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Epithelial cell-targeted transgene expression enables isolation of cyan fluorescent protein (CFP)-expressing prostate stem/progenitor cells

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Abstract

To establish a method for efficient and relatively easy isolation of a cell population containing epithelial prostate stem cells, we developed two transgenic mouse models, K5/CFP and K18/RFP. In these models, promoters of the cytokeratin 5 (*Krt5*) and the cytokeratin 18 (*Krt18*) genes regulate cyan and red fluorescent proteins (CFP and RFP), respectively. CFP and RFP reporter protein fluorescence allows for visualization of K5⁺ and K18⁺ epithelial cells within the cellular spatial context of the prostate gland and for their direct isolation by FACS. Using these models, it is possible to test directly the stem cell properties of prostate epithelial cell populations that are positively selected based on expression of cytoplasmic proteins, K5 and K18. After validating appropriate expression of the K5/CFP and K18/RFP transgenes in the developing and adult prostate, we demonstrate that a subset of CFP-expressing prostate cells exhibits stem cell proliferation potential and differentiation capabilities. Then, using prostate cells sorted from double transgenic mice (K5/CFP + K18/RFP), we compare RNA microarrays of sorted K5⁺K18⁺ basal and K5[−]K18⁺ luminal epithelial cells, and identify genes that are differentially expressed. Several genes that are over-expressed in K5⁺ cells have previously been identified as potential stem cell markers. These results suggest that FACS isolation of prostate cells from these mice based on combining reporter gene fluorescence with expression of potential stem cell surface marker proteins will yield populations of cells enriched for stem cells to a degree that has not been attained by using cell surface markers alone.

Keywords

Prostate; Adult stem/progenitor cells; Transgenic mice; Cytokeratins

Introduction

Normal prostate gland epithelium consists of two major epithelial cell types, basal cells and secretory luminal cells. The lineage relationship of these cell-types has long been of interest. Several years ago, a stem cell driven hierarchical mechanism for renewal of the epithelial cells of the prostate was postulated (Bonkhoff et al. 1994; Isaacs 1987). As this model has evolved, it is generally thought that a subset of the basal cells are the progenitor or stem cells of the prostate, and that these stem cells give rise to basal cells which in turn give rise to secretory luminal cells and neuroendocrine cells (De Marzo et al. 1998). Strong evidence for this model derives from the fact that androgen independent basal cells that populate the regressed prostate upon androgen withdrawal are capable of restoring the intact prostate cellular structure upon androgen re-administration. The fact that cells having properties of stem/progenitor cells have been isolated based on the expression of proteins expressed on the cell surface of basal cells, including $\alpha 2\beta 1^{\text{hi}}$, ScaI, CD49f, CD44, CD133, CD117, and Trop2, is additional support for the stem cell hierarchical mechanism for the development and maintenance of the prostate epithelium (Goldstein et al. 2008; Lawson et al. 2007; Leong et al. 2008; Richardson et al. 2004).

In contrast to these findings, a few studies suggest that basal cells are not essential for prostate regeneration, and a small population of luminal cells may have stem/progenitor cell properties. Urogenital sinus, the embryonic origin of the adult prostate, isolated from basal cell marker p63 knockout mice can develop into prostate tissue containing only luminal and neuroendocrine cells, but not basal cells, when grafted into adult male mice. The grafted basal cell-devoid prostate can regress and regenerate in response to changes in androgen, indicating that cells other than basal cells have stem/progenitor cell capabilities (Kurita et al. 2004). Cell kinetic studies following androgen-induced cell regeneration in the prostates of castrated rats suggest that basal and secretory cells derive from *two* independent lineages, thus providing further evidence that challenges the hierarchical stem cell theory (English et al. 1987; Evans and Chandler 1987). A more recent study showed that a sub-population of Nkx3.1-expressing luminal cells display stem/progenitor cell properties (Wang et al. 2009).

Given the unsettled state of our understanding of prostate stem cells and cell lineages and the potential role that stem cells may play in cancer initiation, the isolation and characterization of prostate stem/progenitor cells from the mouse prostate is of interest and has been the focus of recent studies. These studies have relied on the concurrent use of multiple antibodies to selected cell surface proteins for removal of non-epithelial and differentiated epithelial cells from prostate cell preparations by fluorescent activated cell sorting (FACS) (Goldstein et al. 2008; Lawson et al. 2007; Leong et al. 2008). While this approach has yielded cell populations that are highly enriched for stem/progenitor cells, technical limitations associated with antibody-based isolation have made it difficult to achieve pure stem/progenitor cell populations.

Aiming to improve upon our ability to isolate a pure population of prostate stem/progenitor cells, we have developed two transgenic mouse models, K5/CFP and K18/RFP, for efficient and easy isolation of epithelial basal cells and luminal cells, respectively. In K5/CFP mice, cyan fluorescent protein (CFP) is regulated by the promoter of the bovine cytokeratin 5 gene (*Krt5*), while in K18/RFP mice the promoter of the murine cytokeratin 18 gene (*Krt18*) regulates red fluorescent protein (RFP). Cytokeratin 5 (K5) and cytokeratin 18 (K18) are cytoplasmic proteins; K5 is a conventional marker for basal cells of the prostatic epithelium, while K18 is highly expressed in prostatic luminal cells. Fluorescence of CFP and RFP reporter proteins allows for visualization of K5⁺ and K18⁺ fluorescing cells within the cellular spatial context of the prostate gland and for their direct isolation by FACS.

