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# Isolation and functional interrogation of adult human prostate epithelial stem cells at single cell resolution

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ABSTRACT

Using primary cultures of normal human prostate epithelial cells, we developed a novel prostasphere-based, label-retention assay that permits identification and isolation of stem cells at a single cell level. Their *bona fide* stem cell nature was corroborated using *in vitro* and *in vivo* regenerative assays and documentation of symmetric/asymmetric division. Robust WNT10B and KRT13 levels without E-cadherin or KRT14 staining distinguished individual stem cells from daughter progenitors in spheroids. Following FACS to isolate label-retaining stem cells from label-free progenitors, RNA-seq identified unique gene signatures for the separate populations which may serve as useful biomarkers. Knockdown of *KRT13* or *PRAC1* reduced sphere formation and symmetric self-renewal highlighting their role in stem cell maintenance. Pathways analysis identified ribosome biogenesis and membrane estrogen-receptor signaling enriched in stem cells with NF-κB signaling enriched in progenitors; activities that were biologically confirmed. Further, bioassays identified heightened autophagy flux and reduced metabolism in stem cells relative to progenitors. These approaches similarly identified stem-like cells from prostate cancer specimens and prostate, breast and colon cancer cell lines suggesting wide applicability. Together, the present studies isolate and identify unique characteristics of normal human prostate stem cells and uncover processes that maintain stem cell homeostasis in the prostate gland.



## 1. Introduction

The adult prostate gland contains a simple columnar epithelium composed of luminal secretory and underlying basal cells with a scarce neuroendocrine cell component. These epithelial cells are derived from a rare, relatively quiescent stem cell population that maintains glandular homeostasis throughout life (Leong et al., 2008). While prostate epithelial stem cells and their progeny have been investigated in rodent models and humans, their unique characteristics and lineage hierarchy remain a topic of debate. Credible evidence suggests that there may be a common precursor stem cell for all lineages while other findings support distinct basal and luminal stem cell populations within the adult prostate. These two scenarios are not mutually exclusive as emerging data indicates inherent plasticity and stage/context-specific utilization of stem and progenitor cell populations. In rodent models, prostate homeostasis appears to be maintained by both luminal and basal unipotent progenitor cells as well as bipotent stem/progenitor cells that exist in both compartments (Ousset et al., 2012; Toivanen et al., 2016; Wang et al., 2015; Wang et al., 2013; Xin et al., 2007). Although stem cell traits and lineage hierarchy for the human prostate epithelium are less studied, lineage tracing techniques using mitochondrial mutations have clearly demonstrated that basal, luminal and neuroendocrine cell lineages in the adult prostate are derived from a common precursor stem cell (Blackwood et al., 2011; Gaisa et al., 2011). Most current evidence from human prostate tissues suggests that normal stem cells primarily reside within the basal cell compartment (Goldstein et al., 2008; Zhang et al., 2016).

Advances in prostate cancer research have identified resident cancer stem-like cells that are intrinsically resistant to standard treatments and reseed tumor growth following ablative therapies (Chen et al., 2016; Collins et al., 2005; Yun et al., 2016). Furthermore, gene profiling analysis has shown that prostate cancer increases in a stem-like state as it progresses from organ-confined to metastatic disease (Smith et al., 2015). Consequently, it is imperative to develop therapeutic modalities that target the prostate cancer stem-like population for effective disease management. Although prostate cancer stem cells will be distinct from

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normal prostate stem cells (Chen et al., 2016), similarities could be capitalized on for therapeutic advantage. Thus a fundamental understanding of normal human prostate stem cell properties and the factors that modulate their self-renewal and lineage commitment may provide new insights into the origin and treatment of prostate cancer.

Approaches for isolating prostate stem cells have primarily utilized flow cytometry and 3D spheroid culture (Hu et al., 2011; Leong et al., 2008; Xin et al., 2007). However, a detailed characterization has been hindered by their lack of specificity and selectivity. Utilization of FACS with different antibodies against multiple surface antigens have yielded variable results (Collins et al., 2005; Vander Griend et al., 2008; Williamson et al., 2013), raising questions on the identity of the isolated cells. While resident stem cells are typically growth quiescent *in vivo*, when placed in 3D matrix culture without niche restraints, they undergo asymmetric division, generating progenitor cells that rapidly proliferate and lineage commit. Whereas the prostasphere (PS) culture system has been useful to enrich stem and progenitor cell populations, the resulting spheroids are a heterogeneous mixture of these cells types (Fig. 1C), making the identification of unique stem cell properties inconclusive. Clearly, improved assays to recognize and separate prostate stem cells are essential to move the field forward. Towards that end, the overall goal of the present study was to develop a system that permits clear identification and isolation of purified stem cells from human prostate specimens and conduct robust downstream analysis of their functional properties.

