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Isolation of stem-like cells from 3D spheroid cultures

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Sept 23, 2019

Dear Dr. Myers,

According to the comments and suggestions from JOVE editorial team, I am submitting our REVISED JOVE methods manuscript entitled "Isolation of stem-like cells from 3D spheroid cultures". We described in step-by-step the detail protocol of a novel biomarker-free method of functional characterization of stem-like cells by a spheroid-based label-retention assay.

Thank you very much for the invitation and your kind consideration.

Sincerely

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TITLE:
Isolation of Stem-like Cells from 3-Dimensional Spheroid Cultures

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KEYWORDS:
stem cells, progenitor cells, cell isolation, spheroids, prostate, cancer specimens

SUMMARY:
Using human primary prostate epithelial cells, we report a novel biomarker-free method of functional characterization of stem-like cells by a spheroid-based label-retention assay. A step-by-step protocol is described for BrdU, CFSE, or Far Red 2D cell labeling; three-dimensional spheroid formation; label-retaining stem-like cell identification by immunocytochemistry; and isolation by FACS.

ABSTRACT:
Despite advances in adult stem cell research, identification and isolation of stem cells from tissue specimens remains a major challenge. While resident stem cells are relatively quiescent with niche restraints in adult tissues, they enter the cell cycle in anchor-free three-dimensional (3D) culture and undergo both symmetric and asymmetric cell division, giving rise to both stem and progenitor cells. The latter proliferate rapidly and are the major cell population at various stages of lineage commitment, forming heterogeneous spheroids. Using primary normal human prostate epithelial cells (HPrEC), a spheroid-based, label-retention assay was developed that permits the identification and functional isolation of the spheroid-initiating stem cells at a single cell resolution.

HPrEC or cell lines are two-dimensionally (2D) cultured with BrdU for 10 days to permit its incorporation into the DNA of all dividing cells, including self-renewing stem cells. Wash out commences upon transfer to the 3D culture for 5 days, during which stem cells self-renew

through asymmetric division and initiate spheroid formation. While relatively quiescent daughter stem cells retain BrdU-labeled parental DNA, the daughter progenitors rapidly proliferate, losing the BrdU label. BrdU can be substituted with CFSE or Far Red pro-dyes, which permit live stem cell isolation by FACS. Stem cell characteristics are confirmed by in vitro spheroid formation, in vivo tissue regeneration assays, and by documenting their symmetric/asymmetric cell divisions. The isolated label-retaining stem cells can be rigorously interrogated by downstream molecular and biologic studies, including RNA-seq, ChIP-seq, single cell capture, metabolic activity, proteome profiling, immunocytochemistry, organoid formation, and in vivo tissue regeneration. Importantly, this marker-free functional stem cell isolation approach identifies stem-like cells from fresh cancer specimens and cancer cell lines from multiple organs, suggesting wide applicability. It can be used to identify cancer stem-like cell biomarkers, screen pharmaceuticals targeting cancer stem-like cells, and discover novel therapeutic targets in cancers.

INTRODUCTION:

The human prostate gland contains luminal epithelium with secretory function and basal cells underlying it along with an unusual neuroendocrine cell component. The epithelial cells, in this case, are generated from a rare population of prostate stem cells that are relatively quiescent in vivo and act as a repair system to maintain glandular homeostasis throughout life[1]. Despite many advances, the identification and functional isolation of prostate stem cells remains a major challenge in the field. Stem cell biomarkers, including cell surface marker-based methodologies combined with flow cytometry are commonly used for stem cell research[2–4]. However, results for enrichment and isolation vary widely as a function of marker combinations and antibody specificity[5,6], raising questions about the identity of the isolated cells. Another widely used approach for stem-like cell enrichment is three-dimensional (3D) spheroid culture[2–4]. While resident stem cells are relatively quiescent in vivo with niche restraints, they undergo cell division in 3D matrix culture (both symmetric and asymmetric), generating both stem and progenitor cells that rapidly reproduce toward the lineage commitment[7,8]. The formed spheroids are a heterogeneous mixture containing both stem cells and progenitor cells at various stages of lineage commitment, including early and late stage progenitor cells. Thus, assays using the whole spheroids are not stem cell exclusive, making the identification of unique stem cell properties inconclusive. Therefore, it is critical to create assays to identify and separate prostate stem cells from their daughter progenitors. Towards this end, the goal of the current protocol is to establish an assay system that allows for the efficient identification and isolation of stem cells from human prostate tissues followed by robust downstream analysis of their biological functions.

Long-term 5-bromo-2'-deoxyuridine (BrdU) label-retention is widely used for in vivo and in vitro lineage tracing of stem cells based on their prolonged doubling time[9,10]. The current approach for prostate stem cell identification and isolation described herein is based on their relative quiescent characteristic and label-retention properties within a mixed epithelial population. Furthermore, based on the immortal strand DNA hypothesis, only stem cells can undergo asymmetric cell division. The stem cell represents the daughter cell that contains the older parental DNA while the progenitor cell, which is a committed daughter cell, receives the newly synthesized DNA. The unique stem cell property described above is exploited to perform BrDU labeling in parental stem cells in primary cultures and then track their label following BrdU-

washout upon transfer to 3D anchor-free spheroid culture. While the majority of primary prostate epithelial cells retain a basal and transit amplifying phenotype in 2D culture, there is also a rare population of multipotent stem cells replenishing and maintaining the epithelial homeostasis as evidenced by formation of spheroids or fully differentiated organoids with corresponding culture media upon transfer to 3D systems[3,12]. In our current protocol, by using HPrEC prostate spheroids or prostasphere-based BrdU, CFSE, or Far Red retention assays followed by fluorescence activated cell sorting (FACS) sorting, we identify label-retaining stem cells in spheroids at a single cell level[13].

Importantly, we further confirmed the stem cell characteristics of label-retaining cells within early-stage spheroids compared to the progenitor cells with lineage commitment. These include stem cell asymmetric division, in vitro spheroid formation ability and in vivo tissue regeneration capacity, elevated autophagy activity, augmented ribosome biogenesis and decreased metabolic activity. Subsequently, RNAseq analysis was performed. Differentially expressed genes in label-retaining spheroid cells were observed that may serve as novel biomarkers for human prostate stem cells. This spheroid-based label-retaining approach can apply to cancer specimens to similarly identify a small number of cancer stem-like cells, thus providing translational opportunities to manage the therapeutic resistant populations[13]. Presented below is the prostasphere-based label-retention assay using human primary prostate epithelial cells (HPrEC) cells as an example.

PROTOCOL:

All cell handling and media preparations should be performed with aseptic technique in a Class II biological safety cabinet (BSC).