In this study, we subject FACS-isolated epithelial prostate cell populations to a number of stringent immunostaining and functional biological tests and demonstrate that a subpopulation of CFP⁺ cells has properties consistent with the presence of K5-expressing stem cells in the basal compartment. A comparative analysis of the microarray gene activity profiles of CFP⁺RFP⁺ and CFP⁻RFP⁺ FACS-isolated cells identified several putative stem cell markers that are over-expressed in CFP-expressing cells as compared to CFP-non-expressing cells, adding further support for the presence of a stem/progenitor cell population in the basal compartment. These results suggest that FACS isolation of prostate cells from K5/CFP mice based on combining CFP fluorescence with potential stem cell surface marker protein expression will yield enriched stem cell populations to a degree that has not been possible to attain using cell surface markers alone.

Materials and methods

DNA constructs

pK5/CFP was constructed as previously described (Peng et al. 2007). pK18/mRFP was constructed using pCX/mRFP, a gift from Anna-Katerina Had-jantonakis (Memorial Sloan-Kettering Cancer Center, New York) and pK18iresEGFP, a gift from Robert G. Oshima (UCSD). Fragments of mRFP and IRES were amplified by PCR. The primers used in pK18/mRFP cloning are: IRES 5' GGTACCCCGCGGGCCCCTCTCCCTCCCCCCCC, IRES3' CTCGGAGGAGGCCATTATTATCATCGTGTGTTTTCAAAGGAAAACC, mRFP 5' TTGAAAAACACGATGATAATAATGGCCTCCTCCGAGGACCTCATC, and mRFP 3' NNGCGGCCGCTTTAGGCGCCGGTGGAGTGGCGGCC. IRES 3' end and mRFP 5' end were overlapped. The PCR product was mixed and run five cycles without primers to extend the overlapped part. Then, IRES 5' primer and mRFP 3' primer were added to amplify IRESmRFP fragment. The PCR fragment was cloned into SacII and NotI digested pK18iresEGFP to replace IRESEGFP.

Animals and preparation of prostate epithelial cells

K5/CFP transgenic mice were generated as previously described (Peng et al. 2007). Following the breeding of K5/CFP heterozygotes to C57BL/6 J mice (Jackson Laboratory) for more than 10 generations, heterozygotes were bred each other to generate a K5/CFP homozygous transgenic line. To generate K18/RFP transgenic mice, pK18/mRFP was digested with *Xba*I and *Ngo*MIV and the 12 kb transgene fragment containing K18 and the monomeric red fluorescent protein (mRFP) was purified and microinjected into nuclei of B6C3F2 fertilized mouse oocytes as described (Hogan et al. 1986). Adult mice used in immunostaining or cell sorting experiments were 8 weeks of age or older. Following dissection of prostates under a dissecting microscope, prostate cell suspensions were prepared as previously described (Sawicki and Rothman 2002).

Athymic nude mice and timed pregnant Sprague–Dawley rats used in renal transplantation experiments were purchased from Harlan Laboratories. FVB/NJ mice were purchased from The Jackson Laboratory.

All procedures using mice and rats were done in accordance with protocols approved by the Lankenau IACUC.

Cell sorting and analysis

Prostate cells were suspended in PCT prostate epithelium medium Cn-T52 (Millipore). Cell sorting was performed using a BD FACS AriaII cell sorter (BD Biosciences). Viable cells were identified by trypan blue exclusion.

Prostate colony-forming assay

1×10^5 irradiated Swiss 3T3 fibroblast cells were plated onto vitrogen-fibronectin (Cohesion Technology and Sigma) coated 24-well plates and cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) overnight. Two hundred to 1,000 sorted cells/well were seeded onto the 3T3 feeder layer and cultured in William's medium E (Invitrogen) plus supplement (Morris et al. 1990). After 7 days, colonies were viewed using a microscope and counted.

Prostate sphere-forming assay

Sorted cells were counted and mixed in 100 ml 1:1 Matrigel (BD Bioscience) and CnT-52 medium (Millipore). The mixture was placed into the center of a 35 mm petri dish (Falcon) 5–10 min until the mixture solidified, after which 3 ml CnT-52 medium was added. The cells were incubated at 37°C in 5% CO₂, balance air. After 7 days, spheres were counted.

Recombinant cell renal grafts

Urogenital sinus mesenchyme (UGM) cells used in tissue regeneration were obtained from day 18 pregnant SD rats. Urogenital sinuses (UGS) were collected from E18 embryos and digested in 1% trypsin at 4°C for 70 min. UGM were separated from UGS epithelia (UGE). UGM from 2 embryos were mixed with sorted prostate epithelial cells and resuspended in 20 ml of collagen Type I (BD Biosciences), which was pre-treated with NaOH in PBS according to the manufacturer's protocol. Upon gelation at room temperature, 3 ml William's medium was added. The cell mix was incubated overnight at 37°C in 5% CO₂, balance air. The next day, the cells were grafted beneath the kidney capsule of male athymic nude mice. After 8 weeks, mice were euthanized and the grafts were photographed in situ using a Nikon SMZ 1500 dissecting microscope with a Nikon digital camera DXM 1200F. They were then excised and processed for immunohistochemical analysis.