The approach for stem cell identification utilized herein is functional, based on the relative quiescence and thus label retention property of stem cells within a mixed epithelial population. Long-term 5-bromo-2′-deoxyuridine (BrdU) retention has been previously used to label stem cells *in vivo* and *in vitro* based on their prolonged doubling time (Cicalese et al., 2009; Klein and Simons, 2011). In addition, the immortal strand DNA hypothesis suggests that as stem cells undergo asymmetric division, the older parental DNA segregates into one daughter stem cell while the other daughter cell receives newly synthesized DNA and becomes a committed progenitor cell (Cairns, 1975). This unique situation allows the opportunity to BrdU-label DNA in parental stem cells within primary cultures and monitors their properties following BrdU-washout upon transfer to 3D spheroid culture. In the present studies, this pulse-chase approach was applied to primary prostate epithelial cultures derived from healthy organ donors, as opposed to benign regions from patient specimens, to ensure lack of a modifying disease field effect. While primary prostate epithelial cells adapt a basal and transit amplifying phenotype in 2D culture, they also contain the rare multipotent stem cells as evidenced by formation of fully differentiated organoids or differentiated spheroids upon transfer to 3D systems (Hu et al., 2011; Karthaus et al., 2014). By using PS-based BrdU/CFSE/Far red retention assays followed by FACS sorting, we herein identify label-retaining spheroid cells at a single cell resolution. Importantly, they exhibit stem cell characteristics including asymmetric cell division with segregation of parental DNA in daughter stem cells, *in vitro* serial

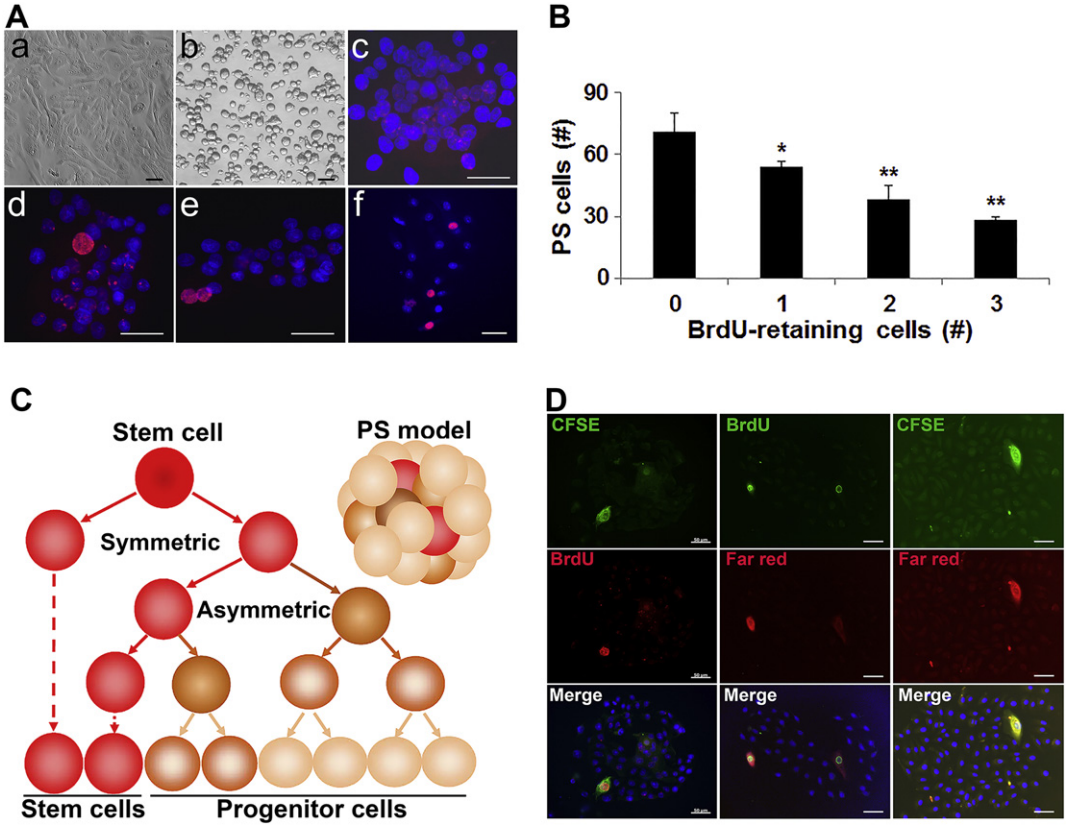


**Fig. 1.** Prostate stem cell identification by prostasphere-based label-retention assay. (A): 2D primary PrEC (a) were BrdU labeled and transferred to 3D culture with PS harvested on day 5 (b). BrdU immunostaining (pink) identified stem-like cells with retention of parental DNA (c-f). Any BrdU label in rapidly dividing progenitor cells (DAPI, blue) was diluted and lost (c-f). Representative images of whole PS with different numbers of BrdU$^+$ label-retaining cells (0-c, 1-d, 2-e, 3-f). (B): Graph shows an inverse relationship between BrdU$^+$ cells and PS cell number. *P < 0.05, **P < 0.01 *vs* zero BrdU$^+$ cell group. N = 12, 33, 29 and 12 for spheres with 0, 1, 2 and 3 BrdU$^+$ cells, respectively. (C): Proposed model for stem cell hierarchy as a PS is formed. In response to the stem cell niche, quiescent prostate stem cells (solid red) undergo symmetric self-renewal or asymmetric cell division. Symmetric self-renewal yields two daughter stem cells that can remain quiescent (left) or undergo asymmetric division (right). Asymmetric division generates one daughter stem cell (red) and one early stage progenitor cell (dark brown). As progenitor cells divide and lineage commit, they give rise to middle (partial brown) and late (light brown) stage progenitor cells. (D): Fluorescent pro-dyes CFSE and Far-red exclusively label BrdU-retenting PS cells. PrEC cells labeled with BrdU were treated with CFSE or Far-red and transferred to label-free PS culture. Day 5 PS stained for BrdU plus CFSE (green) or Far-red (red) showed signal co-localization upon fluorescence imaging. Representative images show BrdU/CFSE (left panel), BrdU/Far-red (middle panel) and CFSE/Far-red (right panel) co-labeling in a single PS cell. Scale bars = 50 μm.