1. Culture and maintenance of HPrEC Cells in 2D

1.1 Coat 100 mm culture dishes with 2 mL of 2.5 µg fibronectin solution overnight at room temperature (RT). Aspirate the solution and let the culture dishes air dry in the BSC for 45 min. Fibronectin-coated culture dishes can be stored 2–4 weeks at 4 ˚C.

1.2 Add 9 mL of the prostate epithelial cell growth medium (e.g., PrEGM) to a fibronectin-coated 100 mm culture dish and keep plates warm in a CO_2 incubator at 37 ˚C.

1.3 Thaw one vial of frozen HPrEC cells (1 x 10^5/mL) in a 37 ˚C water bath and resuspend the cells in 10 mL of warm medium.

1.4 Centrifuge the cell suspension at 500 x *g* for 5 min at RT. Aspirate and discard the supernatant.

1.5 Thoroughly resuspend the cells in 1 mL of the warm culture medium. Seed cells by pipetting 1 mL of the cell suspension directly into the fibronectin-coated 100 mm culture dish containing 9

mL of the prewarmed medium (total volume of medium and cells is 10 mL). Place culture dishes back into a CO_2 incubator at 37 ˚C.

1.6 Replenish the medium every 3 days. To do so, carefully aspirate all media and add 10 mL of fresh warm medium.

1.7 In about 5 days, ensure that the cultures are ~70–80% confluent. Perform the enzymatic digestion by incubating the cells with 3 mL of 0.05% Trypsin/EDTA at 37 ˚C for 5 min.

1.8 Stop the digestion by adding 3 mL of warm PBS containing 10% FBS.

1.9 Collect the cells into a 50 mL centrifuge tube and centrifuge at 500 x *g* for 5 min at RT. Aspirate and discard the supernatant.

1.10 Resuspend the cell pellet in 30 mL of warm medium and dispense it equally into three new fibronectin-coated 100 mm culture dishes for the next passage.

NOTE: Primary HPrEC cells can be passaged ~3–5x without altering their basal epithelial cell characteristics. Epithelial phenotype is tested by expressions of basal epithelial cell markers cytokeratin 5 and p63 with immunocytochemistry/immunofluorescent (ICC/IF) assays.

2. Labeling HPrEC with BrdU, CFSE, or Far Red pro-dyes

NOTE: The cells can proceed either to step 2.1 or step 2.2 followed by the transfer to 3D prostasphere culture as described in step 3.1.

2.1 Single labeling of cells with BrdU

2.1.1 Culture 1 x 10^5 HPrEC cells in fibronectin-coated 100 mm culture dishes with 10 mL of warm medium containing 1 µM of BrdU. Culture the cells for 10 days (over two passages) to ensure labeling of all cells including prostate stem and progenitor cells.

2.1.2 After 10 days, carefully aspirate the PrEGM medium containing the BrdU and wash the cells with 5 mL of warm PBS. Repeat 1x.

2.1.3 Perform the enzymatic digestion using 0.05% trypsin/EDTA as described in steps 1.7–1.8. Centrifuge the cells at 500 x *g* for 5 min at RT. Aspirate and discard the supernatant.

2.1.4 Resuspend the BrdU-labeled pelleted cells (5 x 10^4) in 500 µL of ice-cold (1:1) basement membrane matrix/medium and transfer to a 3D prostasphere culture (described in step 3.1) for 5 days to permit BrdU washout during the spheroid formation (~6 cell cycles).

2.2 Double labeling of cells with BrdU and CFSE or Far Red pro-dyes

2.2.1 If co-labeling for live cells is desired, co-label the BrdU-labeled cells from step 2.1 with 5 µM CFSE or Far Red live cell fluorescent pro-dyes for 30 min. Perform the co-labeling at Day 10.

2.2.2 Carefully aspirate the medium containing BrdU and CFSE or Far Red pro-dyes. Wash the cells with 5 mL of warm PBS. Repeat the wash 1x.

2.2.3 Perform enzymatic digestion using 0.05% trypsin/EDTA as described in steps 1.7–1.8. Centrifuge the cells at 500 x *g* for 5 min at RT. Remove and discard the supernatant.

2.2.4 Resuspend the co-labeled cells (5 x 10^4) in 500 µL of ice-cold (1:1) basement membrane matrix/medium and transfer to a 3D prostasphere culture (described in section 3.1) for 5 days to permit BrdU and CFSE or Far Red washout during the spheroid formation (~6 cell cycles).

2.3 Perform detection of label-retaining cells in spheres (Day 5 prostaspheres) as described in steps 4.1, 4.2, 4.3, or 5.

NOTE: Carboxyfluorescein diacetate, succinimidyl ester (CFSE), and Far Red are long-lasting fluorescent pro-dyes and are well retained within the labeled cells. Intracellular esterase in live cells cleave the acetate groups that activate the green or red fluorescent molecules that are now membrane impermeant.

3. Prostasphere formation in the 3D basement membrane culture system

3.1 Prostasphere culture in the 3D basement membrane matrix system

3.1.1 Thaw the basement membrane matrix at 4 ˚C overnight and keep on ice before use.

3.1.2 Gently add 1 mL of ice-cold basement membrane matrix into an equal volume of the ice-cold culture medium. Slowly mix by pipetting up and down, avoiding bubbles.

NOTE: Pre-coat the bottom of 12 well culture plate wells with 100 µL of this solution. This will minimize the number of cells that fall through the matrix and grow as a monolayer.

3.1.3 Resuspend 5 x 10^4 HPrEC cells in ice-cold (1:1) basement membrane matrix/culture medium mix in a total volume of 500 µL.

3.1.4 Pipette 500 µL of this cell solution at the bottom rim of each well.

3.1.5 Swirl the plate to evenly distribute the mixture around the rim of the well. Place the plate in a CO_2 incubator at 37 ˚C for 30 min to allow the matrix to solidify.

3.1.6 Once the matrix has solidified, cover with 1 mL of warm culture medium per well. Do not disturb the basement membrane matrix ring. Carefully aim the pipette tip and dispense the culture medium to the center of the well.

NOTE: The culture medium must be warmed to 37 ˚C when added to the solidified basement membrane matrix. The basement membrane matrix will lose its integrity and dissolve when exposed to cold/cool media.

3.1.7 Replenish medium every 3 days. Carefully aspirate ~500 µL of spent medium and add 500 µL of fresh warm culture medium.

3.1.8 Monitor the prostasphere formation and growth for 5–7 days using an inverted microscope.

3.2 Passaging of prostaspheres

3.2.1 Harvest prostaspheres from the matrix by carefully aspirating off as much media as possible. Avoid disturbing or picking up floating pieces of the solidified matrix.

3.2.2 Add 1 mL of dispase solution (basement membrane matrix:dispase at a 1:2 ratio). Mix thoroughly by pipetting up and down several times.