Castration experiment

Three-month old K5/CFP-K18/RFP double transgenic mice were castrated. Two weeks later, some mice were euthanized, their prostates were removed, and frozen sections prepared. Other mice received daily subcutaneous injections of testosterone [60 mg in 100 ml peanut oil (Sigma)] for 2 weeks, after which they were euthanized and frozen sections of their prostates were prepared. Sections were viewed under fluorescence using a Zeiss Axiovert 200 M microscope and images were taken with an AxioCam digital camera.

Immunohistochemistry and immunocytochemistry

Sorted cells were fixed for 10 min in 2% paraformaldehyde, washed with 1.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), then smeared on glass slides and air dried. Cells were permeabilized with 0.25% Triton-X100 in PBS for 10 min. Following quenching of endogenous peroxidase, cells were incubated with primary antibody at 4°C overnight, then with biotinylated secondary antibody for 30 min at room temperature, followed by streptavidin-CY3 for 20 min at room temperature. Images were taken using a Zeiss Axiovert 200 M microscope equipped with an AxioCam digital camera.

K5/CFP prostate tissue and recombinant cell grafts were fixed in formalin (Fisher) for 2 h and processed for paraffin embedding. Four micrometer sections were deparaffinized, and antigen retrieval was performed by steam heating 30 min followed by endogenous peroxidase quenching with 3% H₂O₂/methanol for 20 min. K18/RFP prostate tissue was fixed in 4% paraformaldehyde (Electron Microscopy Science) for 5 min and then embedded in O.C.T. compound (Tissue-Tek). Four micrometer sections were treated with 0.1% pepsin in 0.01 N HCl at room temperature for 5 min, followed by endogenous peroxidase

quenching with 0.3% H₂O₂/PBS for 10 min. Both paraffin and frozen sections were incubated with primary antibody at 4°C overnight, biotinylated secondary antibody for 30 min at room temperature, and streptavidin-HRP for 20 min at room temperature. Signals were amplified and visualized using the TSA-Plus Fluorescence System (Perkin Elmer) according to manufacturer's instructions. Images were taken using a Zeiss Axiovert 200 M microscope or a Zeiss LSM510 META confocal fluorescent microscope.

The antibodies used in immunostaining were as follows: K5 (Covance), K8 (Covance), CFP (Clontech, EGFP antibody), AR (C-19) (sc-815, Santa Cruz), synaptophysin (clone sy38, Millipore), and CD49f (BD Biosciences).

Microarray

Total RNA from sorted CFP⁺ and CFP⁻ epithelial cells was prepared using a PicoPure RNA Isolation Kit (Arcturus) according to the manufacturer's protocol. Samples were sent to Phalanx Biotech Group (CA) for amplification and OneArray™ Microarray service, including single color labeling, three replications, hybridizations, scanning, signal extractions, and basic data analysis. The mouse OneArray™ consists of 29,922 mouse genome probes and 1,880 experimental control probes (see <http://www.phalanxbiotech.com> for more information). Genes that were differentially expressed between the CFP⁺ and CFP⁻ cell populations were evaluated for enriched biological pathways and gene ontology terms using Ingenuity Pathway Analysis and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) at <http://david.abcc.ncifcrf.gov>. DAVID was also used to perform functional clustering which organizes genes into groups containing similar functional descriptions. Common themes among the highlighted functional categories are growth and proliferation, DNA replication, cell signaling and adhesion, and cancer pathways.

Single cell one-step RT-PCR

One-step RT-PCR of single cells was performed using the AmpliGrid system (Advalytix, Beckman Coulter) according to the manufacturer's protocol. In brief, cells were FACS-sorted directly onto an AmpliGrid slide, and then air dried. Following deposition of 0.5 µl of cell extraction reaction mix on each cell, 5 µl of sealing solution was added to each sample. The AmpliGrid slide was placed on the AmpliSpeed slide cycler and heated (5 min @ 75°C, 2 min @ 95°C). 1 µl of RT-PCR master mix, made from AmpliGrid Single Cell RT-PCR Kit, was added to each sample through the sealing solution, and the amplification program was run for 40 cycles using an annealing temperature of 64°C. PCR results were assessed on FlashGels (Lonza). The primers used for K5 and K18 were: K5 5' TGCCCTGATGGACGAGATCAACTT, K5 3' GTTGGCACACTGCTTCTTGACGTT, K18 5' AGTATGAAGCGCTGGCTCAGAAGA, K18 3' TCCAGGGCATCGTTGAGACTGAAA.

Statistics

Statistical significance of differences in the ability of different cell populations to form clones was determined using the unpaired two-tailed student *t* test.

