1. Background

Prostate cancer (PC) is the most common cancer affecting men and the second leading cause of cancer-related deaths in males in the developed world [1,2]. The disease progresses from intra-epithelial neoplasia or de novo, locally invasive carcinoma to metastatic cancer that advances to hormone refractory prostate cancers (HRPCs). HRPCs contribute virtually to all PC associated deaths [3,4]. Although the exact mechanisms responsible for PC initiation and progression remain elusive, PCSCs are widely regarded to be the origin of PC [16,17]. These cells derived from a patient with advanced PC were able to generate prostate tumors that resembled the original tumor [17]. These observations supporting the candidacy of CD44+ PCSCs, it remains to be demonstrated whether CD44+ CD133+ cells isolated from primary PC tissues are capable of initiating tumors in immuno-compromised mice. Therefore, to date, bona-fide

Several lines of evidence suggest the existence of PCSCs. A small population of primary PC cells expressing the surface antigenic profile CD44+α2β1hiCD133+ was suggested to be a candidate for PCSCs [8]. This possibility is supported by several reports. Cancer stem cells are known to share similar surface antigens with their tissue stem cell counterparts [9–13]. Consistent with this concept, human α2β1hiCD133+ prostate epithelial cells possess the properties of prostate stem cells [14], and a single cell in the mouse prostate defined as Lin Sca-11CD133+CD44+CD117+ is capable of generating an intact prostate [15]. Additionally, primary human prostate epithelial cells and malignant cells that ectopically express human telomerase reverse transcriptase (hTERT) displayed the CD44+α2β1hiCD133+ surface profile [16–18]. Furthermore, hTERT-transformed primary prostate epithelial cells derived from a patient with advanced PC were able to generate prostate tumors that resembled the original tumor [17]. These observations are consistent with a report that CD44+ subpopulations isolated from several cultured PC cell lines were more tumorigenic in nude mice than the isogenic CD44− population [19]. However, despite these observations supporting the candidacy of CD44+α2β1hiCD133+ cells as PCSCs, it remains to be demonstrated whether CD44+α2β1hiCD133+ cells isolated from primary PC tissues are capable of initiating tumors in immuno-compromised mice. Therefore, to date, bona-fide
PCSCs have not been isolated from primary PC tissues. This is largely due to the challenging nature of PC tissue, limited access to clinical samples, highly heterogeneous PC materials, and frequent infiltration into surrounding non-neoplastic prostatic tissue.

Although in vitro PC cells with the properties of cancer stem cells (CSC) have been studied, these cells have not been thoroughly investigated. Different systems have been used to culture PCSCs, including using suspension, low adherence culture on a layer of agar [16,18], or use of low adherence plates [17,20]. In general, two types of media are used to culture these stem-like PC cells that contained either fetal bovine serum (FBS) [17,19], or supplemented with epidermal growth factor (EGF; 10 or 20 ng/ml) and basic fibroblast growth factor (bFGF; 10 or 20 ng/ml) [16,20]. Although these stem-like PC cells have been demonstrated to have CSC properties, these cells have not been cultured for an extended period of time. Therefore, whether these cells constituted as progenitors or bona-fide PCSCs remains to be determined. Furthermore, the essential pathways that regulate PCSCs have not been examined until very recently. Dubrowska and colleagues recently reported that the PI3K-AKT pathway is activated, which is required for isolation and maintenance of DU145-derived PCSCs [20]. We have characterized a sub-population of DU145 cells that propagate as spheres under defined serum-free conditions and which display stem-like properties. Approximately 1.25% of DU145 monolayer cells are able to form spheres in a serum-free medium supplemented with EGF (10 ng/ml). Spheres are enriched in sphere-forming cells, since approximately 26% of sphere-derived cells are capable of forming secondary spheres. bFGF inhibits the formation of primary spheres and the propagation of secondary spheres. Although EGF significantly promotes the generation and propagation of spheres, these stem-like cells are able to form spheres without external EGF. DU145 sphere cells initiated xenograft tumors with increased ability compared to monolayer cells. Unlike a recent report, the PI3K-AKT pathway does not play a major role in the generation and maintenance of DU145 spheres.

2. Materials and methods

2.1. Tissue cultures and knockdown of PTEN

DU145 prostate cancer cells, 293 T, Caco-2 and PC12 cells were obtained from ATCC, and were cultured and maintained according to ATCC instructions.

Short-hairpin PTEN targeting (shPTEN) vector was constructed by inserting the PTEN targeting sequence (GTTGAGCCTGCGAGATAA) into the pRHI retroviral vector as a hairpin, according to our published conditions [21]. High titres of pRHI (empty vector: EV) and pRHI/shPTEN were produced using 293 T cell as described previously [21]. DU145 cells were subsequently infected with EV or shPTEN retrovirus. Infected cells were selected in hygromycin (0.5 mg/ml) and then examined for PTEN expression using western blot.