3.2.3 Incubate the culture plates in a CO_2 incubator at 37 ˚C for 30 min.

3.2.4. Take images of prostaspheres that are at the bottom of the culture dish for sphere number counting and size measurements.

3.2.5 Collect the sphere mixture into a 15 mL tube. Centrifuge the suspension at 500 x *g* for 5 min at RT to pellet the spheres. Aspirate and discard the supernatant.

3.2.6 Disperse the spheres into single cells by digesting with 500 µL of warm 0.05% trypsin/EDTA and transferring the suspension into a 1.5 mL microcentrifuge tube.

3.2.7 Incubate the microcentrifuge tube in a CO_2 incubator at 37 ˚C for 5 min.

3.2.8 Stop the trypsin action with 500 µL of warm PBS containing 10% FBS. Pass the sphere suspension through a 1 mL syringe with a 26 G needle 5x to dissociate the spheres into single cells.

3.2.9 Centrifuge the suspension at 500 x *g* for 5 min to pellet the cells at RT. Aspirate the supernatant and discard.

3.2.10 Resuspend the cell pellet in 1 mL of warm culture medium and filter through a 40 µm pore size nylon cell strainer.

3.2.11 Centrifuge the cell suspension at 500 x *g* for 5 min to pellet the cells at room temperature. Aspirate and discard the supernatant.

3.2.12 Resuspend the cell pellet in 1 mL of ice-cold (1:1) basement membrane matrix/culture medium and subculture in a 3D basement membrane matrix for the second passage of the prostasphere.

4. Identification of BrdU-retaining prostate stem cells by immunofluorescent staining

4.1 Outgrow the attached prostaspheres with immunofluorescent (IF) staining for prostate stem cell 2D imaging.

4.1.1 Harvest prostaspheres by dispase digestion as described in steps 3.2.1–3.2.3. Centrifuge the spheres in a 1.5 mL microcentrifuge tube at 500 x *g* for 5 min. Discard the supernatant.

4.1.2 Resuspend spheres in 1 mL of warm culture medium.

4.1.3 Incubate ~50 prostaspheres per well in 8 well chamber slides in 200 µL of culture medium in a 37 ˚C incubator overnight to allow for the attachment and outgrowth of spheres.

4.1.4 On Day 2, aspirate and discard the culture medium. Wash the outgrown spheres with 200 µL of PBS for 5 min.

4.1.5 Fix the spheres in 200 µL/well of ice-cold methanol at -20 ˚C for 20 min.

4.1.6 Wash the spheres with 200 µL/well of PBS for 5 min. Repeat 2x.

4.1.7 Acid wash spheres with 200 µL/well of 2N HCl for 30 min at RT.

4.1.8 Wash the spheres with 200 µL/well of PBS for 5 min. Repeat 3x.

4.1.9 Add 100 µL of blocking solution containing 5% normal goat serum in PBST (PBS with 0.25% Triton X-100) and incubate for 30 min at RT.

4.1.10 Aspirate off the blocking solution and add 100 µL of PBST containing 2% normal goat serum and primary mouse anti-human BrdU antibody (1:200) to each well and incubate in a humidified box at 4 ˚C overnight.

NOTE: Mouse IgG antibody is used as a negative control.

4.1.11 Aspirate and remove the primary antibody solution. Wash the spheres with 200 µL of PBS for 5 min. Repeat 2x.

4.1.12 Add 100 µL of PBST containing 2% normal goat serum and secondary goat anti-mouse Alexa Fluor 488 antibody (1: 500) to each well and incubate at room temperature for 2 h.

4.1.13 Aspirate and remove the secondary antibody solution. Wash the spheres with 200 µL of PBS for 5 min. Repeat 3x.

4.1.14 Mount the slides with ~25-40 µL of aqueous mounting medium containing DAPI.

4.1.15 Obtain images of the stained spheres/cells with a fluorescent microscope and a color digital camera.

4.2 Whole mount IF staining of prostaspheres for prostate stem cell 3D imaging

NOTE: For whole mount IF staining, prostaspheres are handled using 1.5 mL microcentrifuge tubes.

4.2.1 Harvest the prostaspheres by dispase digestion as described in steps 3.2.1–3.2.3. Centrifuge the spheres in a 1.5 mL microcentrifuge tube at 500 x *g* for 5 min. Aspirate the supernatant and discard.

4.2.2 Resuspend and fix the spheres with 1 mL of ice-cold methanol in a -20 ˚C freezer for 20 min. Centrifuge the spheres at 500 x *g* for 5 min. Aspirate the supernatant and discard.

4.2.3 Wash the spheres by resuspending the pellet in 1 mL of PBS. Centrifuge the spheres at 500 x *g* for 5 min. Aspirate the supernatant and discard. Repeat 1x.

4.2.4 Acid wash the spheres by resuspending the pellet in 1 mL of 2N HCl for 30 min.

4.2.5 Wash the spheres with 1 mL of PBS for 5 min and centrifuge at 500 x *g* for 5 min. Aspirate the supernatant and discard. Repeat 3x.

4.2.6 Resuspend ~15–30 spheres in 100 µL of blocking solution containing 5% normal goat serum in PBST (PBS with 0.25% Triton X-100) and incubate for 30 min at RT.

4.2.7 To remove the blocking solution, centrifuge the spheres at 500 x *g* for 5 min. Aspirate and discard the supernatant.

4.2.8 Resuspend the spheres in 100 µL of PBST containing 2% normal goat serum and primary mouse anti-human BrdU antibody (1:200) and incubate at 4 ˚C overnight.

4.2.9 Wash the spheres by resuspending the pellet in 1 mL of PBS. Centrifuge the spheres at 500 x *g* for 5 min. Aspirate the supernatant and discard. Repeat 1x.

4.2.10 Resuspend the spheres in 100 µL of PBST containing 2% normal goat serum and secondary goat anti-mouse Alexa Fluor 488 antibody (1:1,000) and incubate at RT for 2 h.

4.2.11 Wash the spheres by resuspending the pellet in 1 mL of PBS. Centrifuge the spheres at 500 x *g* for 5 min. Aspirate the supernatant and discard. Repeat 1x.

4.2.12 Resuspend the spheres in 30–50 µL of aqueous mounting medium containing DAPI. To prevent flattening the spheres, dispense them into a well created from a 35 mm uncoated culture dish with a cover glass bottom. Then add a coverslip to the culture dish, covering the well.

4.2.13 Acquire whole sphere Z-stack images using a transmitted light inverted fluorescent confocal microscope. Convert these Z-stack images into 3D images using an imaging software with freeform drawing capabilities.

NOTE: Mouse IgG antibody is used as a negative control.

4.3 Paired-cell analysis (4 day protocol)

4.3.1 Culture the BrdU-labeled spheres to Day 5.

4.3.2 Day 1: Harvest the spheres from a basement membrane matrix with 1 mL of dispase digestion. Disperse into single cells by 500 µL of warm 0.05% trypsin/EDTA in a 1.5 mL microcentrifuge tube as described in steps 3.2.1–3.2.11.

