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
Prostate cancer stem cells

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 metastasize 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 α2β1hi in prostate cancer tissue [10], CD44, CD133, integrin α2β1hi, CD24, AlDH1 in human prostate cancer cell lines [11-16] and Lin-Sca-1+, CD49fhi, 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 μg/l to 370 μg/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 mm3), 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 μg/cm2).

Needle biopsy samples were cut into small pieces (about 1 mm3), and the pieces seeded in the 6-well plate coated by collagen I (10 μg/cm2) 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-
Prostate cancer stem cells

Table 1. Antibodies used for immunocytochemical Staining (ICCS)

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

CK, Cytokeratin; AR, Androgen Receptor.

Table 2. Primer sequences for real-time PCR experiments

<table>
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<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
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<td>GAPDH</td>
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</tr>
<tr>
<td>ABCB1</td>
<td>5′GGCAGGGAAAGTCTGCTGCTG3 and 5′CACCAGCTGTTGTCCTCAG3'</td>
</tr>
<tr>
<td>ALDH1</td>
<td>5′GTGGCCGACATGGGACTTCAAA3 and 5′ACGGCCCTGGACATTTGCA3'</td>
</tr>
<tr>
<td>BMI</td>
<td>5′CGACCACTTTTGTCAGCTCA3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>HES1</td>
<td>5′AGATGACGGGCTCAGTGGA3 and 5′GCACACCTCCTATTAACGC3'</td>
</tr>
<tr>
<td>ITGA2</td>
<td>5′CGACCACTTTTGTCAGCTCA3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>KRT19</td>
<td>5′CGACCACTTTTGTCAGCTCA3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>KRT7</td>
<td>5′CGACCACTTTTGTCAGCTCA3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>ASCL2</td>
<td>5′CGGACCGCGATGCTGCTGCTG3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>CD133</td>
<td>5′CGGACCGCGATGCTGCTGCTG3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>TRIM32</td>
<td>5′CGGACCGCGCGCGCAGTTGCG3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
<tr>
<td>ZEB1</td>
<td>5′CGGACCGCGCGCGCAGTTGCG3 and 5′AGGGGAGATTCAATGTGTGGT3'</td>
</tr>
</tbody>
</table>

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 Accutase (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...
SYBR Green PCR Master Mix (Toyobo). The relative expression values were calculated relative to GAPDH by using the $2^{-\Delta\Delta CT}$ method. 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 μl PBS and mixed with 50 μl Matrigel (Becton Dickinson) at a 1:1 ratio and held on ice. The
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...
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
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 log₂ 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 prostasphere from PPrCC increased tumorigenic potential in vitro, we performed soft-agar assay. As the size of prostasphere from PPrCC grew beyond 100μm in diameter, the prostasphere were enzymatically dissociated and then replanted as single-dissociated cells. Colonies were counted after 3 weeks. Though colony formation efficiency (CFE) of prostasphere from PPrCC (0.72%, Figure 4) was lower than CFE of LNCaP (3.47%, Figure 4), CFE of prostasphere from PPrCC was about 5-fold more than CFE of adherent cells from PPrCC (0.14%, Figure 4).

Prostasphere from PPrCC failed to give rise to form tumor in vivo

To further address whether prostasphere from PPrCC enriched for PrCSC, we tested whether prostasphere from PPrCC also showed enhanced tumor forming potential in vivo, by injection of cells from prostasphere or/thor 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 prostasphere 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 3rd and/or 4th passed 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 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 prostasphere by culture prostate cancer cell line in serum-free medium or harvest enough of PrCSC from 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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