTGTTGCTCCAT3'
ALDH1	5'GCTGGCGACAATGGAGTCAA3' and 5'ACGGCCCTGGATCTTGTCAG3'
BMI	5'CTGTGAGAAGGAATGGTCCAC3' and 5'GGGCTGTGGCCTTGCTCACT3'
HES1	5'AGATGACGGCTGCGCTGAG3' and 5'GCGCACCTCGGTATTAACGC3'
ITGA2	5'CCTCTGTGGGCACAGCAATG3' and 5'AAGGGCAGGGCTGAGTTGC3'
KRT19	5'GAGCATGAAAGCTGCCTTGA3' and 5'CCGCTCACTATCAGCTCGCA3'
KRT7	5'GGACGACCTCCGGAATACCC3' and 5'CTTGGCAGCTGGTTCTTGA3'
ASCL2	5'GCGCCCTCGACCTATGAGCCTCAGC3' and 5'CGGCATTGGTCAGGCTGGGCCG3'
CD133	5'AGCAGCAGTCTGACCAGCGTGA3' and 5'CCACGGGTGGAAGCTGCCTCAG3'
TRIM32	5'AGCCGCGGGCGGTGAGGAAT3' and 5'CCCGAGGGCATCCAGGTTCAAGT3'
ZEB1	5'ACCTGCCAACAGACCAGACAGTG3' and 5'TCCTTCTGTGCATCCTCCAGC3'

Sigma), 5 µg/ml insulin (Sigma) and 1×N2 nutrition (Stemcell Technologies Inc.).

Prostate cancer cells were incubated in a humidified atmosphere with 5% carbon dioxide at 37 °C for 10 days, and collected by gentle centrifugation. The pelleted cells were enzymatically dissociated with Ac-

clonal anti-cytokeratin 8 rAb (Abcam ab9023, 1:200), rabbit polyclonal anti-androgen receptor Ab (Santa Cruz Biotechnology sc-13062, 1:200), Goat anti-Rabbit-Cy3 Ab (Jackson 111-165-045, 1:1000), Goat anti-Mouse-Alexa488 Ab (Invitrogen A-11029, 1:1000). At last, cells were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma D-8417, 1:8000) in PBS, were washed twice with PBS and were mounted with Vectashield (Vector Laboratories) before viewing under a fluorescence microscope. Fluorescence detection and imaging were carried out on Leica TCS SP5 confocal microscope.

### *In vitro tumorsphere formation assay*

Prostate cancer cells were counted and planted in ultra-low dishes at a constant density of 50000 viable cells per milliliter. Cells were grown in serum-free DMEM/F12 medium (Gibco), which was supplemented with 20 ng/ml epidermal growth factor (EGF, Sigma), 10 ng/ml basic fibroblast growth factor (bFGF, Sigma), 0.4% bovine serum albumin (BSA,