4.3.3 Resuspend the cells in 1 mL of warm culture medium.

4.3.4 Plate the dispersed single cells (~300–500 per well) in 8 well chamber slides and culture in 200 µL/well culture medium overnight to allow for attachment.

4.3.5 Day 2: Add 200 µL of 2 µM cytochalasin D in the culture medium and incubate overnight at 37 ˚C to permit one cell division that pauses at late metaphase to anaphase.

4.3.6 Day 3–4: Aspirate and discard the medium. Fix cells in 200 µL/well of ice-cold methanol at -20 ˚C for 20 min. Follow the immunostaining protocol for BrdU as described in steps 4.1.5–4.1.14.

4.3.7 Take images of paired cells with a fluorescence microscope. Ensure that the distance between the two nuclei is greater than 30 µm. Based on BrdU-retention, identify the paired stem cells as undergoing symmetric or asymmetric cell division.

NOTE: BrdU label-retaining prostate stem cells undergo symmetric division to give rise to two daughter stem cells retaining an equal amount of BrdU, whereas stem cells undergoing asymmetric division give rise to one daughter stem cell retaining all the BrdU and the other daughter progenitor cell which has lost the BrdU label.

5. Isolation of the CFSE label-retaining prostate stem cells by FACS sorting

5.1 Day 5: Harvest the CFSE-labeled prostaspheres growing in 6 well culture plates in 3D culture by replacing the medium with 2 mL of dispase (basement membrane matrix: dispase at a 1:2 ratio). Pipette up and down several times to mix thoroughly.

5.2 Incubate the plates in a CO_2 incubator at 37 ˚C for 30 min to digest the matrix.

5.3 Collect the sphere mixture into a 15 mL centrifuge tube. Centrifuge the spheres at 500 x *g* for 5 min at RT. Aspirate the supernatant and discard.

5.4 Resuspend the sphere pellet in 500 µL of warm 0.05% trypsin/EDTA, and transfer into a 1.5 mL microcentrifuge tube.

5.5 Incubate the spheres in a CO_2 incubator at 37 ˚C for 5 min. Add 500 µL of warm PBS containing 10% FBS.

5.6 Pass the spheres through a 1 mL syringe with a 26 G needle 5x to completely dissociate the spheres into single cells.

5.7 Centrifuge the cells at 500 x *g* for 5 min at RT. Aspirate and discard the supernatant.

5.8 Resuspend the cells in 1 mL of warm culture medium containing 1 µg/mL propidium iodide (PI) to stain dead cells. Incubate for 1 min at RT.

5.9 Centrifuge the cells at 500 x *g* for 5 min at RT. Aspirate and discard the supernatant.

5.10 Resuspend the cells in 1 mL of warm culture medium. Centrifuge the cells at 500 x *g* for 5 min at RT. Aspirate and discard the supernatant.

5.11 Resuspend the cells in 500 µL of warm culture medium and filter through a 40 µm pore size nylon strainer. Collect the cells in 5 mL polystyrene round bottom tubes with caps.

5.12 Perform the analysis of trypsin-dispersed CFSE-labeled prostasphere cells with a single-channel FACS analyzer.

5.13 Gate the subpopulations of fractionated $CFSE^{Hi}$, $CFSE^{Med}$, and $CFSE^{Lo}$ cells. Use the negative and positive controls for gating.

NOTE: The presence of FACS sorted CFSE label-retaining prostate stem cells (see Hu et al.[13]) can be confirmed by green fluorescence microscopy and their larger cell size compared to non-retaining cells. This larger cell size is a property of prostate stem cells relative to progenitor cell populations. This may be different with other tissue types.

REPRESENTATIVE RESULTS:

Primary normal human prostate epithelial cells are placed into fibronectin-coated culture dishes and cell growth is maintained in 2D culture (**Figure 1A**). Upon transfer into 3D culture with a basement membrane matrix, differentiated epithelial cells slowly die out. Only prostate stem cells can survive in an anchor-free culture and form spheroids in 5 days (**Figure 1B**).

Dual labeling of prostate epithelial cells in 2D culture followed by spheroid formation in 3D culture indicates the colocalization of BrdU, CFSE, and Far Red in the same label-retaining cells (**Figure 2A–I**).

Label-retaining cells show stem cell characteristics in Day 5 spheroids. Dual immunostaining shows that label-retaining cells exhibit lower levels of cytokeratin protein KRT14; decreased cell junction protein E-cadherin[14]; increased stem cell early marker proteins Wnt10B[13,15,16] and ALDH1A1; increased autophagy protein LC3, an indicator of autophagy flux activity[17]; and increased myosin IIB (**Figure 3A–F**).

A spheroid-based label-retention assay also successfully detects cancer stem-like cells in prostate cancer specimens (**Figure 4**). This will enable the discovery of true biomarkers for cancer stem-like cells and has the potential to identify novel therapeutic targets for prostate cancer.

FIGURE LEGENDS:

Figure 1: Maintenance of HPrEC in 2D culture and spheroid formation in 3D culture. (**A**) A 2D primary culture of HPrEC was BrdU-labeled and transferred to 3D culture with prostasphere formation on Day 5 (**B**). Scale bars = 400 μm.

Figure 2: Identification of long-term label-retaining cells in primary prostaspheres. Double labeling of BrdU (red) and CFSE (green); CFSE (green) and Far Red (red); BrdU (green) and Far Red (red) identified the same stem-like cells with retention of parental DNA. Any BrdU, CFSE, or Far Red labels in rapidly dividing progenitor cells (DAPI, blue) were diluted and lost (**A–I**). Representative images show BrdU/CFSE (**A–C**) (upper panel), CFSE/Far Red (**D–F**) (middle panel), and BrdU/Far Red (**G–I**) (lower panel) co-labeling in single prostasphere (PS) cells. Scale bars = 50 μm.

Figure 3: Label-retaining PS cells exhibiting stem cell properties. As compared to non-label-retaining progenitor cells, BrdU or CFSE label-retaining stem cells exhibit (**A**) lower levels of cytokeratin 14 (KRT 14), (**B**) decreased levels of E-cadherin, (**C**) elevated levels of Wnt10B, (**D**) higher levels of ALDH1A1, (**E**) increased LC3, and (**F**) increased myosin IIB proteins. Scale bars = 50 μm.

Figure 4: Using the sphere-based label-retaining assay for identification of cancer stem-like cells. CFSE label-retaining cancer stem-like cells in spheroids derived from human prostate cancer specimens exhibited reduced E-cadherin protein relative to the non-labeled progenitor cells. Scale bars = 50 μm.