2.2. Isolation and propagation of PCSC

DU145 monolayer cells were enzymatically-dissociated in trypsin (0.25%/EDTA) and resuspended at clonal (10 cell/ml) or sub-clonal density in a serum-free medium (DMEM/F12: 3:1 mixture) containing 0.4% BSA, 0.2×B27 lacking Vitamin A (Invitrogen) and supplemented with recombinant EGF at 10 ng/ml (Sigma-Aldrich). When examining the effect of bFGF on sphere formation, recombinant bFGF (Invitrogen) was added at a concentration of 10 ng/ml, in addition to heparin at 4 μg/ml (Sigma-Aldrich). Typical spheres formed in 10 to 12 days. Spheres formed were sub-cultured in trypsin and then resuspended in the above medium at clonal density.

To examine the sphere forming capacity of DU145 monolayer cells, cells from sub-confluent (~80%) cultures were resuspended in the above SFM at a density 5×10^3 or 10^4 cells/ml, followed by dispensing 1 mL of this cell suspension into individual wells of a 24-well culture plate (4 wells per treatment). The number of spheres that formed after 12 days of culture was counted. To assay the sphere forming capacity of secondary or established spheres, sphere cells were individualized by trypsinization and plated in 96-well plates at a density of 1 cell/well (120 wells in total). Wells containing only single cells were monitored. The number of spheres that formed was counted after 12 days of culture. Triplicate experiments were conducted. Experiments were repeated three times.

2.3. Anchorage-Independent Growth Assay

DU145 monolayer and sphere cell cultures were plated in individual wells of six-well plates at a density of 10^4 cell/well in 1.5 mL of media containing 0.25% agarose. Each treatment was conducted in triplicate wells. After 8 weeks, colonies were counted under a phase-contrast microscope in 5 random fields per well. Digital images of plates were taken using a Sony Cybershot® (DSC-N2) camera, and mean colony area (pixels) was determined using ImagePro Plus 5.0 software. Duplicate experiments were conducted.

2.4. Cell Proliferation assay

DU145 monolayer cells and sphere cells were seeded in triplicate in a 96-well plate at clonal density. Cell number was measured using Roche Cell Proliferation kit 1 (MTT) according to the manufacturer's instructions.

2.5. Western blot analysis

Preparation of cell lysates and performance of western blotting were carried out according to our published procedure [22]. Antibodies used were goat anti-AKT (Santa Cruz, C-20, 1:1000), rabbit anti-phospho-AKT (AKT-P; Ser473 phosphorylation) (Cell Signaling, 1:1000), mouse anti-PTEN (Santa Cruz, clone A2B1, 1:500) and goat anti-β-actin (Santa Cruz, C-11, 1:1000). Densitometric analysis of bands was conducted using Scion Image software.

2.6. Immunofluorescence and Immunohistochemistry

Immunofluorescent (IF) staining of monolayer cell cultures was performed by fixing cells with 4% paraformaldehyde (20 min) or methanol:acetic (3:7) solution for 10 min, followed by staining with the specified primary antibodies. For spheres, intact spheres were fixed, washed three times with PBS and allowed to settle in a 30% sucrose solution overnight at 4 °C. Sucrose solution was removed and intact spheres were added to embedding medium for frozen tissue sections (Sakura Tissue-Tek O.C.T. compound). Frozen sections (4 μm) were prepared, and IF staining was carried out using the following antibodies: rabbit polyclonal anti-p16INK4A (Santa Cruz, C-20, 4 μg/ml), rabbit polyclonal anti-BMI1 (Santa Cruz, H-99, 4 μg/ml), mouse anti-CD133/1 (Miltenyi Biotec, clone AC133, 5 μg/ml), mouse anti-human CD44 (BD Biosciences, clone G44-26, 2 μg/ml), mouse anti-CD24 (Neomarkers, clone SN3, 2 μg/ml), mouse anti-cytokeratin 18 (CK18; Santa Cruz, clone DC-10, 2 μg/ml), mouse anti-integrin αvβ3 (Chemicon, clone BHA2.1, 0.5 μg/ml), mouse anti-human cytokeratin HMW (DakoCytomation, clone 34βE12, 1 μg/ml), mouse anti-Synaptoxin (Millipore, clone SVP-38, 1:100), IgG was used as a negative control. Slides were subsequently washed in 1× PBS and then incubated with secondary antibodies (donkey anti-mouse IgG-FITC or donkey anti-mouse IgG-PE, 1:200 dilution; Jackson Laboratories) for 1 hour. Slides were mounted with a coverslip using Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc., H-1200), and images were captured using a Zeiss Axiosvert 200 M inverted fluorescence microscope using AxioVision 3.1 software. Images were processed using CorelDraw 14.
For dual IF staining of DU145 xenografts, paraffin-embedded tissues were sectioned at 3 μm thickness, deparaffinized, rehydrated, subjected to antigen retrieval and endogenous peroxidase quenching as indicated for immunohistochemistry (IHC) staining. Sections were blocked for 1 hour at room temperature in 3% goat serum and 3% BSA in TBST, followed by overnight incubation at 4°C with rabbit anti-human CD44 (Abcam, ab51037, 1:100) and mouse anti-human cytokeratin HMW (Dako, clone 34ßE12) antibodies. Slides were subsequently washed in PBS and then incubated with secondary antibodies (goat anti-rabbit IgG Alexa Fluor 594 and goat anti-mouse IgG Alexa Fluor 488, 1:200; Invitrogen) for 1 hour. Sections were counterstained with DAPI.

