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Enhanced enrichment of prostate cancer stem-like cells with miniaturized 3D culture in liquid core-hydrogel shell microcapsules

Wei Rao^{a,b}, Shuting Zhao^{a,b}, Jianhua Yu^{c,d}, Xiongbin Lu^f, Debra L. Zynger^e, and Xiaoming He^{a,b,c,*}

^aDepartment of Biomedical Engineering, The Ohio State University, Columbus, OH 43210, USA

^bDavis Heart and Lung Research Institute, The Ohio State University, Columbus, OH 43210, USA

^cComprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

^dDivision of Hematology, The Ohio State University, Columbus, OH 43210, USA

^eDivision of Genitourinary Pathology, The Ohio State University, Columbus, OH 43210, USA

^fDepartment of Cancer Biology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, USA

These authors contributed equally to this work.

Abstract

Cancer stem-like cells (CSCs) are rare subpopulations of cancer cells that are reported to be responsible for cancer resistance and metastasis associated with conventional cancer therapies. Therefore, effective enrichment/culture of CSCs is of importance to both the understanding and treatment of cancer. However, it usually takes approximately 10 days for the widely used conventional approach to enrich CSCs through the formation of CSC-containing aggregates. Here we report the time can be shortened to 2 days while obtaining prostate CSC-containing aggregates with better quality based on the expression of surface receptor markers, dye exclusion, gene and protein expression, and *in vivo* tumorigenicity. This is achieved by encapsulating and culturing human prostate cancer cells in the miniaturized 3D liquid core of microcapsules with an alginate hydrogel shell. The miniaturized 3D culture in core-shell microcapsules is an effective strategy for enriching/culturing CSCs *in vitro* to facilitate cancer research and therapy development.

Keywords

Cancer stem-like cells; Miniaturized 3D culture; Alginate; Hydrogel; Microcapsule

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*Corresponding author. Department of Biomedical Engineering, The Ohio State University, 1080 Carmack Road, Columbus, OH, USA. Tel.: +1 614 247 8759; fax: +1 614 292 7301. he.429@osu.edu (X. He)..

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.06.011>.

1. Introduction

Prostate cancer is one of the most common and lethal malignancies affecting men in the United States and worldwide [1]. Although localized prostate cancer at an early-stage can be well treated by radical prostatectomy with good prognosis [2], the treatment of advanced prostate cancer using conventional therapies could easily fail with recurrence and metastasis [3-6]. One of the major contributors to this challenge is prostate cancer stem-like cells (CSCs), rare (usually < ~1%) subpopulations of prostate cancer cells that are highly drug resistant and capable of differentiating into multi-lineages of resident cells in the tumor to re-initiate tumor growth post conventional therapies [6-12]. Therefore, the capability of effectively enriching and culturing CSCs *in vitro* is important to the understanding of tumorigenesis and development of efficacious therapies for cancer treatment by eliminating the CSCs.

Conventionally, hanging drops [13,14], gyratory rotation and spinner flask [15,16], NASA rotary cell culture system [17,18], and cultivation in ultralow attachment plate (ULAP) [19,20] have been used to enrich CSCs from cancer cells by keeping them in suspension in CSC culture medium. This is because most non-stem cancer cells would die of anoikis (i.e., apoptosis induced by deprivation of attachment to substrate or extracellular matrix) when suspended in CSC medium while CSCs could survive and proliferate to form the CSC-enriched aggregates [21,22]. More recently, bulk synthetic hydrogel [23] and fibrous scaffold [24] are proposed to prevent cell attachment and induce anoikis of non-stem cancer cells for culturing CSCs. Among the various approaches, the cultivation in ULAP has been the most widely used probably because it is similar to conventional cell culture except the use of ULAP allowing negligible cell attachment [25]. However, these approaches are usually time consuming (~10 days), of high cost (e.g., expensive ULAP), and/or with low efficiency of forming CSC-containing aggregates. Therefore, a more effective approach for enriching and expanding CSCs is very much in need.

In recent years, microencapsulation of living cells including stem cells in homogeneous microscale hydrogels of various biomaterials for 3D culture has been studied intensively [26-31]. Besides homogeneous hydrogel microcapsules, microencapsulation of ovarian follicles containing totipotent precursor cells (i.e., oocytes) in the miniaturized 3D collagen core of microcapsules with a hydrogel shell has been shown to significantly facilitate the follicle development [32]. Moreover, culture of mouse embryonic stem cells in the miniaturized 3D liquid core of core-shell microcapsules (CSMCs) with a hydrogel shell has been shown to significantly better maintain the stemness (or pluripotency) of the pluripotent cells compared to conventional culture in open bulk medium [33]. However, such investigation has not been reported for CSCs.

In this study, we developed a semi-closed, miniaturized 3D culture approach to enrich CSCs by encapsulating PC-3 human prostate cancer cells in the aqueous liquid (i.e., CSC culture medium) core of CSMCs with an alginate hydrogel shell. Alginate was used to make the hydrogel shell of CSMCs due to its excellent biocompatibility as well as mild gelling condition using divalent cations [32-35]. The resultant prostaspheres enriched with CSCs in the liquid core of CSMCs were characterized by expression of CSC surface receptor

markers, dye exclusion, gene and protein expression, and *in vivo* tumorigenicity. The results were further compared to that of prostaspheres obtained using the well-established conventional approach by culturing PC-3 cells in open bulk CSC medium in ULAP to illustrate the advantage of the semi-closed, miniaturized 3D culture in CSMCs for enriching/culturing CSCs.