DISCUSSION:

Flow cytometry using multiple stem cell surface markers is a commonly used approach for stem cell research despite lacking both specificity and selectivity[1,5,6]. While spheroid formation in a 3D culture system is another useful method in enriching the rare stem cell population from primary epithelial cells, including HPrEC, the resulting spheroids are still a heterogeneous mixture of stem and progenitor cells[2–4]. In prostaspheres, compared to the rapidly proliferating progenitor cells, the relatively quiescent character of stem cells allows them to be identified by long-term label-retention assay.

The methods summarized in this paper describe the labeling of HPrEC using BrdU, CFSE, and/or Far Red pro-dyes[13]. While BrdU, CFSE, and/or Far Red pro-dyes in rapidly proliferating cells are all quickly diluted by each cell division, there is an additional mechanism that accounts for the loss of BrdU known as immortal strand DNA segregation[11]. This is when the stem cell undergoes asymmetric division, giving rise to one daughter stem cell and a progenitor cell. The daughter stem cell retains all the old parental DNA with BrdU label, while the daughter progenitor cell receives the newly synthesized DNA without the BrdU label. Therefore, a label-retention assay using BrdU allows for clearer and easier identification of label-retaining stem cells by immunofluorescent staining. This is especially useful to confirm stem cell biomarkers by IF double staining using two different antibodies. One major limitation of BrdU labeling is that cells must be fixed for IF staining, thus preventing further functional studies following BrdU labeling and cell fixation.

To solve this issue, two fluorescent pro-dyes for live HPrEC labeling were tested in label-retaining assays. Results indicated that there was a complete overlap of BrdU, CFSE, and Far Red labeled sphere cells. BrdU label could be substituted with CFSE or Far Red in sphere-based label-retaining assays to allow visualization of live cells for imaging and separation by FACS, promoting functional characterization of isolated live stem cells using both in vitro cell culture assays and in vivo xenograft assays[13]. CFSE or Far Red label-based FACS sorted live stem and progenitor cells could also be used for RNA-seq including single cell RNA-seq, which is very powerful in identifying novel stem cell gene markers and signaling pathways as well as epithelial cell lineage hierarchy.

The novel biomarker-free method of functional characterization of stem cells by a spheroid-based label-retention assay presented here can identify cancer stem-like cells from primary patient specimens and cancer cell lines. Thus, it provides an avenue for discovering novel biomarkers of cancer stem-like cells and developing effective therapeutics targeting cancer[13].

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DISCLOSURES:
The authors do not have financial relationships to disclose.