Results

Transgenic model validation

We observed CFP and RFP fluorescence in the different lobes of the adult prostate and in the developing prostate of K5/CFP transgenic mice and of K5/CFP-K18/RFP double transgenic mice (Fig. 1). Fluorescent cells were localized to the epithelium of the adult prostate and to the urogenital epithelium lining the urogenital sinus in fetuses at day 16 and

18 of gestation. The spatial distribution of CFP and RFP fluorescence in both adult prostate and embryonic urogenital sinus (UGS) epithelium is consistent with the expected higher expression of CFP in K5⁺ basal cells and RFP in K18⁺ luminal cells. At day 18, CFP⁺ cells and weakly positive RFP-expressing cells were also observed in prostatic buds, the first well-defined structures of the developing prostate. In the immature prostate of 2-week old mice, both CFP and RFP expressing cells were observed in the epithelium of developing ascini. CFP-expressing cells were present in the epithelium of all organs known to express K5 that we examined in adult K5/CFP (Fig. S1) K18/RFP mice (Fig. S2). The skin of newborn transgenic pups fluoresced brightly under UV illumination making them easy to identify (Fig. S3). In contrast to RFP expression in the skin of adult mice, many cells in the hair follicles of newborn mice express RFP (data not shown), thus accounting for the observed strong red fluorescence of perinatal skin.

The usefulness of the K5/CFP and K18/RFP transgenic models is dependent upon the correct expression of each of the transgenes; that is, the cells that express CFP and RFP must be those cells that express K5 and K18, respectively. To validate transgene expression, we have used conventional fluorescent microscopy as well as confocal microscopy to examine immunostained sections of prostates. Following dual immunostaining for K5 and CFP of sections prepared from adult prostate, using conventional fluorescent microscopy, we observed that nearly all CFP-expressing cells also expressed K5 (Fig. 2a). Z-stack analyses of confocal images of dual-stained sections confirmed this co-incident expression and highlight the fact that images taken at a single plane, as in conventional fluorescent microscopy, are likely to under-represent cells that express both K5 and CFP (Fig. 2b and Fig. S4a). In other analyses, we confirmed co-incidence between K5 immunostained cells and cells that display CFP fluorescence (Fig. S5a). We also determined an exact correlation between those cells that immunostained positive for CFP and cells that displayed CFP fluorescence (Fig. S5b). To validate appropriate expression of RFP in prostates of K18/RFP mice, we confirmed that RFP fluorescence is observed in luminal cells that display high nuclear expression of the androgen receptor (AR) as detected in immunostained sections (Fig. 2c). This approach was necessitated by the fact that the K18/RFP transgene contains the entire coding sequence for human K18, and an antibody that can distinguish native mouse K18 and K18 transgene expression does not exist.

To further validate the models, we immunostained FACS-sorted prostate cells from adult K5/CFP mice. One-hundred percent (40/40) of CFP-expressing cells also expressed K5 (Fig. S6a). Nearly 100% (157/170, 92 ± 1.8%) of CFP-expressing cells also expressed CD49f, a cell-surface protein used to enrich for stem cells (Lawson et al. 2007) (Fig. S6b). No K5 or CD49f staining was observed in the CFP⁻ population.

Castration of double transgenic K5/CFP + K18/RFP mice provided even further evidence of appropriate expression of both transgenes (Fig. 3). Upon castration of these mice, the prostate involuted. While the number of CFP⁺ prostate cells in the involuted prostate remained the same as in the prostate of intact non-castrated mice, large RFP⁺ cells were no longer present in acini. Upon administration of testosterone to castrated males, the prostate returns to its normal size and large RFP⁺ cells were again evident in acini. These observations are expected if RFP⁺ cells are androgen-dependent luminal cells and CFP⁺ cells are androgen-independent basal cells.

Stem cell properties of CFP-expressing prostate cells

Proliferative potential—One of the defining characteristics of stem cells is their high proliferative potential when placed in culture. We FACS-sorted cells from the adult prostate of K5/CFP mice and placed them in culture with irradiated Swiss 3T3 feeder cells to test their ability to form clones (200–1,000 cells each of CFP⁺ and CFP⁻ cells). Four to 8% of

CFP⁺ cells had the ability to form clones as compared to 0.03–0.06% of CFP[−] cells. The amount of CFP expressed by cells in all clones was variable (Fig. 4a). When unsorted cells were plated, only 0.08% of them formed clones. Cells in clones derived from CFP⁺ sorted cells continue to express CFP even after dissociation and replating (Fig. 4b). These results demonstrate that FACS, based on the expression of K5, a cytoplasmic protein, can be used to isolate a prostate cell population having clonal potential that is characteristic of stem cells.

We also tested whether additional sorting of CFP⁺ cells for expression of three potential prostate stem cell markers CD133 (Richardson et al. 2004), CD117 (Richardson et al. 2004), and aldehyde dehydrogenase (ALDH) (Ginestier et al. 2007; Huang et al. 2009; Powsic et al. 2007) would have an effect on clonogenicity. Populations of CFP⁺ CD133⁺ or [−], CFP⁺ CD117⁺ or [−], and CFP⁺ ALDH⁺ or [−] cells were sorted, placed in culture, and the resulting clones were counted (Fig. 4c). In every case, cells that expressed both CFP and the potential stem cell marker gave rise to significantly more clones than cells that only expressed CFP [1.7-fold increase for CD133⁺ ($P = 0.0003$), 1.7-fold increase for CD117⁺ ($P = 0.0026$), and 3.9-fold increase for ALDH⁺ ($P < 0.0001$)]. This result provides additional evidence that a subset of K5-expressing prostate cells is stem/progenitor cells.