IHC staining of the DU145 xenografts was performed as described previously [34]. Briefly, sectioned slides were deparaffinized, antigen retrieval was performed and sections were incubated with the following primary antibodies: anti-Chromogranin A (Dako, clone DAK-A3, 1:100), anti-human cytokeratin HMW (Dako, clone 34ßE12, 1:100), anti-Cki8 (Santa Cruz, clone DC-10, 1:100), anti-E-cadherin (Dako, clone NCH-38, 1:200), and anti-Synaptophyins (Dako, clone SY38, 1:25). Biotinylated goat anti-mouse IgG and avidin-biotin complex (ABC) were then added (Vectastain ABC kit, Vector Laboratories). The chromogen reaction was carried out with diaminobenzidine, and counterstaining was done with hematoxylin. Images were captured using an Olympus BX41 light microscope.

2.7. RNA extraction and Reverse Transcriptase–PCR (RT-PCR)

RNA was extracted from cells using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. RT-PCR was carried out using SuperScript III reverse transcriptase (Invitrogen) and the designed primers. β-Actin was used as an internal control. Primers and RT-PCR conditions are listed in Supplementary Table 1.

2.8. Xenograft tumor formation

DU145 monolayer and sphere cells were resuspended in MEM/Matrigel mixture (1:1 volume), followed by implantation of 0.1 ml of this mixture subcutaneously (s.c.) into flanks of 8-week-old male NOD/SCID mice (The Jackson Laboratory). Mice were inspected for tumor appearance and palpation, and tumor growth was measured weekly using a caliper. Tumor volume was determined using the standard formula: \(V = \frac{L \times W^2 \times 0.52}{2}\), where \(L\) and \(W\) are the longest and shortest diameters, respectively. The presence of each tumor nodule was confirmed by necropsy. All animal work was carried out according to experimental protocols approved by the McMaster University Animal Research Ethics Board.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows software. Data were presented as mean ± SE (standard error) unless otherwise specified. All Student’s t-tests performed were two-tailed. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Generation and propagation of DU145 sphere cells

A variety of evidence suggests that human prostate stem cells reside in the basal cell layer of the human prostate [23,24] and basal cells express the EGF receptor [25]. Based on this knowledge, we formulated a medium composed of DMEM/F12 (3:1), 0.4% BSA, 0.2× B27 supplemented with EGF at 5, 10, or 20 ng/ml and examined their ability to support the growth of DU145 stem-like cells as suspension spheres. Supplementation of 10 ng/ml of recombinant EGF to the serum-free medium (SFM + EGF) was sufficient to support DU145 cells to grow as spheres (Fig. 1A). Individual spheres were most likely derived from single cells, as spheres were formed at clonal (10 cells/μl) density (Fig. 1A). Neural spheres were routinely generated at the densities of 10 or 50 cell/μl [26] and at 10 cells/μl, neural spheres have been shown to derive from single cell [27]. Approximately 1.25% of DU145 monolayer cells are able to form primary spheres (Fig. 1C) and spheres contain an average of 100–150 cells (data not shown).

We subsequently examined whether DU145 sphere cells possess self-renewal capacity using a well-established assay [28]. When sphere cells were seeded at single cell/well in a 96-well plate, typical spheres were formed during the course of 12 days (Fig. 1B). In the total of 360 single-cell wells (120 single-cell wells per experiment in the total of three repeats) analyzed, 26.1 ± 2% of sphere cells formed subsequent spheres (Fig. 1C). This is consistent with 20% of neural [28] and brain cancer [9] sphere cells being able to form new spheres. Collectively, these observations suggest that DU145 spheres are composed of a heterogeneous population of cells with (stem-like cells) or without (progenitor cells) self-renewing capacity. In comparison to monolayer cells, sphere cells display high levels of proliferation potential when cultured in SFM + EGF (Fig. 1D). This is consistent with the reported high level of proliferation potential associated with brain cancer stem cells, when cultured in a defined serum-free medium [9].