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Figure 1

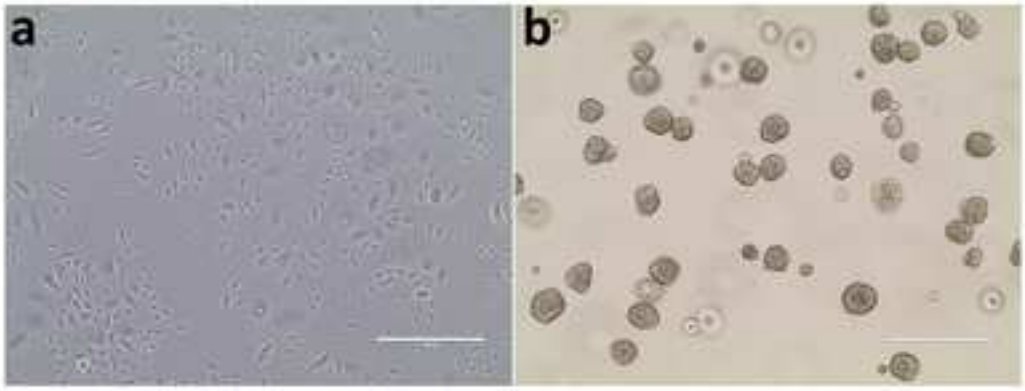


Figure 2

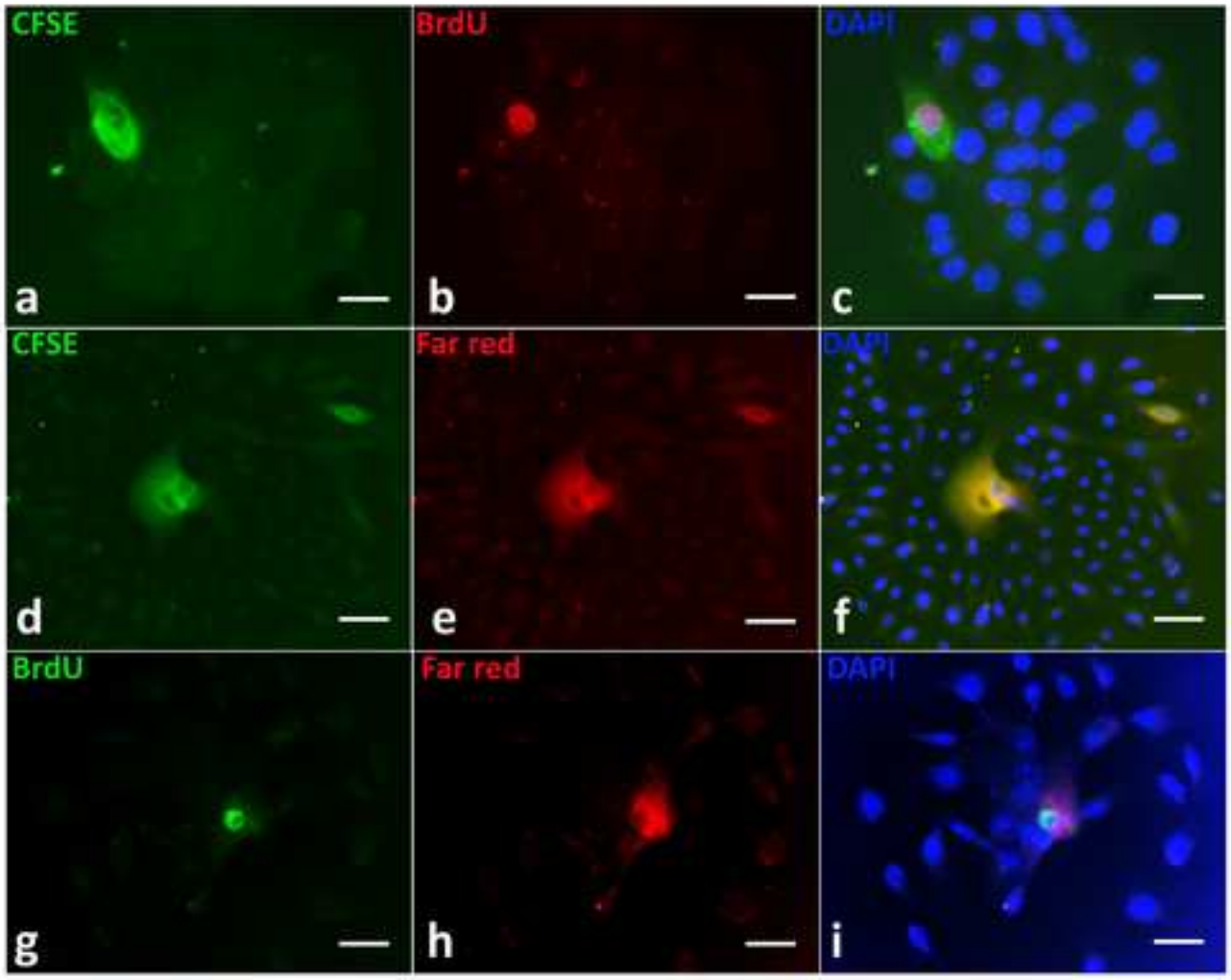


Figure 3

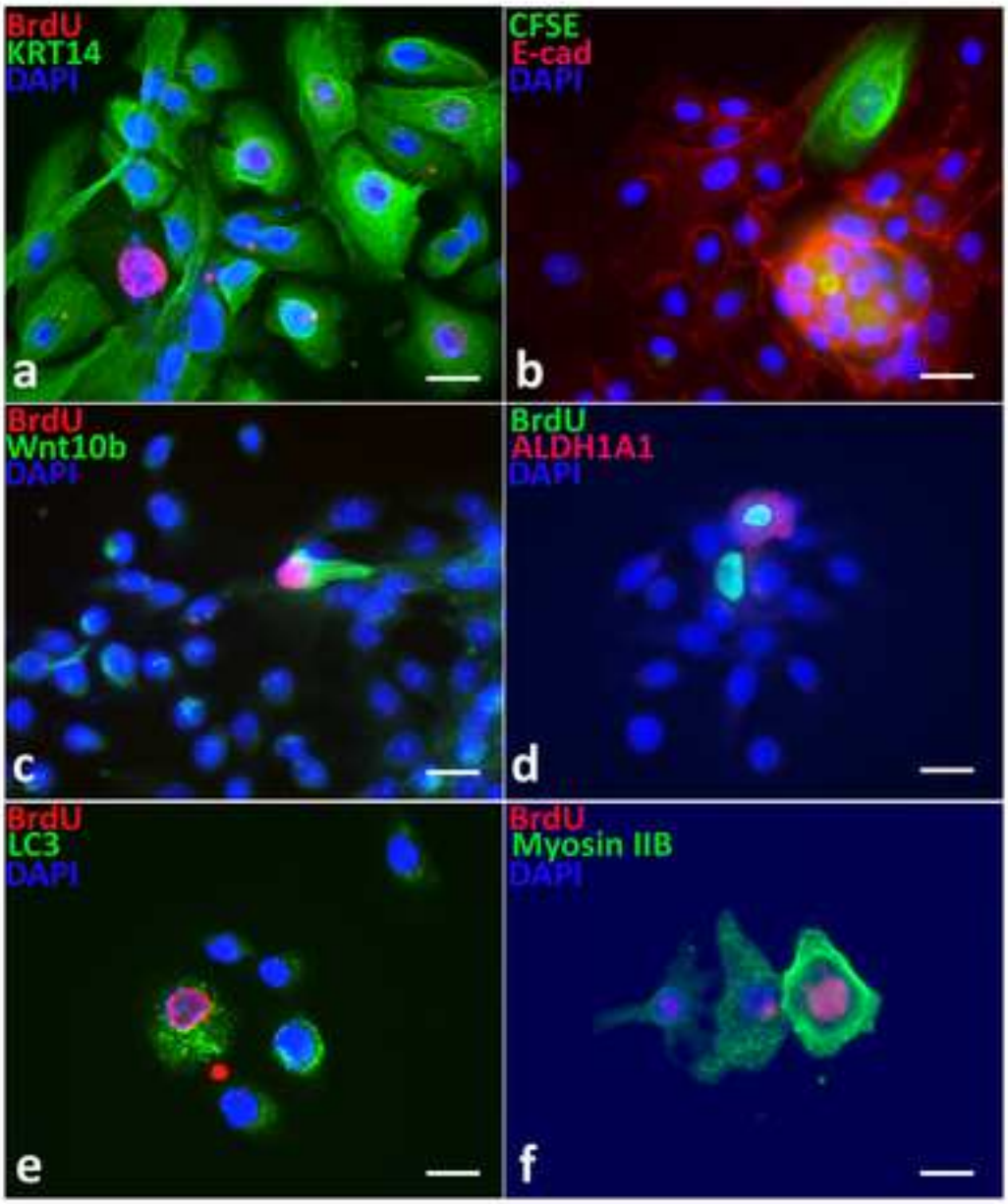
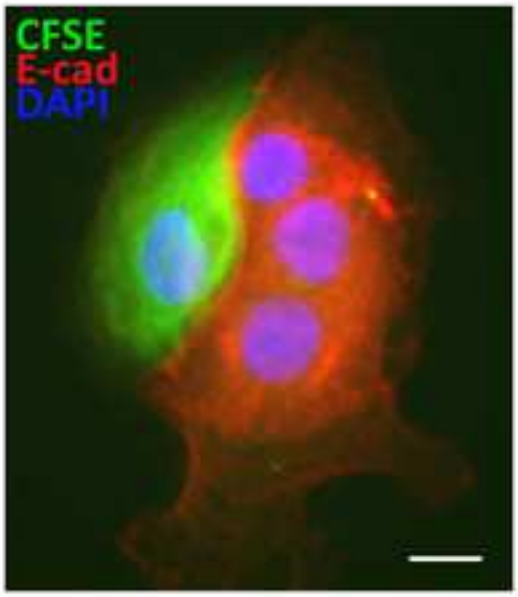


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.05% Trypsin-EDTA	Gibco	25300-054	
1 mL tuberculin syringes	Bectin Dickinson	BD 309625	
1.5 mL microcentrifuge tubes, sterile			
100 mm culture dishes	Corning/Falcon	353003	
12-well culture plate	Corning/Falcon	353043	
15 mL centrifuge tubes	Corning/Falcon	352097	
22 x 22 mm coverslips, sq	Corning	284522	For MatTek 35 mm culture dish
24 x 50 mm coverslips	Corning	2975245	
26G x 1.5 inch hypodermic needle	Monoject	1188826112	
2N HCl			
35 mm culture dish with cover glass bottom	MatTek Corp	P35G-0-10-C	Glass bottom No. 0, uncoated, ɣ irradiated
40 µm pore nylon cell strainer	Corning	352340	
5% CO_2 culture incubator, 37 °C	Forma		
50 mL centrifuge tubes	Corning/Falcon	352098	
5mL Polystyrene Round-Bottom Tube with cap	Corning	352058	
6-well culture plates	Corning	353046	
8-well chamber slides	Millipore Sigma	PEZGS0816	
Aqueous mounting medium containing DAPI	Vector Laboratories	H-1200	A nuclear fluorescent dye
Biological safety cabinet, Level 2 certified			