To further evaluate proliferative potential, we tested the ability of CFP⁺ and CFP[−] cells FACS-isolated from the prostates of K5/CFP mice to form spheroid structures when grown in a Matrigel matrix. Approximately 25% of CFP⁺ cells formed spheroids after a week in culture, whereas only ~0.2% of CFP[−] cells had this ability. When spheroids derived from CFP⁺ cells were dissociated into single cells, 20–35% of the cells formed spheroids upon replating, the percent varying with the number of cells plated/well. Thus, based on the results of two assays, clonal growth and the ability to form spheroids, we conclude that a subset of CFP⁺ cells has a much greater proliferative potential than do CFP[−] cells.

Many, but not all, cells in spheroids derived from CFP⁺ cells expressed CFP (Fig. 4d). To explore the identity of the CFP[−] cells in spheroids, we tested CFP⁺ cells isolated from the prostates of double transgenic K5/CFP + K18/RFP mice in the spheroid assay. Spheroids formed in the usual way, and individual cells appeared to be either CFP⁺RFP[−] (blue) or CFP[−]RFP⁺ (red) when observed under fluorescent light. Interestingly, with increased time in culture, the spheres grew larger and the CFP[−]RFP⁺ cells became centrally located, while the CFP-expressing cells were localized to the periphery of spheres (Fig. 4e). The spatial distribution of these cells in acini in spheroids appears to recapitulate the spatial distribution of cells in the acini in the adult prostate, and suggests that CFP⁺ cells have differentiated and become luminal cells.

Differentiation potential—The ability of a single cell or a defined population of cells to proliferate and differentiate into multiple cell types and give rise to an organized structure that resembles the cellular structure of the adult organ is perhaps the most stringent test to evaluate stem cell function. We used the renal capsule recombinant cell transplantation assay (Cunha et al. 1987) to test the ability of FACS-isolated CFP⁺ cells from K5/CFP adult mouse prostate to differentiate into adult prostate structures. Urogenital mesenchymal (UGM) cells from fetal rat were combined with CFP⁺ or CFP[−] mouse prostate cells and grafted beneath the kidney capsule of nude mice. Control cell transplants were prepared with rat UGM only. Only (CFP⁺ + UGM) cell transplants gave rise to tissues (7/10 transplants). Differential Hoechst staining of rat and mouse cells in paraffin sections of the resulting recombinant tissues confirmed the presence of murine cells in structures that histologically resemble prostate gland (Fig. S7). Both K5⁺ and K8⁺ cells were present in these structures as detected by dual immunostaining using antibodies to K5 and K8 (Fig. 5a, b). K5⁺ cells appeared to reside in the basal layer of prostatic acini epithelial structures, while the K8⁺ cells resided on the luminal aspect and had a columnar shape typical of luminal cells.

Additional immunostaining of paraffin sections using a GFP antibody that recognizes CFP confirmed that CFP-expressing cells reside in the basal epithelium (Fig. 5c, d), and that CFP co-localizes with K5 (Fig. 5e, f). Dual immunostaining of sections using the GFP antibody to detect CFP and an antibody to the androgen receptor (AR), a marker strongly expressed in the nucleus of luminal cells, showed that CFP-expressing cells had low level AR expression, and CFP⁻ cells had high AR expression (Fig. 5g, h). This interpretation was confirmed by the dual staining of cells for cytoplasmic K8 and nuclear AR, thus providing further evidence that K8⁺ cells are luminal cells (Fig. 5i, j). The identification of synaptophysin-expressing, K5⁻ cells is evidence that the grafted CFP⁺ cells have the ability to differentiate into neuronal cells as well (Fig. 6a). Colocalization of p63 and K5 as shown by immunostaining (Fig. 6b) is further evidence that all three cell types in the prostatic epithelium (basal, luminal, and neuroendocrine) are present in grafts derived from CFP⁺ cells. Taken together, the analysis of recombinant cell implants demonstrates that FACS-isolated K5-expressing cells from the prostate of K5/CFP transgenic mice have the ability to differentiate into luminal K8- and AR-expressing cells and synaptophysin-expressing neuroendocrine cells that no longer express K5.

Basal cells express K5 and K18—FACS analysis of cells prepared from the prostates of adult double transgenic K5/CFP + K18/RFP mice indicates that >98% of all K5 cells also express and K18 (Fig. 7a). This result was somewhat surprising since K8 and K18 are generally accepted as markers for luminal cells. To investigate this observation further, we performed RT-PCR on cDNAs prepared single prostate cells from the double transgenic mice using primer pairs for specific amplification of K5 and K18 (Fig. 7b). All cells that were subject to RT-PCR expressed CFP and were therefore K5⁺. However, because of the technical limitations of this assay, only 31% of the cells yielded a positive PCR result for K5 (Table 1). While some single cells tested positive for both K5 and K18, a K18 PCR product was only observed in 28% of the cells. If one assumes that the rate of false negatives is the same for K5 and K18, it is reasonable to conclude that most K5⁺ cells also express K18.

In complementary experiments, we also applied an enhanced immunostaining technique to sections of fetal urogenital sinus and the prostate of adult nontransgenic mice to study the co-localization of K5 and K8 expression. Using conventional fluorescent microscopy to examine the stained sections, we observed co-localization of K5 and K8 expression in many cells in both fetal and adult prostates (Fig. 7c, d), thus adding further support for co-expression of K5 and K8/18 in basal cells in the prostate epithelium.