2. Experimental

2.1. Animals and materials

Immunodeficient NOD/SCID mice were purchased from National Cancer Institute–Frederick Laboratory and were maintained on a 16:8 h light–dark cycle. All animal use procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University and all efforts were made to minimize animal suffering. Sodium alginate was purchased from Sigma and purified by washing in chloroform and charcoal and dialyzing (MWCO: 50 kD) against 1 L deionized water for 20 h with 3 times water change, followed by freeze-drying to remove water. Fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (Logan, UT, USA). The F-12K and DMEM/F-12K cell culture medium were purchased from ATCC (Manassas, VA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless specifically mentioned otherwise.

2.2. Cell culture

PC-3 human prostate cancer cells (ATCC, Manassas, VA, USA) were cultured in F-12K supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in 75 cm² T-flasks at 37 °C in humidified air with 5% CO₂. Medium was changed every other day. Cells between passage 5 and 20 at 70% confluence were detached for passaging and/or further experimental use.

2.3. Enrichment of CSCs by the conventional bulk suspension culture in CSC culture medium in ULAP

To obtain prostaspheres enriched with prostate cancer stem-like cells (CSCs) using the conventional approach (Fig. 1A), single PC-3 cells obtained from 2D culture were suspended in 6-well ULAP (Corning, Lowell, MA) at a density of 20,000 cells/mL in 1 mL CSC medium consisting of serum-free DMEM/F12-K supplemented with 5 µg/mL insulin, 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), 1 × B27 (Invitrogen, Carlsbad, CA, USA), 0.4% (w/v) bovine serum albumin (BSA), and 100 U/mL penicillin and 100 µg/mL streptomycin. A total of 125 µL of medium per well was added every other day. After designated days, the prostaspheres were collected for further experimental use.

2.4. Enrichment of CSCs by miniaturized 3D culture in CSC culture medium in the semi-closed core of CSMCs

A cross-sectional view of the coaxial needle used for preparing microcapsules with a core–shell structure is illustrated in Fig. 1B. The coaxial electrospray system consists of two syringe pumps that push the core fluid with cancer cells and shell fluid of sodium alginate

through the concentric inner (28 G) and outer (21 G) lumens in the coaxial needle, respectively. Under an open electrostatic field from a voltage generator, the two coaxial fluids at the needle tip were broken up into microdrops and sprayed into the gelling bath containing 100 mM calcium chloride solution to instantly gel (or crosslink) alginate in the shell fluid to form calcium alginate hydrogel before the core and shell fluids could mix.

To prepare the core fluid, detached single PC-3 cells in physiological saline were centrifuged and a total of 20,000 cells were re-suspended at various densities (0.2, 1, 5, and 10 million cells/mL) in 0.25 M aqueous mannitol solution containing 1% (w/v) sodium carboxymethyl cellulose (1:1 mixture of high and medium viscosity ones from Sigma). The shell fluid consisted of 2% purified alginate (w/v) in 0.25 M aqueous mannitol solution. After careful optimization for the purpose of this study, the flow rate of the core and shell fluids was set at 47 and 60 $\mu\text{L}/\text{min}$, respectively, and the electrostatic field was set at 1.5 kV to generate CSMCs with a liquid core of cells and alginate hydrogel shell. Afterward, the cell-laden CSMCs were collected and washed with 0.5 M mannitol solution for 7 min. The cells encapsulated in the CSMCs were further suspended in 1 mL CSC medium and cultured in regular 6-well plates (Fig. 1B). During culture, a total of 125 μL of medium per well was added every other day. After designated days, the prostaspheres were collected for further experimental use.

2.5. Morphology and growth of prostaspheres

The morphology of prostaspheres was monitored by taking images using a Zeiss (Oberkochen, Germany) Axio Observer.Z1 microscope. To quantify the size of prostaspheres, the images were processed using NIH ImageJ by converting them into 8-bit gray scale for automatic identification of the boundary of the prostaspheres using an automated threshold function built in the software. The area of a prostasphere was then measured and the equivalent diameter of the prostasphere was calculated by assuming a spherical geometry. Images of at least 60 prostaspheres were analyzed for each data point.

2.5.1. Immunohistochemical staining—For immunohistochemical staining with two CSC surface receptor markers (CD44 and CD133), prostaspheres were collected and fixed in 4% paraformaldehyde. The fixed samples were then incubated in 3% BSA in $1 \times \text{PBST}$ ($1 \times \text{PBS}$ and 0.05% Tween 20) at room temperature for 1 h to block potential non-specific binding, followed by overnight incubation at 4 °C with primary antibodies CD44 (1:40 dilution, Abcam, Cambridge, MA, USA) and CD133 (1: 11 dilution, Miltenyi Biotec Ltd., Surrey, UK). The aggregates were then washed 3 times and incubated in dark at room temperature for 1 h with secondary antibodies (Abcam) diluted in 3% BSA in $1 \times \text{PBST}$ (1:50 dilution). The samples were then washed and further stained for nuclei using Hoechst 33342 (5 μM) for further examination using an Olympus FV1000 confocal microscope.