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
BrdU (5-bromo-2'-deoxyuridine)	Sigma-Aldrich	B5002	1 mM stock solution in DMSO
Centrifuge for 1.5 mL microcentrifuge tubes	Eppendorf		
Centrifuge for 15 mL tubes	Beckman Coulter		Allegra 6
CFSE (carboxyfluorescein succinimidyl ester)	Thermo Fisher Scientific	C34554	5 mM stock solution in DMSO
cytochalasin D	Thermo Fisher Scientific	PHZ1063	
Dispase 1U/mL	StemCell Technologies	07923	
FACS CellSorter MoFlo XDP	Beckman Coulter	s	
Far-Red pro-dye	Thermo Fisher	C34564	5 mM stock solution in DMSO
Fetal Bovine Serum (FBS)			
Fibronectin	Sigma-Aldrich	F0895	For coating 100 mm culture dishes
Fluorescent microscope with color digital camera	Carl Zeiss		Axioskop 20 fluorescent microscope; color digital Axiocamera
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher	A-11029	
HPrEC (Primary normal human prostate epithelial cells)	Lifeline Cell Technology	FC-0038	Pooled from 3 young (19-21yr od) disease-free organ donors; 1 x 10^5 cells/mL; stored in liquid
ice bucket and ice			
Inverted microsope with digital camera			
Matrigel, low growth factor, phenol-red free	Corning	356239	
Methanol	Corning	A452-4	
Mouse anti-BrdU antibody	Cell Signaling	5292S	
Mouse IgG antibody (negative control)	Santa Cruz Biotechnology	sc-2025	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Normal goat serum	Vector Laboratories	S-1000	
Phosphate Buffered Saline (PBS), pH 7.4	Sigma-Aldrich	P5368-10PAK	
Pipettors and tips, various sizes			
PrEGM (ProstaLife Epithelial Cell Growth Medium)	Lifeline Cell Technology	LL-0041	
Propidium Iodide (PI)	R & D Systems	5135/10	10 µg/mL PI in PBS stored at 4 °C in the dark
Serological pipets, various sizes			
Software for sphere counting and size measurements			
Software: 3D images using Imaris an imageing software with freeform drawing capabilities			
Triton X-100	Millipore Sigma	T8787	
Water bath, 37 °C			
z-stack images using a transmitted light inverted fluorescent confocal microscope			

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Author(s): Wen-Yang Hu and Gail S. Prins

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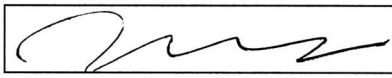
Signature:	
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Sept 23, 2019

Ref: **JOVE60357R2**

Dear Dr. Nguyen,

According to the comments and suggestions from JOVE editorial team, I am submitting our REVISED JOVE methods manuscript entitled "**Isolation of stem-like cells from 3D spheroid cultures**". Below are the point-by-point responses to the editor's and reviewers' comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Authors' response: Thanks for the comments. We have corrected all the spelling and grammar errors.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Lifeline, ProstaLife, Sigma-Aldrich, Matrigel, etc.

Authors' response: We removed all commercial language.

3. Please sort the Materials Table alphabetically by the name of the material.

Authors' response: We updated and sorted the Materials Table alphabetically by name of the material.

4. Please be consistent with the panel labeling: Figure 1 has capital letters and Figure 2/3 have lower case letters.

Authors' response: We changed the labels to lower case letters in Figure 1.

5. The preparation step listing the materials and equipment for each step (x.1) can be merged into the Table of Materials.

Authors' response: We merged the preparation steps into the Table of Materials.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Authors' response: We added more details to our protocol steps in the revised version.

7. Please specify all culture conditions.

Authors' response: We have specified all the culture conditions in the revised version.

8. 2.2.2: How is the dispersion done? Transfer with what?

Authors' response: We added the detail of cells dispersing and transferring in the revision.

9. 3.2: How thin is the thin layer of Matrigel?

Authors' response: A thin coating of matrigel of 100 µL per well in a 12-well culture plate.

10. Please specify what happens after centrifugation? Aspiration?

Authors' response: Correct, aspirate and remove the supernatant after centrifugation.

11. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Authors' response: We highlighted the essential steps of the protocol for the video recording.

12. Please do not abbreviate journal titles.

Authors' response: We spelled out all the words in the title.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Hu and Prins summarize an ex vivo approach to identifying label-retaining prostate epithelia using Brdu and CFSE in flow cytometry or IF in 2D culture followed by serial passaging of 3D spheroids. This is a valuable resource for the community.

Authors' response: Thank you for the positive comments.

Major Concerns:

no major concerns

Minor Concerns:

English grammar needs correction throughout.

Authors' response:

Thanks for the comments. We have corrected all the spelling and grammar errors.

Reviewer #2:

Manuscript Summary:

This article describes a biomarker-free method of functional characterization of stem-like cells by a spheroid-based label-retention assay with two fluorescent pro-dyes.

Authors' response: Thank you for the positive comments.

Major Concerns:

None

Minor Concerns:

Additional copy-editing would increase the readability of the manuscript. And why did the authors highlight some texts in yellow?

Authors' response:

Thanks for the comments. We have edited the text and corrected the spelling and grammars. The texts highlighted in yellow are essential steps of the protocol for video recording required by the JOVE.

Reviewer #3:

Manuscript Summary:

The manuscript by Hu and Prins describes a procedure of isolation of stem-like cells from 3D spheroid cultures. This assay may allow isolation of slowly stem/progenitor-like cells cycling cells which do not have a defined stem cell signature. Unfortunately, its applicability to study cancer stem cells is unclear, because such cells can be fast cycling in some cancers.

Major Concerns:

103- How many different batches of HPrECs cells were purchased and tested?

Authors' response: HPrECs from three healthy donors (cells of each donor are from one same batch) were purchased and pooled for the experiments tested in this study.

121- How long does it take to reach a 70-80% confluence at passage 1?

Authors' response: The 1st passage of HPrECs reaches 70-80% confluence in about 5 days after inoculation at a cell density of 3,000 cells/cm^2.

124- How are primary HPrEC tested for basal epithelial cell characteristics?

Authors' response: The phenotype of primary HPrEC cells are tested using CK5 and TP63 basal epithelial cell markers.

135- HPrECs are labeled with BrdU for 10 days. How was the label efficiency tested?

Authors' response: We and others have tested and confirmed that 10 days incubation time with BrdU allows nearly 100% label efficiency (Hu et al 2017).