As we observed in renal transplantation assays of K5⁺ cells from K5/CFP mice, following renal capsule recombinant cell transplantation using FACS-isolated K5⁺K18⁺ prostate cells from double transgenic mice, tissue growths developed that had fully differentiated prostate structures as determined histologically and by immunostaining with antibodies to selected cell lineages (Fig. S8).

Expression profiles of CFP⁺ and CFP⁻ prostate epithelial cells

We used FACS to isolate CFP⁺RFP⁺ and CFP⁻RFP⁺ prostate epithelial cells from double transgenic K5/CFP + K18/RFP mice, then prepared mRNAs and cDNAs from each population and subjected the cDNAs to microarray analysis. As noted above, all CFP⁺ basal cells in double transgenic mice also express RFP. Use of the double transgenic mice in this study allowed for isolation of CFP⁻ cells and ensured that they were luminal epithelial cells because they were also RFP⁺, and not CFP⁻RFP⁻ stromal or neuroendocrine cells. Each cDNA was tested in triplicate against each of the gene probes. We filtered the data and identified genes that were differentially expressed between CFP⁺ and CFP⁻ cells using a *t* test ($P \leq 0.05$). Differentially expressed genes having ≥ 4 -fold increase or decrease in CFP⁺

cells relative to CFP⁻ cells are listed in Tables S1 and S2, respectively. Cytokeratin 5 (*Krt5*) had the second highest median ratio (CPF⁺/CFP⁻), 4.96, thus further validating the appropriate expression of CFP in K5/CFP transgenic mice. Genes that have been identified in the literature as potential stem cell markers that are expressed at higher levels in CFP⁺ cells as compared to CFP⁻ cells are listed in Table 2. Since stem/progenitor cells are a subset of the CFP⁺ cells, the fact that some of these potential stem cell markers have a high median ratio suggests that their expression in stem cells is quite robust.

Functional analysis was performed to provide more information for future study. An adjusted p value limit of 1×10^{-5} and 2-fold change cut off was used to identify 1,280 differentially expressed genes for functional analysis. The functional clustering of genes having prostate epithelial-related functions are listed in Table S3. Genes in prostate-related KEGG pathways are listed in Table S4.

Discussion

In this study, we developed two transgenic mouse models, K5/CFP and K18/RFP, in which promoter sequences of the *Krt5* and *Krt18* genes regulate activity of cyan and red fluorescent proteins, respectively. We extensively validate the appropriate expression of the transgenes in the embryonic and adult prostate, and then use cell-specific cytoplasmic expression of CFP in K5-expressing cells to isolate cells of the basal epithelium of the mouse prostate, a population that is generally believed to contain prostate stem/progenitor cells. While there is considerable evidence that prostate stem cells reside in the basal compartment, a cell population for which K5 has become a standard marker, this is the first study in which the stem cell properties of a population of prostate epithelial cells is isolated on the basis of which cytokeratins they express. In attempts to identify and purify prostate stem cells, others have sorted cell suspensions directly with antibodies having reactivity to potential stem cell proteins expressed on the cell surface (e.g., prominin and TERT) (Tsujimura et al. 2007) or purified cells by functional assays (e.g., collagen-binding or $\alpha 2$ -integrin 'bright' cells) (Collins et al. 2001). Still others have used a combination of antibodies to cell surface proteins expressed by non-epithelial cells to first remove these cells from prostate cell suspensions, and then reacted the remaining cells with antibodies that recognize potential stem/progenitor cell markers to FACS-select stem cells (e.g., Lin⁻ cells) (Lawson et al. 2007). In contrast to these approaches, the K5/CFP transgenic model makes it possible for the first time to test directly the stem cell properties of an epithelial cell population that is positively selected based on expression of a cytoplasmic protein, K5. Importantly, use of the K5/CFP transgenic mice allows for the visualization of CFP⁺ cells in the spatial cellular context of the prostate, FACS isolation of K5⁺ epithelial cells, and for their subsequent analysis to interrogate their stem cell properties and gene expression profile. We note that CFP expression was observed in the epithelium of multiple organs in adult K5/CFP mice, suggesting the broader utility of this model for the analysis of epithelial stem cells in other organs.

Both the bovine K5 promoter and the human K18 sequence used in this study have been used successfully by others to target expression of other transgenes, including EGFP, appropriately to K5- and K18-expressing cells in mice (Brien et al. 2004; Liang et al. 2009; Wen et al. 2003). We chose to generate transgenic models using K5 and K18 regulatory sequences to control CFP and RFP because these reporter proteins are spectrally distinct, thus allowing for their combined functionality in double transgenic mice. We used conventional and confocal microscopy together with fluorescence to evaluate the coincidence of CFP with K5 and RFP expression with the androgen receptor (AR) in prostate sections and found no discordance. The dynamics of CFP- and RFP-expressing cells in the involuted prostate following castration and the reconstituted prostate following

testosterone administration provided further evidence that expression of the transgenes is faithful to expression of the endogenous *Krt5* and *Krt18* genes.