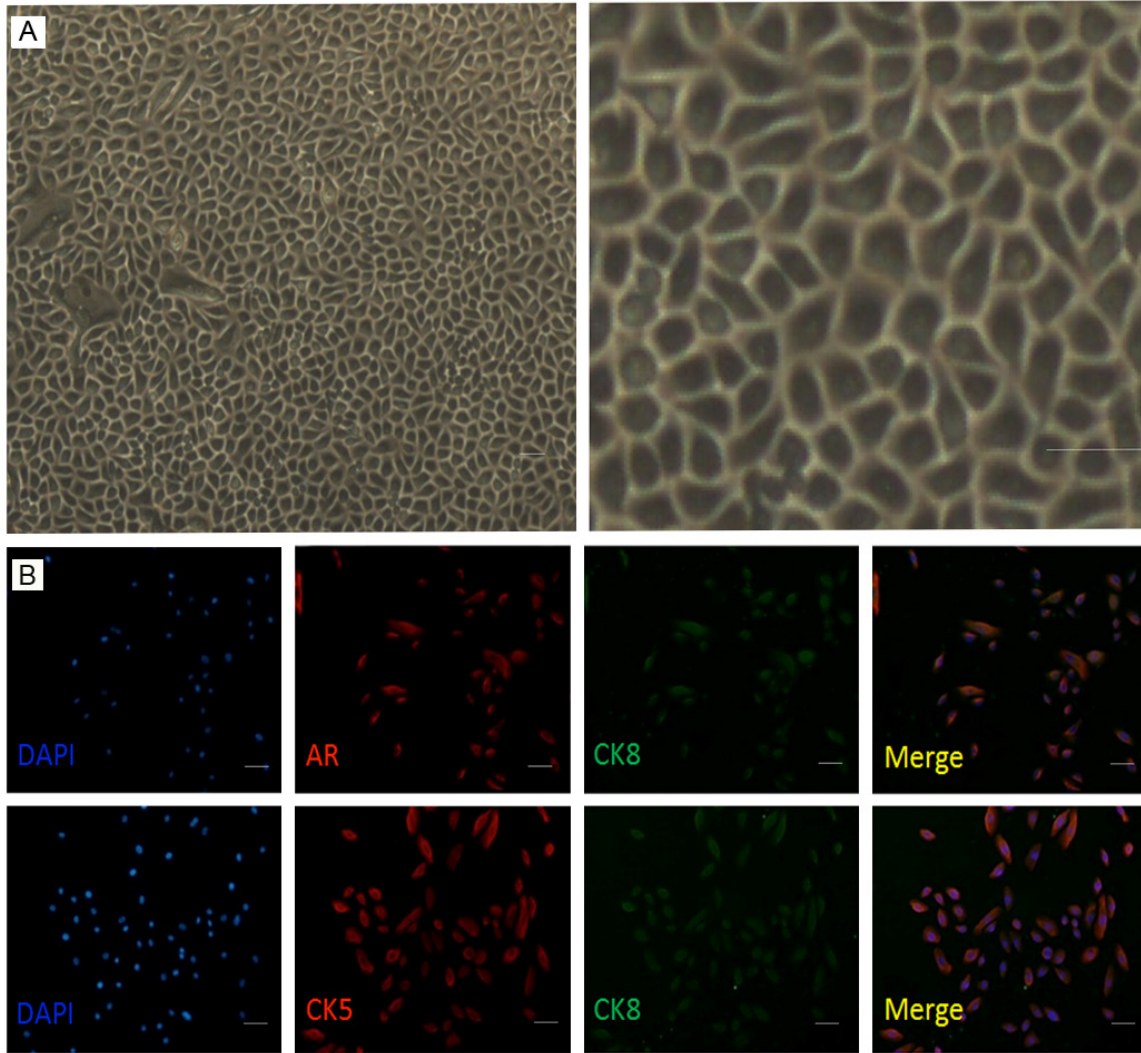
Innovative Cell Technologies) for 10 minutes at 37 °C, and mechanically dispersed by gently pipetting through a 23-gauge sterile needle. The single cells from prostasphere were used for soft agar assay *in vitro* and tumorigenesis assays *in vivo*.

### *Real-time PCR analysis*

Total RNA from adherent cells and prostasphere was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. 1 µg of total RNA was used to generate cDNA using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. 1 µl of cDNA was used for a single PCR reaction to determine the expression of self-renewal markers and stem cell markers. Part length cDNA fragments of the coding region for human genes were amplified by polymerase chain reaction (PCR) using the primer sets as described in **Table 2**.

Quantitative real-time PCR was performed on the Eppendorf MasterCycler RealPlex2 using

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**Figure 1.** Adherent cells from PPrCC. A: Adherent cells from PPrCC were observed under invert phase contrast microscope. Cells are tight attachment to the bottom of the plate, which coated by collagen. Cells are belong to a kind of squamous epithelium cell, have polygonal, flat morphology and have compact structure with one another. B: Adherent cells of passaged PPrCC showing immunofluorescence staining for androgen receptor (AR, red), cytokeratin 5 (CK5, an epithelial cell marker, red) and cytokeratin 8 (a luminal cell marker, green). 4',6-diamidino-2-phenylindole (DAPI, blue) was used to stain nuclei. Scale bar=20  $\mu$ m.

SYBR Green PCR Master Mix (Toyobo). The relative expression values were calculated relative to GAPDH by using the  $2^{-\Delta CT}$  methods. Results were calculated and normalized relative to the GAPDH control using the Microsoft Excel program.