138- What is the frequency of BrdU labeled HPrECs sphere-forming cells (SFCs) after 5 days in 3D?

Authors' response: The sphere-forming frequency in 3D culture is about 1~2% at day 5.

139- How was the Wash-out time of 5 days established?

Authors' response: We and others have shown differentiation and double layer formation of spheres after 7~10 days culture. Day 5 is an early stage of spheres containing prostate stem cells and early progenitor cells, which is an ideal stage to enrich and isolate prostate stem cells.

145- What is the frequency of double labeled HPrECs sphere-forming cells (SFCs) after 5 days in 3D?

Authors' response: The frequency of double labeled sphere-forming cells is about 38%.

149- How many prostaspheres develop outgrowths overnight?

Authors' response: All the spheres develop outgrowths after overnight attachment culture.

154- Input number of spheres dispersed to single cells for FACS? What is the percentage of CFSE labeled single cells collected by FACS compared to the total number of sorted cells?

Authors' response: Input ~50,000 dispersed single sphere cells for FACS sorting, ~1% of CFSE-Label retaining cells are collected (Hu et al, 2017).

157- Do the authors use a specific FACS sorting buffer which prevents bleaching out of far-red fluorescent pro-dyes?

Authors' response: PBS solution is used for FACS sorting.

227- What is the sub-culture ratio for the 2nd passage of Prostaspheres.

Authors' response: Sphere-forming efficiency at 2nd passage can be as high as 65% for CFSE-labeling retaining cells.

340- How many 6-well sphere cultures are harvested for one FACS experiment?

Authors' response: Spheres from one well of 6-well plate are harvested for FACS.

373- Has the larger cell size of human prostate stem cells been reported previously? If yes, please indicate the reference.

Authors' response: Yes, we have shown the larger size of prostate stem cells (Hu et al, 2017).

Minor Concerns:

413- (f) This image shows only 3 cells. Do authors have images with a higher number of cells?

Authors' response: Increased myosin IIB in label retaining cells was performed by paired cell assay. The three cells shown are daughters cells from one mother stem cell. One mother stem cell undergoes asymmetric cell division giving rise to one daughter stem cell (BrdU++/myosin IIB++) and another daughter progenitor cell, the daughter progenitor cell further divides into two daughter cells. Only label retaining cell assay of the whole sphere shows many cells in cluster.

Reviewer #4:

Manuscript Summary:

The submission uses normal human prostate epithelia (HPrEC cells) and chemical stains for Brdu, CFSE and immunostains for commonly used markers of poorly differentiated (CK14) and well differentiated epithelia (Ecad) Immunostains of markers associated with stemness (ALDHJ) and autophagy (LC3). The authors present these data in normal 2D + 3D cultures of HPrEC cells

and a single 3D picture of cells form a prostate cancer to suggest that positive Brdu staining offers a superior method to a slow cycling population of cells with stem cell features.

Major Concerns:

Brdu has been studied extensively in normal and cancerous epithelia from human and mouse prostate cancer models. It has been studied in the context of 2D and 3D prostate cultures extensively both in prostate and many other lineages. Despite this, the authors attempt to test that Brdu provides superior means to identify slow cycling cells having stem cell qualities. Several major issues are presented:

Authors' response: Thank you for your reviewer comments. BrdU labeling has been widely used for the tracking of slow cycling populations including long-term adult stem cells in both in vitro and in vivo models. Sphere-based BrdU label-retaining assay is a novel methodology established in our lab recently for identification and isolation of prostate stem cells (Hu et al, 2017).

(1) No functional studies were completed to show that brdu+ cells are more stem like compared to Brdu- cells (e.g. self-renewal studies, reconstitution studies),

Authors' response: We have shown the stem cell characteristics of BrdU label retaining prostasphere cells using functional studies including both in vitro and in vivo assays in our previous publication (Hu et al, 2017). JoVE journal only accepts methodology studies describing the detail of experimental protocols, therefore, we are not including any functional studies here.

(2) No validation studies were done to show that Brdu+ cells are similar to CFSE+ cells in terms of functional readouts of stemless,

Authors' response: We have demonstrated that BrdU label-retaining sphere cells overlap 100% with CFSE and Far Red label-retaining sphere cells and validated their stem cell characteristics using functional studies (Hu et al, 2017).

(3) No differentiation was made between CFSE-hi vs CFSE-mid vs CFSE-low stains in terms of stemless or label retention,

Authors' response: In our recent publication, we have shown that CFSE-hi prostasphere cells represent prostate stem cells and CFSE-med and CFSE-low are early and late stage progenitor

cells respectively (Hu et al, 2017).

(4) No comparison of Brdu is made with well surface antigen frequently used to isolated stem cells (e.g. CD49f, CD44.....) yet the authors are making this claim.

Authors' response: We have intensively studied prostate stem/progenitor cells using 3D cultured prostasheres over the past ten years (Hu et al, 2011, 2012, 2017, Prins et al 2014, 2015). We found that prostaspheres contain both prostate stem cells as well as early/late stage progenitor cells (Hu et al, 2011, 2012). We also found that prostasphere cells increasingly express several stem cell markers including Nanog, OCT4, CD49f, Trop2, ABCG2, as well as recently identified KRT13 and PRAC1. However, no single stem cell marker is able to identify prostate stem cells from their daughter progenitor cells in the prostaspheres. Our novel sphere-based BrdU label-retention assay is able to clearly identify prostate stem cells in the mixed progenitor population (Hu et al, 2017).

(5) The curious inclusion of a final image (Fig 4) of cells derived from human prostate cancer without any validation of cancerous origin or methodological context with the previous images making use of HPrEC cells. Again, no evidence of that the CFSE stained cells are actually "cancer stem-like cells"

Authors' response: Following the detail description of sphere-based label retention assay in identifying prostate stem cells in normal HPrEC cells, we found the assay can similarly identify cancer stem-like cells in primary culture of prostate cancer specimen. In addition, we have shown the labeling of cancer stem-like cells using several cancer cell lines. More detail of the study can be found in our previous publication (Hu et al, 2017).

Minor Concerns:

Problems with scale bars and image quality throughout. (1) The spheroids (B) appear only marginally large than some of the individual cells (A) yet the scale bar measure 400 uM for both panel. (2) several of the brdu+ cells many be dying or apoptotic (Fig 2+3, lower left).

Authors' response: The single individual cells are attached and spread out in 2D culture while spheres are anchor free in suspension 3D culture. Different sizes of spheres contain different number of cells from ~50 to ~100. The sphere cells are immunostained with different antibodies against specific marker proteins. For example Fig 3e shows the staining of LC3

which only light up the autophagosomes in the cytoplasm, although the whole morphology structure of the cells are not clear, all the cells are healthy.

Thank you very much for the invitation and your kind consideration.

Sincerely

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