Using two different cell proliferation assays (clonal growth and spheroid development), we have determined that the number of K5-expressing cells having a high proliferative potential is ~130-fold greater than the number of K5-non-expressing cells with this potential, thus supporting the presence of stem/progenitor cells in the K5⁺ cell population isolated by FACS. Nevertheless, the presumptive stem/progenitor cells as measured by the clonal growth assay are only a small subset of the K5⁺ cells (4–8%), in agreement with the findings of others (Goldstein et al. 2008). When cells were isolated based on expression of both CFP (i.e., a pure epithelial cell population) and a potential cell surface stem cell marker (CD133, CD117, or ALDH), the number of cells that gave rise to clones was significantly increased as compared to cells isolated on the basis of CFP expression only, thus supporting the presence of a subset of cells having stem cell properties within the K5⁺ cell population. The fact that nearly 25% of K5⁺ cells have the ability to form spheroids suggests that this in vitro assay is a less stringent test for accessing proliferative capacity of cells in a population, perhaps due to culture conditions that make it difficult to distinguish between stem cells and other basal cells that also have high proliferative potential (e.g., progenitor cells).

Following grafting to the kidney capsule of CFP⁺ mouse prostate cells combined with rat urogenital mesenchymal cells, prostate structures composed of basal, luminal, and neuroendocrine cells developed. The ability of CFP⁺ cells to differentiate is perhaps the strongest evidence for the presence of stem/progenitor cells in the CFP⁺ population.

It is generally accepted that K5 and CK14 are markers for basal cells and K8 and K18 are markers for luminal cells in the prostate epithelium. Interestingly, we observed that upon FACS analysis of prostate cells from adult double transgenic K5/CFP + K18/RFP mice, nearly all CFP⁺ cells also express RFP. Enhanced immunostaining of prostate sections and RT-PCR of cDNA prepared from single prostate cells of non-transgenic mice confirmed this finding. Co-expression of K5 and K18 in at least a subset of murine prostate cells that appear to be in the basal compartment has previously been reported, with K5 expression much stronger than K18 expression (Bhatia et al. 2005; Garraway et al. 2003; Tran et al. 2002; van Leenders et al. 2000) (Verhagen et al. 1992). K5 and K18 have also recently been shown to be co-expressed in basal cells human prostate (Goldstein et al. 2010).

We present the results of a microarray analysis in which we compare gene expression in CFP⁺ basal cells and CFP[−] luminal cells derived from whole prostates of K5/CFP + K18/RFP double transgenic adult mice. Inclusion of the K18/RFP transgenic mouse model in this analysis was essential as it ensured that FACS-isolated CFP[−] cells were indeed epithelial cells and not stromal or neuroendocrine cells. This analysis provides for the first time molecular signatures for mouse prostate basal and luminal cells. Several genes that have previously been identified as potential stem cell markers in stem cells from various organs are over-expressed in CFP⁺ cells relative to CFP[−] cells.

In conclusion, we have developed two transgenic mouse models, validated transgene expression, and shown the utility of these models for isolating specific populations of epithelial cells in the prostate, including basal cells having stem/progenitor cell characteristics. This study lays the groundwork for future studies in which FACS isolation of prostate stem/progenitor cells is enhanced based on combining CFP fluorescence with antibodies to presumptive cell surface stem cell markers. We expect microarray analyses of these populations will yield a refined molecular signature of normal prostate stem cells. Given the substantial evidence showing similarity between normal stem cells and cancer stem cells (Gupta et al. 2009; Pece et al. 2010), a better understanding of gene expression in

normal prostate stem cells is likely to advance our understanding of the molecular mechanisms underlying the transformation of these cells. This knowledge will help us design new strategies that target cancer stem cells, resulting in more sustained therapeutic outcomes than are achieved using current therapies. The K5/CFP and K18/RFP transgenes are expressed appropriately in the epithelium of multiple organs in adult mice, suggesting their broader utility for stem cell analyses in other organs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. CFP and RFP fluorescence in fetal, perinatal, and adult prostate of K5/CFP and K5/CFP-K18/RFP double transgenic mice. Frozen sections of urogenital region of **a** Day 16 and **b** day 18 fetuses. **c** Whole mount of the urogenital system of a D18 fetus. **d** Frozen section of prostate of a 14-day old mouse. **e-h** DAPI-stained frozen sections of different lobes of adult prostate. CFP fluorescence is *light blue*, RFP fluorescence is *red*, and DAPI fluorescence (nuclei) is *dark blue*. UGM = urogenital mesenchyme; UGS = urogenital sinus; UGE = urogenital epithelium; PrB = prostatic buds; AP = developing anterior prostate. Bar in **a, b, d, e-h** = 100 μ m; bar in **c** = 1 mm

Fig. 2.

Co-incident expression of CFP and K5, and RFP and androgen receptor (AR), in prostate cells from adult K5/CFP (**a, b**) and K18/RFP (**c**) transgenic mice, respectively. **a** Images of separate and combined fluorescent signals of dual immunostaining for K5 (*red*) and CFP (*green*) as viewed with conventional fluorescent microscopy. Co-incident fluorescence appears as *yellow* signal. **b** Z-stack analysis of confocal images of 1 μm sections from a single prostate acini, dual immunostained for K5 (*red*) and CFP (*green*). Co-incident signal is *yellow*. **c** RFP fluorescence, AR immunostaining, DAPI nuclear staining, and overlaid RFP, AR, and DAPI images from a frozen section of K18/RFP prostate. The *bar* indicates 20 μm

Fig. 3.