### Soft-agar assay

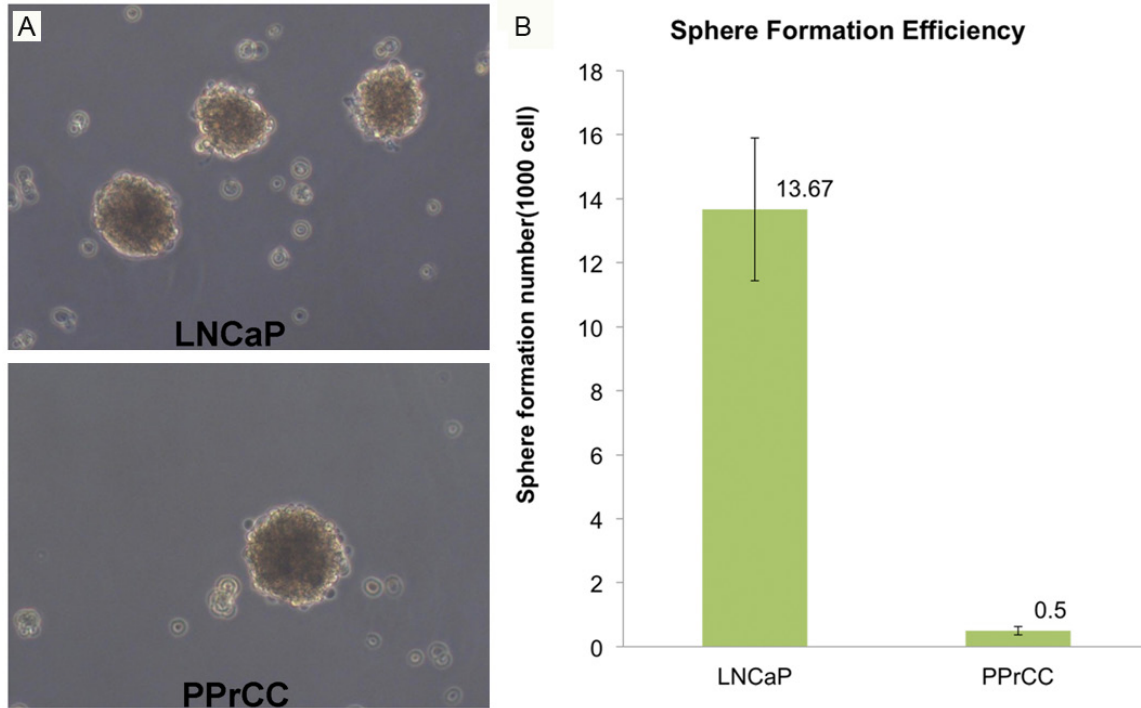
Prostate cancer adherent cells and/or single cells from prostasphere were resuspended in Dulbecco's modified Eagle medium (DMEM; 4.5 g/L D-glucose, Invitrogen) supplemented with 10% FBS and 1% antibiotic containing 0.35% agarose prostasphere (5 000 cells per

35-millimeter well). Cells were grown on tissue culture plates containing a 1-millimeter layer of solidified 0.6% agarose in a DMEM supplemented with 10% FBS and 1% antibiotic. After 3 weeks, the plates were then scanned and photographed, and number of colonies was quantified. For visualization, foci were methanol-fixed and stained with 0.1% trypan Blue.

### *In vivo tumorigenesis assays*

Prostate cancer adherent cells and/or single cells from prostasphere were resuspended in 50  $\mu$ l PBS and mixed with 50  $\mu$ l Matrigel (Becton Dickinson) at a 1:1 ratio and held on ice. The





**Figure 2.** Cells from passaged PPrCC form prostasphere in the suspension medium. A: Morphological examination of prostasphere from passaged cells of PPrCC and/or prostate cell line (LNCaP) cultured in suspension medium. Prostatasphere were maintained in the suspension medium (DMEM/F12 medium containing 10 ng/ml bFGF, 20 ng/ml EGF, 0.4% BSA and 5 µg/ml Insulin and N2 nutrition for 10 days. B: The number of prostasphere was counted which were seeded into ultra-low attachment 6-well plates. The Y-axis shows prostasphere per one thousand seeded cells. Both PPrCC and LNCaP form typical smooth-edged tumorsphere in the supplemented medium. Scale bar=100 µm.

entire 100 µl sample was injected into each flank of 6-8 weeks old Nude mice and/or NOD/SCID mice anesthetized with isoflurane according to the animal protocol approved by the Shanghai Institute of Biology Sciences committee for research in vertebrate animals. 8~12 weeks before that, the same confirmed prostate cancer tissues were injected into flank of NOD/SCID mice, xenograft tumors were observed and histological examination was underwent.