3.2. DU145 sphere-propagating cells express stem cell factors, basal and luminal epithelial cell markers

Transcriptional factors Nanog, Oct4 and Sox2 are known to express in stem cells [29–31]. Consistent with this observation, DU145 sphere cells express Nanog, Oct4, and Sox2 (Supplementary Fig. 1A). While both Nanog and Oct4 were expressed at comparable levels in both monolayer and sphere cells (Supplementary Fig. 1A), Sox2 expression was enhanced in sphere cells (Supplementary Fig. 1A). BMI1 plays an important role in the self-renewal of neuronal and haematopoetic stem cells [32,33]. We have recently shown that BMI1 facilitates prostate tumorigenesis [34], and more recently it has been shown that BMI1 is a critical regulator of murine adult prostate stem cell self-renewal [35]. Consistent with these observations, BMI1 was expressed exclusively in the nuclei of sphere cells (Supplementary Fig. 1B). Taken together, these observations support the concept that DU145 spheres contain cells with stem-like properties. Consistent with the above observations, sphere cells also express candidate cancer stem cell surface markers. PCSCs have been indicated to be CD44+α1ß1CD133+ [16–18]. CD24 is associated with several CSC or cancer-initiating cells [36]. To determine the cell surface antigen profile of DU145 sphere cells, we examined the expression of CD44, CD24, integrin α2β1 and CD133. While the majority of sphere cells are positive for CD44, CD24, and integrin α2β1 (Fig. 2), we could not convincingly demonstrate the AC133 epitope of CD133 in DU145 spheres (data not shown). Furthermore, in more than 20 primary PC specimens examined with Gleason scores ranging from 6–8, we also failed to detect CD133 using the AC133 antibody (data not shown). This may not result from our staining procedure, as the AC133 antibody clearly detected the AC133 epitope of CD133 in AC133-positive Caco-2 cells [37] (Supplementary Fig. 2). The difference between our observation and that of others may be due to the limited specificity of reagents used to detect AC133 (see Discussion for details).

As evidence indicates that prostate stem cells reside in the basal layer of human prostate [23,24], we have investigated the expression of prostate basal, luminal and neuroendocrine (NE) markers in DU145 spheres. Using immunofluorescence, spheres were shown to be immunoreactive for basal epithelial high molecular-weight cytokeratins (CKs; 34ßE12) and the luminal CK18 (Fig. 2). No staining was detected in DU145 spheres for the NE marker synaptophysin, although the antibody clearly detected its expression in synaptophysin-positive PC12 cells (Supplementary Fig. 3). Consistent with these
observations, neuroendocrine markers synaptophysin or chromogranin A could not be detected in xenograft tumors that were derived from either DU145 monolayer cells or spheres, while epithelial marker E-cadherin and prostate lineage-specific markers βE12 and CK18 were detected (Supplementary Fig. 4).

3.3. DU145 spheres exhibit “stemness”

A typical character of SCs and CSCs is their ability to re-populate the original heterogeneous population of cells and from these cells, CSCs can be re-isolated. To demonstrate this property, we found that sphere cells were able to proliferate as monolayer cells in serum (10%) containing medium and furthermore, spheres can be re-generated from sphere-derived adherent cells (SDACs) in SFM + EGF medium. To characterize this process, we have cultured SDACs to passage 20 and analyzed the abilities of SDACs at different passages to form spheres. When 10^3 SDACs were seeded in a 24-well plate at 1 cell/μl, approximately 20% of SDACs formed spheres at passages 0 through 4 and the sphere-forming ability of SDACs significantly declined after passage 4 (Fig. 3A). At passage 20, approximately 2% of SDACs were able to form spheres (Fig. 3A) in comparison to 1.25% of monolayer cells being able to form spheres (Fig. 1C). Therefore, at least 20 passages are required for SDACs to resume a similar sphere-forming frequency as the monolayer cell culture. Furthermore, from these “differentiated” cell populations, spheres could be regenerated and the ability of sphere generation correlates with their degree of differentiation – passage numbers cultured under differentiating media conditions. More importantly, both monolayer and sphere cells formed xenograft tumors in NOD/SCID mice and from these xenograft tumors, spheres could be isolated (Fig. 3B).