2.6. Quantification of CD44⁺CD133⁺ CSC subpopulation

The CD44⁺CD133⁺ CSC subpopulation was quantified by flow cytometry. For prostaspheres cultured in ULAP, they were collected by gravity sedimentation, washed with 37 °C $1 \times \text{PBS}$, detached using trypsin/EDTA, and further dissociated by gentle pipetting. For prostaspheres cultured in the liquid core of CSMCs, they were released out of the core

by incubating in isotonic solution containing 55 mM sodium citrate to dissolve the alginate shell before disassociation. The dissociated prostasphere cells were washed with $1 \times$ PBS and stained with CD44-FITC (Invitrogen, Carlsbad, CA) and CD133-PE (Miltenyi Biotec Ltd., Surrey, UK) antibodies according to the manufacturer's instructions. Lastly, the stained samples were analyzed using a BD LSR-II Flow Cytometer together with BD FACS Diva software (Franklin Lakes, NJ, USA).

2.7. Quantitative analysis of gene expression

To quantify gene expression, total RNAs in cells or prostaspheres were first isolated using RNeasy Mini Kit (Qiagen, Gaithersburg, MD, USA). Reverse transcription was then carried out using a GeneAmp 9700 PCR system to generate cDNAs with iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Finally, qRT-PCR studies were performed with the SYBR Green mix (Bio-Rad) using a Bio-Rad CFX96 real time PCR instrument. Relative gene expression was calculated with the C_t method [36] using the built-in Bio-Rad software. The genes studied and the corresponding primers used are listed in Table S1. Klf4, Nanog, and Oct4 are pluripotent stem cell genes, cytokeratin (CK) 14 is an early epithelial differentiation gene, CK18 is a late (or terminal) epithelial differentiation gene, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference (or housekeeping) gene.

2.8. Detection of protein expression by western blotting

To study protein expression, the cells and prostaspheres were lysed using the Cell Signaling (Beverly, MA, USA) radio immunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor (Roche, Mannheim, Germany) and incubated on ice for 30 min. The lysate was sonicated for 20 s on ice, centrifuged at $12,000 \times g$ for 5 min, and the supernatant collected. The total protein concentration in the lysate was determined using a Bio-Rad colorimetric protein assay reagent. Afterward, a total of 20 μ g of total protein from each sample was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and further transferred to polyvinylidene difluoride (PVDF) membranes with Bio-Rad wet transfer apparatus. The PVDF membranes were then incubated in blocking buffer (5% non-fat dry milk dissolved in $1 \times$ Tris buffered saline with 0.1% tween-20) for 1 h, probed with primary antibodies of Klf4 (1:1000 dilution), Oct4 (1:500 dilution), Nanog (1:300 dilution), and GAPDH (1:1000 dilution) from Cell Signaling (Beverly, MA, USA) in 5% bovine serum albumin dissolved in $1 \times$ Tris buffered saline with 0.1% tween-20 overnight at 4 °C, and further incubated with HRP (horseradish peroxidase)-conjugated secondary antibody (Cell Signaling, 1:1000 diluted with 5% non-fat dry milk) for 1 h at room temperature. HRP activity on the membrane was visualized on an X-ray film by immersing the membrane in Thermo (Waltham, MA, USA) Super Signal West Pico chemiluminescent substrate and processing with a Kodak (Rochester, NY, USA) X-ray developer.

2.9. Analysis of side population that excludes small fluorescence dye

To quantify side population that excludes dye, detached single cells were resuspended in culture medium (1×10^6 cells/mL) and allowed to recover at 37 °C for 1 h. Afterward, the

cells were stained with Hoechst 33342 dye (5 µg/mL, Invitrogen) in dark for 90 min at 37 °C with shaking every 15 min. At the end of incubation, cells were spun down and re-suspended in ice-cold PBS with 5% FBS. The side population was analyzed with a BD LSR-II Flow Cytometer using a dual wavelength analysis (blue at 450 nm and red at 660 nm) after excitation with 405 nm UV light.

2.10. In vivo tumorigenicity

For *in vivo* study, detached cells were suspended at 3×10^4 cells/mL in a mixture (1:1) of PBS and Matrigel. A total of 3000 cells in 100 µL of the mixture were injected subcutaneously into the right mid back areas of each 6-week-old male NOD/SCID mouse (5 mice per group). The progression of tumor occurrence and growth were monitored three times per week. The tumor volume (V) was calculated by the following formula: $V = (L \times W^2)/2$, where L is length and W is width (shorter than length) determined using a caliper. The body weight of the mice was monitored twice a week. The mice were euthanized 52 days after cell injection. Tumors were collected, formalin fixed, and paraffin embedded for further histological analysis.

2.11. Statistical analysis

All data are reported as mean $\pm$ standard deviation from at least three independent runs. The statistical significance in mean values between two groups was determined using Microsoft® Excel based on Student's two-tailed t -test assuming equal variance. For tumor volume study, the statistical significance between the mean values of more than two groups was determined using one-way analysis of variance (ANOVA) and post hoc tests with the Dunnett's multiple comparisons were applied using tumor volumes from the miniaturized 3D culture group as reference. The difference is considered statistically significant when the p value is less than 0.05.