## Results

### Primary prostate cancer cultures (PPrCC)

To develop a method to culture primary prostate cancer cells *in vitro*, we optimized the culture condition. Primary prostatic cancer cells grew well in keratinocyte serum-free medium with epidermal growth factor (EGF) and bovine pituitary extract (BPE), supplemented with leukemia inhibitory factor (LIF), stem cell factor (SCF) and cholera toxin. PPrCC tightly attached to the bottom of the plate coated by collagen I.

Like squamous epithelium cell, PPrCC have a polygonal flat morphology and compact structure with one another (**Figure 1A**).

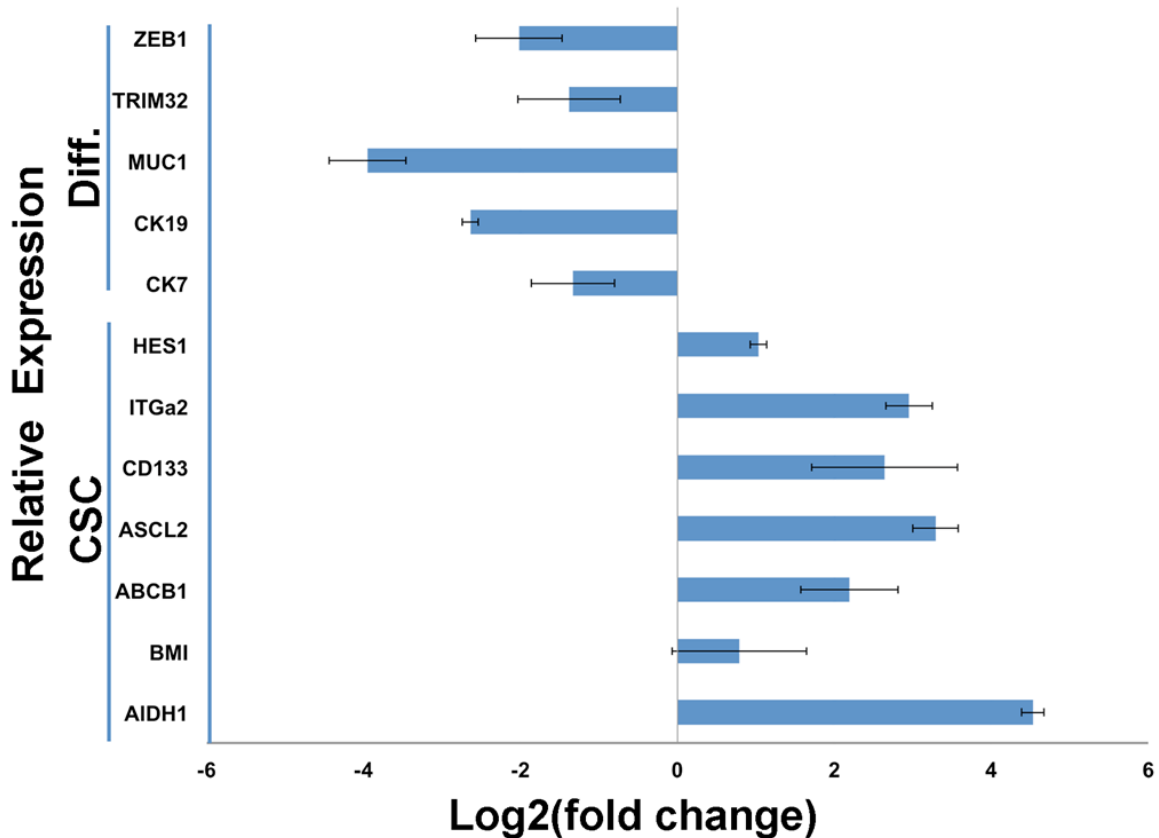
Biopsy samples from 28 patients were used for primary culture, 9 of them were not prostate cancer, and 18 cases (95%) were succeeded to culture prostate cancer cells *in vitro*.

Most prostate cancer cell lines were maintained in regular medium (DMEM or RPMI1640 supplemented with FBS and antibiotic), such as LNCaP, DU145 and PC3. When PPrCC were cultured in regular medium containing FBS, FBS induced cell differentiation (data not shown). When PPrCC were grown in the medium including FBS, cells stop proliferating, the ratio between cytoplasm and cell nucleus increased seriously and cell flat size became much bigger than normal growing cells.