Fig. 1. Isolation and characterization of a stem-like cell population from DU145 cells. A) Stem-like cells can be cultured as suspension spheres. DU145 cells were cultured in SFM [DMEM/F12 (3:1 mixture), 0.4% bovine serum albumin (BSA), 0.2× B-27 supplement lacking Vitamin A, 1% penicillin/streptomycin (P/S) and 10 ng/ml EGF] in a 24-well tissue culture plate for 12 days. Typical floating spheres formed were photographed (bright field images) at 50× (left panel) and 200× magnifications (right panel). B) Sphere cells possess self-renewal capacity. Individual sphere cells were seeded at a density of 1 cell per well, which are capable of forming a single sphere in 12 days. C) The rate of generation of primary and subsequent spheres from monolayer cells and established spheres (>2 passages). Quantification of primary sphere formation was carried out using 24-well plates (10^4 cells/well seeded). To measure self-renewal capacity, individualized sphere cells were plated in 96-well plates at a density of 1 cell per well. A total of 360 wells were counted for three repeats, with 120 wells recorded per experiment. Data were presented as mean±SE. D) DU145 monolayer cells and spheres were enzymatically-dissociated and plated at a density of 10^3 cells/well (96-well plate) in triplicate. Using the MTT assay, cell proliferation capacity was determined after 0, 3, 5, 7, 10 and 12 days of incubation. Representative image of results (mean±SE) conducted in three independent experiments. *p<0.05 (two-tailed, independent t-test).
We further demonstrated that spheres re-populated a heterogeneous cell population based on the profile of cell surface markers of spheres, SDACs, and monolayer DU145 cells. While sphere cells are largely positive for CD44, CD24, integrin α2β1, and CK18 (Fig. 2), approximately 40% or less of monolayer cells express these markers (Fig. 4, Supplementary Fig. 5A). In comparison to sphere cells (Fig. 2), the SDACs at passage 4 expressed CD44 and CD24, CK18, and integrin α2β1 at substantially reduced levels (Fig. 4, Supplementary Fig. 5B). It was surprising to notice that the passage 4 SDAC population consisted of significantly reduced number of CD44-positive or CD24-positive cells compared to the monolayer cell population (Fig. 4, Supplementary Fig. 5A, B). Although the underlying mechanisms for these observations are not clear, these mechanisms may be specific for CD44 and CD24, as the passage 4

![Fig. 2. Spheres are positive for CD44, CD24, integrin α2β1, prostate basal cytokeratins (34βE12) and luminal cytokeratin (CK18). DU145 spheres were immunofluorescently stained with the indicated antibodies (see Materials and Methods for details). Nuclei were counter-stained with DAPI. Typical images are shown. Scale bars represent 50 μm.](image-url)
SDAC population contained more CK18- or integrin α2β1-positive cells than the monolayer cell population (Fig. 4). The passage 20 population of SDACs consists of increasing numbers of CD44-positive or CD24-positive cells and reduced numbers of CK18-positive or α2β1-positive cells (Fig. 4, Supplementary Fig. 5B, C). These changes in general make the passage 20 SDACs more closely resemble monolayer cells than the passage 4 SDACs.

Furthermore, the heterogeneity of the sphere-repopulated cell population could also be demonstrated in xenograft tumors. In both monolayer cell- and sphere cell-derived xenograft tumors, CD44 and βE12 were clearly heterogeneously expressed (Fig. 5A) in comparison to the largely homogenously expression of both markers in sphere cells (Fig. 2). Interestingly, sphere cell-derived xenograft tumors contain more cells that are CD44+, βE12+ and CD44+ βE12+ than monolayer cell-derived xenograft tumors (Fig. 5A, B) (see Discussion about the significance of this observation).

3.4. bFGF attenuates the isolation and propagation of DU145 spheres

EGF was commonly used together with bFGF in culturing PCSCs [16,20]. To examine whether bFGF would enhance the numbers of spheres formed, we seeded DU145 monolayer cells in SFM supplemented with EGF, bFGF (10 ng/ml), or EGF + bFGF at clonal (10 cells/μl) or sub-clonal (5 cells/μl) densities. Surprisingly, in comparison to EGF, bFGF not only significantly reduced primary spheres formed but also significantly inhibited EGF-mediated formation of primary spheres (Fig. 6A). Similar effects were also observed in sphere propagation (Fig. 6B).

A typical characteristic of malignant tumors is their autocrine ability. Since CSCs are responsible for tumor initiation and progression, CSCs may be independent on external growth factors. To investigate this possibility, we performed primary and secondary sphere formation under SFM without addition of EGF or other growth factors.
factors (GF). Although medium lacking GF (GF-) substantially reduced the number of primary and secondary spheres formed compared to the EGF medium, spheres were capable of forming in the GF- medium (Fig. 6A, B). To further consolidate these observations, we took advantage that sphere-derived adherent cells (SDACs) possess a range of sphere-generating capacities (Fig. 3A). When seeded at $10^5$ cells/ml per well (or 1 cell/μl) in a 24-well plate in GF free medium, SDACs generated spheres, although at reduced levels, compared with the addition of EGF (Fig. 6C). Similar to the observations obtained with addition of EGF, the ability of SDACs to form spheres in GF free medium inversely correlates with the length of time (passage) cultured under differentiating media conditions (Fig. 6C). Collectively, the above observations demonstrate that external EGF is not required to support DU145 sphere cultures. Interestingly, when compared to bFGF medium, GF- medium was superior in the propagation of spheres (Fig. 6B). This provides additional evidence that bFGF inhibits the generation and propagation of stem-like DU145 cells, which is consistent with the fact that the basal cells of prostatic glands contain SCs and express EGF receptor [25].