3. Results

3.1. The conventional ULAP and new CSMC culture approaches for enriching CSCs

A schematic illustration of the widely used conventional method and the new approach developed in this study to obtain prostaspheres enriched with CSCs is shown in Fig. 1A and B, respectively. The former is done by culturing a total of 20,000 PC-3 cancer cells in open bulk CSC medium in a ULAP while for the new approach, cancer cells are cultured in the miniaturized 3D space (of CSC medium) enclosed in an alginate hydrogel shell of CSMCs. The CSMCs encapsulated with cancer cells were generated in one step using a coaxial electrospray technology, for which the core solution containing a total of 20,000 PC-3 cells at various densities (0.2, 1, 5, and 10 million cells/mL) and the shell fluid of sodium alginate were injected into the inner and outer lumens of a coaxial needle, respectively. Under an open electrostatic field, drops of the two concentric fluids at the tip of the coaxial needle were broken up into microscale droplets and sprayed into the collection bath with 100 mM calcium chloride solution to instantly gel alginate in the shell fluid by converting sodium alginate solution into calcium alginate hydrogel. Fig. 2A and Fig. S1A show typical micrographs (day 0) of the microcapsules loaded with ~2, 10, 40, and 70 cells (per microcapsule) resulting from coaxial electrospray with cell density in the core solution being

0.2, 1, 5, and 10 million cells/mL, respectively. The resultant four culture conditions are referred as CSMC-2, CSMC-10, CSMC-40, and CSMC-70, respectively (Fig. S1B). The microcapsules have an overall and core diameter of 429.7 ± 39.4 and 314.0 ± 42.5 mm, respectively, and the shell thickness is ~50 mm on average.

3.2. Kinetics of CSC-containing prostasphere formation in ULAP and CSMCs

Typical images taken on days 0, 2, 6, 10 of a total of 20,000 PC-3 cancer cells cultured in CSC medium using the conventional approach in ULAP and in the CSMC core with ~2 (CSMC-2), 10 (CSMC-10), 40 (CSMC-40), and 70 (CSMC-70) cells per microcapsule showing the cell proliferation to form aggregates (i.e., prostaspheres) are given in Fig. 2A and Fig. S1A. The size (equivalent diameter) of the prostaspheres formed under the various culture conditions was further quantified and the data are shown in Fig. 2B and Fig. S1C. Under all the conditions, aggregates were observable starting on day 2 and they further proliferated to form larger prostaspheres on day 10. Although the conventional ULAP culture resulted in the largest prostaspheres on average because of the uncontrolled growth, the prostaspheres obtained from CSMC-40 and CSMC-70 culture conditions were larger than that formed in ULAP on day 2. Besides size, the number of prostaspheres formed on days 2 and 10 for all the conditions was quantified and the results are shown in Fig. 2C. Overall, the semi-closed, miniaturized 3D culture in the alginate hydrogel shell of CSMCs facilitates the formation of significantly more prostaspheres (per 10,000 cancer cells on day 0) compared to the conventional ULAP culture in open bulk medium. The CSMC-40 culture condition with an initial cell number of ~40 cells per microcapsule resulted in the most and second most prostaspheres on day 2 and 10, respectively and both are significantly much more (~8 times on day 2 and 7 times on day 10) than that obtained from the conventional ULAP culture on the corresponding days.

3.3. Expression of CSC surface receptor makers in prostaspheres obtained from ULAP and CSMC culture

To assess if prostaspheres obtained from the semi-closed, miniaturized 3D culture in CSMCs are enriched with CSCs, we first analyzed the prostaspheres with two commonly used prostate CSC surface receptor makers (CD44 and CD133) [37-39]. Typical micrographs of immunohistochemical staining showing high expression of the two markers in the prostaspheres formed on day 2 and 10 under the CSMC-40 culture condition are given in Fig. 3A and B, respectively. Interestingly, CD133 expression in the prostaspheres on day 2 was apparently higher than that on day 10 although no apparent difference was observable in terms of the expression of CD44 between the two days. These data suggest that the expression of CSC markers is dynamic. Therefore, further flow cytometry analyses were performed to quantify CD44⁺CD133⁺ cell subpopulation in prostaspheres obtained on days 2, 6, and 10 from both the miniaturized 3D culture in CSMCs and conventional culture in ULAP. Typical 2-channel flow cytometry data showing the distribution of cells with CD44 and CD133 staining on the three different days are shown in Fig. 4A for prostaspheres obtained using the conventional ULAP and the CSMC-40 culture conditions. By gating the fluorescence intensity so that the cells under 2D culture have 1% CD44⁺CD133⁺ cell subpopulation (Fig. S2), the percentages of CD44⁺CD133⁺ cell subpopulation in prostaspheres obtained under various culture conditions were quantified and the results are

shown in Fig. 4B. It is observable in Fig. 4 that the CD44⁺CD133⁺ cell subpopulation is the least on day 6 for all the culture conditions. Although the CD44⁺CD133⁺ cell subpopulation between days 2 and 10 is not significantly different for the conventional culture in ULAP, it is significantly higher on day 2 compared to day 10 for the miniaturized 3D culture in CSMCs. Moreover, the enrichment under the miniaturized 3D culture enclosed in CSMCs on day 2 is significantly higher than that obtained from the conventional culture in ULAP (~25% versus 9% on average), which is independent of the number of cells encapsulated in the CSMC core on day 0.