### Cell origin of PPrCC

Prostate epithelium consists of basal cells (including stem cells and transient amplifying

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**Figure 3.** Real-time PCR analysis was performed on prostasphere from adherent cells of PPrCC and adherent cells from PPrCC. The log<sub>2</sub> (fold change) in gene expression between the prostasphere cells and adherent cells is shown. Each bar represents the average fold change of three pairs of reaction. Several cancer stem cell (CSC) genes are listed and their expression is correlated with prostasphere cells. Genes associated with differentiation (Diff.) are up regulated in adherent cells. Error bars represent SD from three pairs of reaction.

cells), luminal cells and neuroendocrine cells [17]. Prostate epithelial cells can be distinguished based on expression of a variety of markers [18]. The majorities of basal cells express cytokeratin 5 (CK5), cytokeratin 14 (CK14), p63, CD44, Integrin alpha (ITGa), but no cytokeratin 8 (CK8), cytokeratin 18 (CK18), androgen receptor (AR) or prostate specific antigen (PSA) [17, 18]. In contrast, luminal cells express high levels of AR, PSA, CK8, CK18, but no p63 [17]. Transient amplifying (intermediate) cells express basal marker, CK5 and often co-express the luminal marker, CK8 and prostate stem cell antigen (PSCA) [17, 18].

To determine the expression profile of PPrCC, adherent cultures of passaged PPrCC were immunostained using antibodies against both basal and luminal markers (**Figure 1B**). The basal marker CK5 was strongly expressed in the majority of primary culture cells. Luminal markers, including AR and CK8, were also

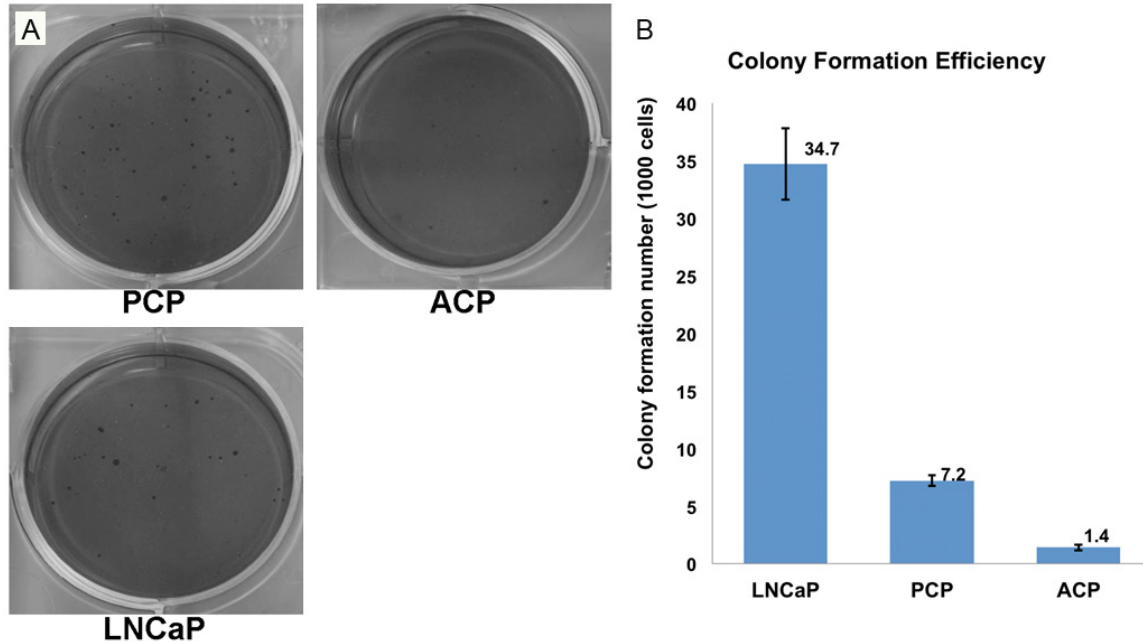
strongly expressed by most of primary culture cells. Co-expression of CK5 and CK8 in almost of all primary culture cells, suggested that primary culture cells mainly were from basal epithelial cells, and after cultured *in vitro*, transient amplifying cells were the dominant cells in the passaged adherent culture condition [24].

### *Tumorigenicity of cells from PPrCC in vitro*

To determine the tumorigenicity of PPrCC *in vitro*, we performed soft-agar assay. Colony formation efficiency (CFE) of adherent cells from PPrCC (0.15%) is 23 times lower than CFE of LNCaP (3.5%).