3.5. Sphere cells proliferate slowly with reduced activation of the PI3K-AKT pathway

Stem cells are normally kept in a quiescent stage or proliferate slowly in their niches. It is not clear whether cancer stem cells including PSCSs retain this property. It has been reported that DU145 stem-like cells proliferate more actively than monolayer cells under sphere-forming SFM supplemented with insulin (4 μg/ml), EGF

![Fig. 4. Spheres are able to re-populate a heterogeneous cell population. Spheres were cultured in complete medium (DMEM, 10% FBS) as a monolayer (adherent culture) for 4 and 20 passages (p4 and p20), respectively, followed by examination for the expression of CD44, CD24, CK18 and integrin α2β1 expression in monolayer, p4 and p20 sphere-derived adherent cells (for typical images, see Supplementary Fig. 5). The percentages of positively-stained cells for CD44, CD24, CK18 and integrin α2β1 markers were derived from randomly counting a total of $10^3$ cells/marker in 5 random fields. Data are presented as mean ± SE. *p<0.05 (two-tailed, independent t-test).

![Fig. 5. Sphere cell-derived xenograft tumors consist of heterogeneous cell populations. A) Expression of CD44 and prostate basal cytokeratin marker 34βE12 in DU145 monolayer cell- and sphere cell-derived xenograft tumors. Detection of CD44 and 34βE12 was performed using anti-CD44 and 34βE12 antibodies. Nuclei were counter-stained with DAPI. Typical images are shown. Scale bars represent 20 μm. B) The percentages of CD44+ 34βE12+ and CD44+34βE12+ cells were derived by randomly counting a total of 103 cells in 5 random fields. Data are presented as mean±SE. *p<0.001 (two-tailed, independent t-test) in comparison to monolayer-derived xenograft tumors.]

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(20 ng/ml), and bFGF (20 ng/ml) [20]. This is consistent with our observation that when cultured in SFM containing only EGF (10 ng/ml), sphere cells show enhanced proliferation potential (Fig. 1D). Since approximately 26% of sphere cells are capable of generating spheres in sphere-forming medium (Fig. 1C), the majority of sphere cells may not actively proliferate, concordant with the profile of sphere cell proliferation in sphere-forming medium (Fig. 1D). Both monolayer and sphere cells proliferate very slowly up to day 4 in sphere-forming medium, but sphere cells begin to proliferate robustly afterwards (Fig. 1D), which may be attributable to the initiation of sphere formation. We therefore reasoned that complete medium supplemented with 10% serum (CM) should be used to compare the proliferation rates of monolayer and sphere cells, as the majority of monolayer and sphere cells actively proliferate in this medium.

To minimize potential differentiation of sphere cells, we examined the proliferation of sphere cells immediately cultured in CM along with monolayer DU145 cells (Fig. 7A). As expected, both sphere and monolayer cells proliferate in CM (Fig. 7A). Monolayer cells exhibit more robust proliferation than sphere cells (Fig. 7A). Furthermore, monolayer cells reached a much higher level of confluence density than sphere cells (Fig. 7A). Taken together, these observations reveal that sphere cells have an intrinsic property of slow proliferation.

The PI3K-AKT pathway promotes cell proliferation. Our observation that sphere cells proliferate slowly indicates that this pathway is not highly activated in sphere cells. To test this possibility, we briefly cultured sphere cells in CM for 18 hours and then examined for serum-induced AKT activation side-by-side with monolayer DU145 cells (Fig. 7B). It is clear that serum induces more robust AKT activation in DU145 monolayer cells than in sphere cells (Fig. 7B), demonstrating that DU145 stem-like cells do not display enhanced activation of the PI3K-AKT pathway compared to monolayer DU145 cells. Consistent with this concept, sphere cells do not express reduced levels of PTEN compared to the monolayer cells (Fig. 7B). Since PTEN is the dominant PIP3 phosphatase, the aforementioned observation thus supports the notion that DU145 stem-like cells do not have their PI3K-AKT pathway activation elevated. As DU145 sphere cells proliferate more actively than monolayer cells in sphere-forming medium observed by others [20] and ourselves (Fig. 1D) and has been reported to display enhanced activation of the PI3K-AKT pathway in sphere-forming medium [20], we compared phosphorylation of Ser 473 of human AKT, a widely used surrogate marker of AKT activation, in both monolayer and sphere cells that were cultured in sphere-forming medium. When cultured in this medium side-by-side for 24 and 48 hours, sphere cells display significantly reduced levels of AKT activation compared to monolayer cells (Fig. 7C). Taken together, the above observations reveal that DU145 sphere cells do not demonstrate elevated activation of the PI3K-AKT pathway.