The data shown in Fig. 4 are exciting because they suggest that the time required for enriching CSCs could be shortened from the conventionally used 10 days to only 2 days while obtaining prostaspheres with significantly (~3 times) higher percentage of CSCs. Since the CSMC-40 culture condition gives the most prostaspheres on day 2 (Fig. 2C), this condition was used for further comparisons between the prostaspheres obtained from the new miniaturized 3D culture in the core of CSMCs and conventional open bulk culture in CSC medium in ULAP.

3.4. Side population in prostaspheres obtained from ULAP and CSMC culture

The subpopulation of cancer cells (often called the side population) with the capability of excluding Hoechst 33342 has been taken as one of the rare CSC subpopulations [20,40,41]. This capability renders the cells drug resistance, which is often associated with increased expression of the ABC (ATP-binding cassette) family of transmembrane glycoproteins that can pump out fluorescence dye or small molecule anticancer drug in the cells [42-48]. Using flow cytometry, we quantified the percentage of side population in prostaspheres obtained using both the CSMC-40 and conventional ULAP culture methods. Typical images of two-channel flow cytometry showing distinct side populations (blue) and the percentage data of three independent runs are given in Fig. 5A and B, respectively. A significantly higher percentage of side population was consistently detected in prostaspheres on days 2, 6, and 10 from the CSMC-40 than ULAP culture and both are significantly higher than that ($0.4 \pm 0.2\%$) in parent PC-3 cells without any enrichment. For the CSMC-40 culture, no significant change was observed for the percentage of side population over time although it is significantly higher on day 10 than 2 for the conventional ULAP culture. Moreover, the side population percentage of the CSMC-40 culture on day 2 is higher than that of the conventional ULAP culture on day 10. These data support the hypothesis that prostaspheres formed on day 2 under CSMC-40 culture contain more CSCs compared to that from conventional culture in ULAP on days 2, 6, and 10.

3.5. Gene and protein expression in prostaspheres obtained from ULAP and CSMC culture

To investigate gene expression, we conducted quantitative (or real-time) RT-PCR analyses of three pluripotency (or stem cell) genes including Oct4, Nanog, and Klf4 that are important in regulating tumor development [49-51] together with two epithelial lineage genes-cytokeratin 14 (CK14 indicating early or basal epithelial differentiation) and 18 (CK18 indicating late or luminal epithelial differentiation). The results are shown in Fig. 6A for the expression of the various genes in prostaspheres obtained from both the CSMC-40 and conventional ULAP culture normalized to that in parent PC-3 cells. In other words, the

expression of the various genes in parent PC-3 cells on the different days is one (dotted line in Fig. 6A). Compared to parent PC-3 cells, the prostaspheres under miniaturized 3D culture in CSMCs on day 2 have significantly much higher expressions of Nanog (624 times), Oct4 (27 times), and Klf4 (13 times). Interestingly, these differences decrease on days 6 and 10 and the decreases are significant between days 2 and 10 for all the three pluripotency genes studied. Moreover, the expression of the three pluripotency genes in prostaspheres obtained from CSMC-40 culture on day 2 is also significantly much higher than that from the conventional culture in ULAP on days 2, day 6 and day 10. The latter are no more than 4 times higher than that in parent PC-3 cells.

Strikingly, the expression of CK14 (a gene marker of early or basal epithelial differentiation) was constantly 0 in prostaspheres obtained from CSMC-40 culture on day 2 (which is significantly lower than that for all the other conditions), but it increased to nearly 900 times higher than that in parent PC-3 cells with longer culture to day 6 and then decreased to 38 times on day 10. The CK14 gene expression in prostaspheres obtained from the conventional culture in ULAP is constantly higher than that in PC-3 cells on days 2 (14 times), 6 (63 times), and 10 (46 times). The expression of the CK18 gene, a marker for terminal differentiation to the luminal epithelial lineage [21,22], is slightly higher in prostaspheres obtained from both the CSMC-40 and conventional ULAP culture compared to parent PC-3 cells although the expression of CK18 gene in prostaspheres under the various conditions is not significantly different. These data are consistent with the literature suggesting that prostate CSCs could be dedifferentiated from more differentiated luminal epithelial cells [52,53].

Due to the high expression of pluripotency genes and zero expression of the basal or early epithelial differentiation CK14 gene in prostaspheres obtained from CSMC-40 culture on day 2, expression of the corresponding three pluripotency and two epithelial differentiation proteins was further detected by western blotting and compared to prostaspheres obtained from conventional ULAP culture on days 10 and 2. The qualitative and quantitative (normalized to that of parent PC-3 cells) results are shown in Fig. 6B and C, respectively. Although the expression of Oct4 and Klf4 proteins is not significantly different, the expression of Nanog protein in the CSMC-40 prostaspheres on day 2 is significantly higher than that from conventional ULAP on both days 2 and 10 and all are 3–7 times higher than that in parent PC-3 cells. Moreover, the expression of the CK18 protein marker of luminal epithelial differentiation in the day-2 CSMC-40 cultured prostaspheres is significantly much (>8 times) lower than that in parent PC-3 cells and prostaspheres obtained from the conventional ULAP culture on both days 2 and 10. Lastly, no expression of CK14 protein marker of basal or early epithelial differentiation was detected in any of the prostaspheres and parent PC-3 cells, which further supports the hypothesis that prostate CSCs could be dedifferentiated from more differentiated luminal epithelial cells.