### *Sphere formation of cells from PPrCC in vitro*

We attempted to employ stem cell suspension culture conditions to establish prostasphere-forming condition by using the cells from passaged PPrCC. By culturing cells in ultra-low



**Figure 4.** *In vitro* tumorigenicity of PPrCC in soft-agar assay. A: Soft-agar analysis of PPrCC and LNCaP cells. PPrCC ( $1 \times 10^4$  cells per 35-mm well) and LNCaP cells ( $1 \times 10^3$  cells per 35-mm well) were re-suspended in DMEM medium supplemented with 10% FBS and 1% antibiotic/antimycotic containing 0.35% agarose. B: Colonies derived from LNCaP and PPrCC were counting after 3 weeks separately. (PCP: Prostasphere cells from PPrCC; ACP: Adherent cells from PPrCC).

attachment dishes, serum-free DMEM/F12 medium, supplemented with EGF, basic fibroblast growth factor (bFGF), bovine serum albumin (BSA), insulin and with/without N2 nutrition were used to enrich PrCSC. According to the prostasphere morphology and the prostasphere number, the optimized suspension medium is serum-free DMEM/F12 medium supplemented with EGF, bFGF, BSA, insulin and N2 nutrition in ultra-low attachment dishes.

Ten days after initial plating, they formed increasingly larger multicellular spheroids. The prostasphere showed well-defined circular shape with evident marginal rims (Figure 2A), which has similar morphological appearance with the prostasphere from LNCaP cells. However, compared with the sphere forming efficiency (SFE) of LNCaP (13.67%), SFE of passaged cells from PPrCC (0.5%) in the same supplemented medium is 27 times lower than SFE of LNCaP (Figure 2B).

*Prostasphere from PPrCC showed increased tumorigenic potential in vitro*

To test whether prostasphere from PPrCC enriched for PrCSC, we first examined the

expression profiles of putative stem cell markers including HES1 (hairy and enhancer of split 1), ITGa2 (integrin alpha 2), CD133 (prominin 1), ASCL2 (achaete-scute complex homolog 2), ABCB1 (ATP-binding cassette, sub-family B, member 1), BMI (BMI1 polycomb ring finger oncogene), ALDH1 (aldehyde dehydrogenase 1) and differentiation markers including ZEB1 (zinc finger E-box binding homeobox 1), TRIM32 (tripartite motif containing 32), MUC1 (mucin 1, cell surface associated), CK19 (cytokeratin 19), CK7 (cytokeratin 7) in prostasphere derived from PPrCC. The  $\log_2$  fold change of gene expression between the cells from prostasphere and adherent cells from PPrCC is shown in Figure 3. Quantitative real-time PCR results revealed that the expression levels of putative stem cell markers are significantly higher in cells from prostasphere than adherent cells, such as expression of ALDH1, ASCL2 and CD133 is 23-fold, 10-fold and 6-fold higher levels separately. Moreover, genes associated with differentiation are down regulated in cells form prostasphere compared to adherent cells, such as EMA and CK19. These gene expression profiles indicate that prostasphere enriched for PrCSC.

To further test whether prostatesphere from PPrCC increased tumorigenic potential *in vitro*, we performed soft-agar assay. As the size of prostatesphere from PPrCC grew beyond 100µm in diameter, the prostatesphere were enzymatically dissociated and then replanted as single-dissociated cells. Colonies were counted after 3 weeks. Though colony formation efficiency (CFE) of prostatesphere from PPrCC (0.72%, **Figure 4**) was lower than CFE of LNCaP (3.47%, **Figure 4**), CFE of prostatesphere from PPrCC was about 5-fold more than CFE of adherent cells from PPrCC (0.14%, **Figure 4**).

*Prostatesphere from PPrCC failed to give rise to form tumor in vivo*

To further address whether prostatesphere from PPrCC enriched for PrCSC, we tested whether prostatesphere from PPrCC also showed enhanced tumor forming potential *in vivo*, by injection of cells from prostatesphere and/or adherent cells from PPrCC into the flank region of Nude male mice and/or NOD/SCID male mice (4-6 weeks). LNCaP were able to form tumors (2/2) in 4 weeks when 500 thousand cells were injected into Nude male mice, but neither cells from prostatesphere or adherent cells from PPrCC could form tumors even in 6 months at a high level cell dose (2.5 million per injection) in Nude male mice or NOD/SCID mice.