To further consolidate this notion, we stably knocked down PTEN in monolayer cells using our pRIH retroviral system [21]. As expected, knockdown of PTEN enhances AKT activation (Fig. 8A). In comparison to empty vector (EV) retrovirus infected cells, knockdown of PTEN did not enhance the rate of primary sphere formation when cells were seeded at two sub-clonal densities (Fig. 8B). Furthermore, we demonstrated that while PTEN remained knocked-down in PTEN shRNA spheres (Fig. 8A), formation of secondary spheres was not enhanced compared to EV spheres (Fig. 8C).
indicating that the PI3K-AKT pathway does not play a major role in maintaining DU145 PCSCs. Taken together, the above observations clearly support the notion that enhanced activation of the PI3K-AKT pathway does not play a major role in the formation and propagation of DU145 sphere cells.

3.6. DU145 spheres display enhanced ability to form xenograft tumors in NOD/SCID mice

CSCs are associated with significantly enhanced tumorigenicity. To examine whether DU145 sphere cells (stem-like cells) possess enhanced capacity of tumorigenesis, we seeded monolayer and sphere cells in soft agar under SFM (without addition of growth factors), SFM + EGF, or CM media (Fig. 9). In SFM, sphere cells produced significantly more numbers and larger colonies than monolayer cells (Fig. 9A, B). Even in CM medium, although monolayer cells generated more colonies than sphere cells (Fig. 9A), they were substantially smaller in size (Fig. 9B). Interestingly, sphere cells grew as efficiently in SFM as in SFM + EGF in soft agar (Fig. 9A), which further supports our observation that sphere cells have autocrine capacity. Taken together, the above observations reveal that sphere cells grow much more efficiently under an anchorage-independent environment compared to monolayer cells, indicative of sphere cells having enhanced capacity for tumorigenesis.

To further consolidate this concept, we performed the “gold standard” experiment to examine the ability of DU145 sphere cells to form tumors in NOD/SCID mice. We were able to show that $10^5$ DU145 monolayer cells generated tumors efficiently in NOD/SCID mice (Fig. 9C), which is consistent with previous reports [38], and that $100$ sphere cells efficiently produced xenograft tumors in NOD/SCID mice (Fig. 9C). In comparison to monolayer cells, sphere cells exhibited a 100-fold increase in tumor formation in NOD/SCID mice, as $10^5$, $10^6$, and $10^7$ sphere cells generated xenograft tumors as efficient as $10^5$, $10^6$, and $10^7$ monolayer cells, respectively (Fig. 9C). This agrees well with our observation that 1.25% of monolayer cells formed spheres in SFM + EGF (Fig. 1C). Taken together, we demonstrate that sphere cells have enhanced tumor-initiating capacity compared to monolayer cells.

4. Discussion

Research on PCSCs is the focal point, not only in our understanding of PC tumorigenesis but also in the development of new therapeutic options to control PC [39–41]. The hedgehog pathway, a well known pathway stimulating PC growth, is activated in PCSCs [42]. Despite being heavily investigated, bona-fide PCSCs have not been identified and isolated from primary PCs. While PC cells with CSC properties have been previously demonstrated [16–19,38], the majority of these cells are unlikely to be PCSCs. This is largely due to the fact that these cells (CD44+ or PC cells ectopically expressing hTERT) consisted of a much larger proportion of the respective cell populations than would be expected for PCSCs [16–19,38]. While CD133+CD44+ cells consist of approximately 1% of monolayer DU145 cells and are enriched to 12.2% in primary spheres [20], these cells have not been thoroughly characterized and whether they can be maintained long-term in SFM supplemented with 20 ng/ml EGF and 20 ng/ml bFGF was not clear [20].

We provide here a systematic investigation of sphere-propagating cells that are derived from DU145 monolayer cell cultures. Although bFGF has been widely used in culturing stem-like PC cells [16,20], we showed that bFGF inhibits the generation and propagation of DU145 spheres. Using bFGF in culturing stem-like PC cells may have been attributed to the lack of long-term maintenance of these cells in published reports. However, we cannot exclude the possibility that our observed inhibition of bFGF on PC sphere formation and propagation is specific for DU145 cell-derived spheres. Although EGF stimulates sphere generation and propagation, addition of EGF is not required (Fig. 6). By using monolayer and sphere-derived adherent cells (SDACs), we were able to produce spheres at different cell densities ranged from 1 cell/μl, 5 cell/μl, and 10 cell/μl (Fig. 6). However, we can not distinguish whether this autocrine-induced effect is attributable to EGF or EGF-mediated autocrine signaling of other growth factors.

Approximately 1.25% of monolayer DU145 cells are able to generate spheres in SFM + EGF and this population is enriched to 26.2 ± 2% in spheres. This is consistent with the percentage of well-characterized neuronal sphere cells and brain cancer stem (sphere) cells [9,28], and also consistent with the consensus that spheres contain both stem and progenitor cells.