3.6. In vivo tumorigenicity

To understand the capability of initiating tumor formation *in vivo* by cells in prostaspheres obtained from CSMC-40 culture for 2 days compared to parent PC-3 cells and prostasphere cells obtained by conventional culture in ULAP for 2 and 10 days, a total of 3000 of the

respective cells were injected into NOD/SCID mice to observe the tumor incidence and growth. The incidence of tumor formation on day 52 is listed in Table 1 and the quantitative data of tumor volume as a function of time showing tumor growth are given in Fig. 7A. The table shows that the prostasphere cells from CSMC-40 culture for 2 days and conventional culture for 10 days in ULAP could induce higher percentage of tumor formation than the prostasphere cells from conventional culture for 2 days and the parent PC-3 cells. Moreover, Fig. 7A shows cells in the day-2 prostasphere of CSMC-40 culture could result in the formation of significantly much larger tumor compared to the parent PC-3 cells and prostasphere cells from the conventional ULAP culture for 2 and 10 days, which is evidenced by the images and weight of the tumors harvested on day 52 for the various conditions shown in Fig. 7B and Fig. S3A, respectively. Light microscopic examination of hematoxylin and eosin (H&E) stained slides of the tumors performed by a genitourinary surgical pathologist revealed no appreciable difference in the histomorphology of the tumors obtained under the four different conditions. The tumors were predominately hypercellular and were composed of sheets of malignant polygonal to spindle cells demonstrating marked pleomorphism, with large, hyperchromatic nuclei, many mitotic figures, and areas of discohesion (Fig. 7C-E). Both geographic necrosis (Fig. 7E and Fig. S3B) and single cell necrosis (Fig. 7C) were identified in all tumors.

4. Discussion

CSCs play an important role in tumor initiation/re-initiation, progression, and metastasis and are reported to be responsible for cancer resistance and recurrence associated with conventional therapies [3-12]. However, CSCs are extremely rare (<~1%) among the parent cancer cell population both *in vitro* and *in vivo* and it is extremely difficult if not impossible to enrich CSCs *in vivo* [54]. Therefore, the capability of enriching/culturing CSCs *in vitro* is of great importance to facilitating drug screening and development of efficacious cancer therapies by eliminating the CSCs. Thus far, suspension culture of parent cancer cells in bulk CSC culture medium in ULAP via the formation of CSC-containing spheres (or aggregates, Fig. 1) is one of the most widely used techniques for enriching CSCs [25]. However, the production of prostate CSCs with this technique is time-consuming (~10 days culture) and costly (the ULAP is more than 10 time more expensive than regular plates). Previous studies have demonstrated the formation of 3D multi-cellular tumor spheroid by microencapsulating differentiated cancer cells in homogeneous hydrogel for culture in regular (i.e., non-CSC) culture medium of cancer cells for anticancer drug screening[55,56]. In order to shorten the time for enriching CSCs and reduce the cost by using regular culture plate instead of the ULAP, we developed a core-shell microcapsule (CSMC) culture technology by encapsulating human prostate cancer (PC-3) cells in the miniaturized 3D liquid (rather than hydrogel) core of CSC medium enclosed in alginate hydrogel shell to facilitate the formation of prostaspheres (Figs. 1 and 2). Our study shows that the CSMC-40 condition with ~40 cells per microcapsule on day 0 gives the most prostaspheres on day 2 with an average size that is bigger than the conventional culture in ULAP for two days.

We tested the effectiveness of this CSMC culture approach for enriching CSCs first by *in vitro* studies (Figs. 3-6) on the expression of CSC surface receptor markers CD44 and CD133 (Figs. 3 and 4), side population that excludes nuclear staining dyes (Fig. 5), and

expression of pluripotency and epithelial differentiation genes and proteins (Fig. 6). Overall, the data in Figs. 3-6 show the dynamic nature of CSCs enriched in the prostaspheres using both the CSMC and conventional ULAP culture approaches. The expression of CSC surface receptor markers (CD44 and CD133) in prostaspheres is the lowest on day 6 for both the CSMC and conventional approaches and more importantly, it is the highest in day-2 prostaspheres obtained using the CSMC approach independent of the initial number of parent PC-3 cells per microcapsule (Fig. 4). Similarly, Fig. 6 shows that the expression of pluripotency genes (Oct 4, Nanog, and Klf4 in Fig. 6A) and protein (Nanog in Fig. 6B) is the highest while the expression of epithelial differentiation gene (CK14 in Fig. 6A) and protein (CK18 in Fig. 6B) is the lowest in the day-2 prostaspheres from CSMC-40 culture. The decrease in CD44⁺CD133⁺ subpopulation on day 6 might be because many CSCs differentiate into transit-amplifying cells (that are highly proliferative and express both the early and late epithelial differentiation cytokeratin markers [57-59]) in the prostasphere, which is supported by the expression of both early (CK14) and late (CK18) epithelial differentiation genes in the prostasphere cells on day 6 (Fig. 6A). The percentage of CD44⁺CD133⁺ subpopulation increases from day 6–10 but it is still lower than that on day 2, which is not completely consistent with the gene expression data from day 6–10 shown in Fig. 6A. This inconsistency is probably because the CD44⁺CD133⁺ subpopulation is only one of the CSC subpopulations, which is supported by the percentage data shown in Fig. 5 for the side population of cancer cells that exclude dye, are drug resistant, and have been well taken as one of the CSC sub-populations [20,40,41]. The percentage of the side population in prostaspheres obtained from the conventional ULAP culture increases from day 2 through day 10 but is constantly lower than that in prostaspheres obtained from the CSMC-40 culture. No significant change was observed for the latter from day 2 through day 10.