### Discussion

#### *Primary prostate culture cells*

For decades, several different primary culture mediums with different supplementary had been used to maintain prostate cancer cells *in vitro*, such as PFMR-4A [25], KSFM [10] and PrEGM [26]. We found that KSFM medium with the supplementary is the best condition medium for us to culture primary prostate cancer cells in collagen-coated tissue culture dishes. And the cells growing from KSFM medium could not proliferate in PrEGM medium or in tissue culture dishes without collagen coating. In order to avoid variation in higher generation cells, we mainly used the 3<sup>rd</sup> and/or 4<sup>th</sup> passaged cells in our test assays, which could enlarge enough cells for different assay, though PPrCC could be subculture over 7 generation for most cancer prostate samples.

Prostate epithelium consists of CK5<sup>+</sup>/CK14<sup>+</sup>/p63<sup>+</sup> / basal cells and CK8<sup>+</sup>/CK18<sup>+</sup> luminal cells [27] as well as neuroendocrine cells. Basal cells preferentially survive androgen ablation and give rise to luminal cell *in vitro*, which support that basal layer is the origin of prostate stem cells. *In vitro*, undifferentiated cells with both basal marker and luminal marker could differentiate into luminal cells and basal cells [28]. *In vitro*, PPrCC express markers normally associated with both basal cells (CK5) and luminal cells (CK8 and AR). Potential uses of the PPrCC here were enrichment of PrCSC, understanding the properties of PrCSC from PPrCC, which was different from prostate cancer cell line cultured *in vitro* for many generation.

The microenvironment is critical for PPrCC growth. Most prostate cell line is adherent *in vitro*, such as PC3, DU145, 22Rv1 and LNCaP, and certain amount of fetal bovine serum is essential for the cells proliferation. However, fetal bovine serum is a strong factor of differentiation for PPrCC growth, which does not only convert the phenotype from epithelium to fibroblast, but also inhibit the proliferation with the result of cell apoptosis or necrosis. The other significant factor in our culture system is the collagen-coated tissue culture dish. PPrCC from collagen-coated dish could passaged for twice more in regular culture dish without coating, but the morphology changed greatly with few typical epithelial cells, most cells grew bigger than before and the cells were not close as before. Obviously, this is different from the culture condition of prostate cancer cell lines, which grow very well in tissue culture dish with/without coating treatment.

#### *Prostate cancer stem cell*

Many prostate cancer cell line have been used to separate PrCSC, such as LNCaP [12-14, 20], PC3 and Du145 [11, 13, 21]. Prostate cancer cell line have more mature culture system than PPrCC, and cells from the former proliferate much more quickly than cells from the latter, which also produce plenty of prostatesphere by culture prostate cancer cell line in serum-free medium or harvest enough of PrCSC by flow cytometry assay basing on cancer stem cell markers, such as CD133/CD44.

However, there are three limitations to separate PrCSC from primary prostate cancer tissue



sample: (1) prostate cancer cells limitation. About 10 000 cancer cells could be harvested from an 18-gauge-needle biopsy sample of prostate [29]. (2) Differentiation inducing by fetal bovine serum (FBS). Typical epithelial cells could be changed to fibroblast type if medium replaced by medium with FBS. Furthermore, the new fibroblast-like cells in our culture condition have limit proliferation in medium with FBS. (3) Slow-proliferation of PPrCC. Cell doubling time of adherent PPrCC *in vitro* is about 3 days, but the cancer cell death rate is close to that of its proliferation *in vivo* (xenograft to mice), net growth (i.e., tumor doubling time) is about one month [30]. Limitation is accompanied with the advantages of PPrCC, which give more variety (similar with *in vivo*) than prostate cancer cell line.

In this study, enriched PrCSC by sphere culture assay were evaluated by both soft agar assay and gene expression profiles of some putative cancer stem cell markers *in vitro*. Putative cancer stem cells (with 50% Matri-gel) from sphere culture assay were subcutaneous into NOD/SCID mice, and none tangible tumors could be detected in 6 months, which is similar to the results from the [30], though half of the biopsy sample could initiate tumor in 3 months by the same type of injection. The result indicates that our putative PrCSC from spheroids are quiescent, further studies on xenograft system are needed to evaluate the property of PrCSC *in vivo*.

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### Disclosure of conflict of interest

None.

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