Sections of prostates from intact (non-castrated) and castrated K5/ CFP + K18/RFP double transgenic mice viewed under fluorescent illumination. In panels on the right, images are of prostates from mice treated for 2 weeks with testosterone 3 weeks following castration. CFP fluorescence (K5⁺ cells) is light blue; RFP fluorescence (K18⁺ cells) is red; DAPI fluorescence is dark blue. The bars in top row indicate 100 μ m, and in bottom row, indicate 20 μ m

Fig. 4.

Clones and spheroid body derived from CFP⁺ cells isolated by FACS. **a** Bright field (*left*), fluorescent (*center*), and combined bright field and fluorescent images (*right*) of a typical clone 1 week after plating. Bar = 100 μ m. **b** A clone, typical of appearance of clones derived from primary clones that were picked, dissociated, and re-plated. CFP continues to be expressed in these secondary clones. Bar = 100 μ m. **c** Number of clones/100 CFP⁺ cells doubly FACS-sorted for CFP and either CD133, CD117, or ALDH. **d** Spheroid derived from CFP⁺ cells. **e** Spheroids derived from the CFP⁺ cell population isolated from K5/CFP + K18/RFP prostates and maintained in culture for 1–5 days

Fig. 5.

Immunostained sections of tissue recombinants deriving from renal capsule-implanted recombinant cells (CFP + murine prostate cells + rat UGM). Low power images (bar = 100 μ m) on *left*, high power (bar = 20 μ m) on *right*. **a, b** Immunostained for K5 (*green*) and K8 (*red*). **c, d** Immunostained for CFP (*green*) and K8 (*red*). **e, f** Immunostained for K5 (*green*) and CFP (*red*). The yellow signal in **f** indicates cells in which K5 and CFP expression is concordant. **g, h** Immunostained for CFP (*green*) and AR (*red*). **i, j** Immunostained for K8 (*green*) and AR (*red*)

Fig. 6. Immunostained sections of tissue recombinants deriving from renal capsule-implanted recombinant cells (CFP + murine prostate cells + rat UGM). **a** synaptophysin (*red*) and K5 (*green*). **b** p63 (*red*) and K5 (*green*). The bar in each panel = 20 μ m

Fig. 7.

Co-expression of K5 and K8 in prostate epithelium. **a** FACS profile of CFP and RFP fluorescing prostate cells from double transgenic K5/CFP + K18/RFP mice. The CFP⁻/RFP⁺ cells in the upper left, and the CFP⁺/RFP⁺ cells are the cell populations whose gene expression profiles were compared by microarray analysis. **b** Single cell RT-PCR analysis of prostate cells from double transgenic K5/CFP + K18/RFP mice. K5 amplicon is 346 bp, and K18 amplicon is 427 bp. **c, d** Sections of: **c** proximal region of ventral prostate lobe from adult FVB/NJ male (bar = 20 μ m), and **d** urogenital sinus from FVB/NJ day 16 fetus, immunostained for both K5 and K8 (bar = 100 μ m). Cells co-expressing K5 and K8 are yellow, some of which are indicated by the arrows in panel c. See Fig. S8 for images of separate fluorescent channels of urogenital sinus

Table 1

Summary of single cell RT-PCR assays performed on prostate cells from double transgenic CK5/CFP + CK18/RFP mice

	CK5	CK18
# cells tested	32	40
# PCR positive cells	10	11
% positive	31.25	27.5
Expected % positive	100 ^a	?

^aBased on CFP fluorescence exhibited by cells

Table 2

Potential stem cell marker genes having higher expression in CFP⁺ epithelial cells relative to CFP⁻ epithelial cells

Gene symbol	Name	Median ratio [*]	Gene ID	Reference
<i>Trp63</i>	Transformation related protein 63 (p63)	3.8	22061	(Yang et al. 1999)
<i>Krt15</i>	Cytokeratin 15	2.8	16665	(Morris et al. 2004)
<i>Wnt7a</i>	Wingless-related MMTV integration site 7A	2.3	22421	(Clevers 2006)
<i>Sox2</i>	SRY-box containing gene 2	1.8	20674	(Avilion et al. 2003)
<i>Smo</i>	Smoothened homolog	1.6	319757	(Zhao et al. 2009)
<i>Itga2</i>	Integrin alpha 2	1.6	16398	(Collins et al. 2001)
<i>Itga6</i>	Integrin alpha 6 (CD49f)	1.4	16403	(Lawson et al. 2007)
<i>Nes</i>	Nestin	1.1	18008	(Gleiberman et al. 2008)
<i>Cd44</i>	CD44 antigen	0.7	12505	(Hebbard et al. 2000)
<i>Gli2</i>	GLI-Kruppel family member Gli2	0.35	14633	(Ahn and Joyner 2005)
<i>Tpm2</i>	Tropomyosin 2, beta (Trop2)	0.3 ^{**}	22004	(Goldstein et al. 2008)

^{*}
 $P \leq 0.05$

^{**}
 $P = 0.07$