We further investigated the effectiveness of the CSMC culture approach for enriching CSCs by *in vivo* xenotransplantation studies, which confirmed the *in vitro* observations that prostaspheres obtained by the CSMC-40 culture on day 2 contain more CSCs than that obtained on days 2 and 10 from the conventional culture using ULAP. As shown in Fig. 7, a total of 3000 prostasphere cells from the CSMC-40 culture (for 2 days) generated significantly larger tumors when compared to tumors generated by the same number of parent PC-3 cells and prostasphere cells obtained from the conventional ULAP culture for 2 and 10 days. Since no apparent histological difference was observed in tumors from the four different groups, the larger tumor obtained for the day-2 CSMC-40 culture might be simply because there were more CSCs enriched in the prostasphere cells obtained with the CSMC-40 culture approach compared to the parent PC-3 cells and the prostasphere cells obtained using the conventional ULAP culture approach.

All these *in vitro* and *in vivo* data show that the miniaturized 3D culture in the liquid core enclosed in a hydrogel shell of the CSMCs is better than the open bulk culture in ULAP for enriching CSCs. This is possibly because the CSMC system resembles the physical configuration of pre-hatching stage embryos where stem cells from the totipotent to pluripotent stage are enclosed in a miniaturized permissive core to grow as an integrated aggregate by a hydrogel-like shell known as the zona pellucida, as illustrated in Fig. S4. In

other words, the miniaturized CSMC culture system mimics the physical niche that nature uses to maintain the stemness of stem cells from the totipotent to pluripotent stage, suggesting that the CSMC system is inductive to stemness. This is supported by the data in this study and a previous study showing that mouse embryonic stem cells cultured in the liquid core of microcapsules with a hydrogel shell have higher expression of several pluripotency genes compared to the conventional 2D open bulk culture [33]. This is also consistent with the report that forced growth of differentiated cells into 3D aggregates induces the differentiated cells to acquire stemness properties [60] because the miniaturized 3D culture in the CSMC core can certainly better force the encapsulated parent PC-3 cells to grow together to form 3D prostaspheres than the open bulk culture in ULAP (Fig. 2). Therefore, it might be able to better induce dedifferentiation of the encapsulated non-stem cancer cells into CSCs by acquiring stemness properties. In keeping with this theory, dedifferentiation of differentiated cancer cells due to an inductive microenvironment in addition to genetic mutation of normal stem cells has been recently proposed as an important possible origin of CSCs [51,54,57,58,61-63].

Lastly and importantly, although the biomimetic nature of the CSMC culture system is shown to be advantageous for maintaining stemness to enrich prostate CSCs, the exact mechanisms at work are still unclear. One possible mechanism is that the encapsulated cells may produce some autocrine molecules that are important for maintaining stemness and can be much better retained around the cells within the miniaturized core by the semi-permeable hydrogel shell compared to open bulk culture. If such crucial autocrine molecules do exist, it is possible that they affect the stemness of the encapsulated cells by regulating the signaling pathways that control the expression of Nanog, CK14, and CK18 rather than Oct4 and Klf4 genes and proteins, according to the data shown in Fig. 6. It is also possible that the mechanical cues provided by the permissive core (that could be remodeled by the encapsulated cells with time) and the hydrogel shell play a significant role to maintain and induce stemness in the encapsulated cells. Further studies are certainly warranted to identify the exact mechanisms by which the miniaturized biomimetic CSMC culture system maintains and/or induces stemness and to optimize the CSMC properties such as permeability and stiffness to achieve the best performance in these regards.

5. Conclusions

In summary, we successfully developed an approach of miniaturized 3D culture in CSMC for efficient and cost-effective enrichment of prostate CSCs. With this approach, the time required for obtaining the CSC-containing prostaspheres could be shortened from the conventional 10 days to only 2 days. Moreover, the prostaspheres obtained on day 2 using the new approach were found to have higher expression of stem cell surface receptor makers, contain more side population cells that exclude dye, and exhibit higher pluripotency than that obtained from conventional ULAP culture, which renders the prostasphere cells higher tumorigenicity *in vivo*. Furthermore, this CSMC technology can be used to produce thousands of cell-laden CSMCs in a matter of minutes and therefore thousands of prostaspheres highly enriched with CSCs in 2 days, which is highly desirable for high throughput and efficient drug screening. Therefore, the miniaturized 3D CSMC culture system developed in this study is valuable to facilitate research of CSC biology and promote

the identification of efficacious therapeutics against prostate cancer by eliminating the CSCs — the root of cancer resistance, recurrence, and metastasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A schematic illustration of conventional culture in open bulk cancer stem cell (CSC) medium in ultralow attachment plate (ULAP) versus the new miniaturized 3D culture in CSC medium in the core of core-shell microcapsules (CSMCs) showing the semi-closed nature of the CSMC culture as a result of the semipermeable hydrogel shell. (A) Conventional culture in ULAP. (B) Miniaturized 3D culture in CSMCs: the cell-laden CSMCs were produced by coaxial electrospray using coaxial needle in one step and transferred into regular 6-well plate for culture in CSC medium. The percentage of CSCs enriched in the resultant prostaspheres is very much dynamic (i.e., time dependent and lowest on day 6) and it is the highest in prostaspheres obtained from the CSMC culture for 2 days.