The notion that our spheres display stem-like properties is based on the following observations: 1) Sphere-generating cells in the monolayer cell population are enriched from 1.25% to 26.2 ± 2% in
A heterogeneous cell population, which can be defined by the profile of cell surface and prostate lineage-specific markers in vitro (CD44, CD24, CK18 and integrin α2β1) and in xenograft tumors (CD44 and 34βE12) (Fig. 5); 3) Spheres could be regenerated from these heterogeneous cell populations that were produced by culturing in vitro in the presence of 10% FCS and in vivo (xenograft tumors) (Fig. 3); 4) Sphere cells initiate xenograft tumors with substantially enhanced ability in comparison to monolayer cells (Fig. 9C); 5) In line with the above observation, sphere cell-derived xenograft tumors might be more advanced than monolayer cell-derived xenograft tumors. This is based on the observations that the former contain more CD44+ cells than the latter (Fig. 5) and that CD44+ DU145 cells have been reported to be more tumorigenic compared to DU145 CD44- cells [19].

The observation that DU145 sphere cells are CD44+CD24+ further suggests that these cells mark both PC progenitor and stem cell populations. Our results are in line with reports that CD44+ PC cells were more tumorigenic than CD44- PC cells [19]. Consistent with a report [18], our sphere cells are CK18+. The observation that sphere cells express both prostate basal (CD44, integrin α2β1, 34βE12-) and luminal epithelial cell (CK18) markers suggests that these stem-like cancer cells were generated via abnormalities that occurred during the differentiation of a prostate stem/progenitor cell. Although PC cells with enhanced tumor-initiating ability have been shown to be CD133+ [16,18,20], evidence also exists that CD133+ DU145 cells are not more tumorigenic than CD133- cells [43]. This would functionally support our observation that DU145 sphere cells are not CD133-positive. Alternatively, our inability to detect CD133 might be caused by our cell culture condition. Since CD133 (prominin-1) is commonly referred as AC133, a specific glycosylated epitope [44,45], our culture conditions may prevent this post-translational modification. Furthermore, the differences between our observed and published results regarding CD133 expression in PCSCs may be attributable to the low specificity of anti-CD133 antibodies. While the AC133 epitope of prominin-1 (CD133) is largely associated with stem cells, the protein itself is widely expressed in differentiated cells [45]. During the differentiation of colon cancer stem cells, the AC133 epitope, but not the CD133 protein, is specifically reduced [46]. CD133 can be detected by several monoclonal antibodies, including AC133. These antibodies have been reported to recognize different CD133 epitopes [47]. Other than AC133, whether other CD133 epitopes associate with the stem cell population remains to be determined [47]. Although the AC133 antibody that recognizes the AC133 epitope was used in our research, this antibody may or may not detect other glycosylated forms of CD133 [46].

It has recently been reported that the PI3K-AKT pathway is activated in DU145 sphere cells and high levels of PI3K-AKT activity are essential for the generation and maintenance of DU145 spheres or the stem-like cell population [20]. Our research, however, is inconsistent with their observations. We have demonstrated, from a variety of perspectives, that DU145 stem-like cells display an intrinsically reduced activation of the PI3K-AKT pathway. This
reduction might keep stem-like cells in a quiescent-like stage or cause them to proliferate slowly. Additional evidence supporting this possibility is our observation that sphere cells express very high levels of p16INK4A exclusively in their nuclei (Supplementary Fig. 6). Although p16INK4A is mutated in DU145 cells [48], its high level expression in sphere cells supports the concept that sphere cells are operating at a slow proliferative rate. Our observation that sphere cells are in a quiescent-like environment is consistent with stem cells being quiescent or cycling slowly in their niches [49]. The discrepancies between our research and the results of Dubrovska et al. may be attributable to different cell populations being investigated. As their cells were maintained in a high level of bFGF, their cultures might be predominantly composed of heavily proliferating progenitor cells. These cells would be expected to have the activated PI3K-AKT pathway. Our observation that DU145 sphere cells proliferate slowly is consistent with a recent report showing that CD24+ CSC cells proliferate slower than the CD24- population [50]. Nonetheless, our research outlines a condition for long-term culture of DU145 stem-like cells. This system will be very useful for the investigation of unique properties of PC stem-like cells in terms of their biology and their specific cell surface marker expression that distinguishes them from normal prostate stem cells. Regarding specific surface markers that are associated with stem-like cells, our current understanding is that potential PCSCs are CD44+α2β1hiCD133+ [16–18,51]. As we have shown that majority of DU145 sphere cells, which were prepared by section of embedded spheres, are positive for CD44, CD24, and integrin α2β1 (Fig. 2), it is thus very likely that CD44+CD24+α2β1+ cells are progenitors as well. Therefore, bona fide PCSC surface markers remain to be identified. Our system will provide a unique opportunity to identify these markers.

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Appendix A. Supplementary data

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References