Fig. 2.

Growth of prostaspheres under the miniaturized 3D culture in CSMCs and conventional culture in ULAP with a total of 20,000 parent PC-3 cells on day 0 showing more efficient formation of prostaspheres with the CSMC than ULAP culture. (A) Typical micrographs showing the morphology and growth on 4 different days of prostaspheres formed in ULAP and in CSMCs with ~10 (CSMC-10) and 40 (CSMC-40) cells per microcapsule on day 0. (B) Quantitative data showing the growth of prostaspheres under the three different conditions. (C) Total number of prostaspheres (per 10,000 cells on day 0) formed on days 2 and 10 under CSMC culture with 2, 10, 40, and 70 cells per microcapsule on day 0 and the conventional ULAP culture. $*p < 0.05$.

Fig. 3.

Immunohistochemical stain of CD44 and CD133 in prostaspheres obtained from CSMC-40 culture showing an apparent decrease in CD133 expression on day 10 compared to day 2.

(A) Prostraspheres obtained on day 2. (B) Prostraspheres obtained on day 10. DIC: Differential interference contrast.

Fig. 4.

Quantitative flow cytometry analyses showing higher CD44 and CD133 expression in prostaspheres obtained on day 2 using the CSMC culture method than that obtained from conventional ULAP culture method on days 2, 6, and 10. (A) Typical two-channel flow cytometry data showing the distribution of cells stained with CD44 and CD133 and the quadrant (Q1) of CD44⁺CD133⁺ cells on days 2, 6, and 10 from prostaspheres obtained under CSMC-40 and the conventional ULAP culture. The gates for CD44 and CD133 were chosen to result in 1% CD44⁺CD133⁺ cells in the parent PC-3 cells (Fig. S2). (B) A summary of the percentage data of CD44⁺CD133⁺ cells in prostaspheres obtained from the conventional ULAP culture and the miniaturized 3D culture in CSMC with ~2, 10, 40, and 70 cells per microcapsule on day 0: The percentage of CD44⁺CD133⁺ cells is very much time dependent and is the lowest on day 6. The percentage of CD44⁺CD133⁺ cells in prostaspheres obtained on day 2 in CSMC is approximately 3 times higher than that in prostaspheres obtained on days 2 and 10 under the conventional ULAP culture. * $p < 0.05$ and ** $p < 0.01$.

Fig. 5. Side population analyses by flow cytometry of cells excluding Hoechst nuclear stain in prostaspheres showing side population is significantly higher in prostaspheres obtained using the CSMC-40 culture compared to the conventional ULAP culture. (A) Typical 2-channel flow cytometry data showing the distinct side population that excludes Hoechst 33342 stain in prostaspheres obtained on days 2, 6, and 10. (B) The corresponding quantitative data of the side population. * $p < 0.05$.

Fig. 6.

Gene and protein analyses of cells in prostaspheres obtained under both the CSMC-40 and conventional ULAP cultures (normalized to that of parent PC-3 cells) on different days showing higher expression of pluripotency genes and proteins in prostasphere cells from the CSMC-40 culture. (A) Real time RT-PCR data of three pluripotency genes (Klf4, Nanog, and Oct4) and two epithelial differentiation genes (CK14 for early or basal epithelial differentiation and CK18 for terminal or luminal epithelial differentiation). (B) Typical qualitative data showing expression of the three pluripotency protein markers and two epithelial differentiation markers. (C) A summary of the quantitative data of expression of four of the five (except CK14 for which it is 0) protein markers. * $p < 0.05$ and ** $p < 0.01$.

Fig. 7.

In vivo tumorigenicity of the prostatesphere cells obtained using CSMC-40 culture on day 2 versus parent PC-3 cells and prostatesphere cells obtained using the conventional ULAP culture on days 2 and 10 showing higher tumorigenicity of prostatesphere cells from the CSMC-40 culture. (A) Tumor volume as a function of time showing tumor growth. (B) Image of tumors harvested on day 52 showing their difference in size and shape. (C–E) Photomicrographs of hematoxylin and eosin stained tumors showing (C) confluent sheets of anaplastic cells with nucleomegaly, prominent nucleoli, and many mitotic figures, (D) areas of discohesive cells and thin-walled blood vessel, and (E) regions of necrosis, pathognomonic for malignancy. $**p < 0.01$.

Table 1

Tumorigenicity (assessed by tumor incidence on day 52 after implanting 3000 cells subcutaneously) of PC-3 cells and cells in PC-3 prostaspheres enriched using the CSMC-40 culture for 2 days and conventional ULAP culture for 2 and 10 days.

Cell type	PC-3 cells	ULAP prostasphere cells on day 10	ULAP prostasphere cells on day 2	CSMC-40 prostasphere cells on day 2
Incidence	3/5	5/5	4/5	5/5
Percentage	60%	100%	80%	100%