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Isolation and Growth of Prostate Stem Cells and Establishing
Cancer Cell Lines from Human Prostate Tumors

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INTRODUCTION

The objective of this proposal was to isolate, grow, and characterize normal prostate stem cells and establish new prostate cancer cell lines from fresh human prostate tissues. The hypothesis is that prostate stem cells express defined stem cell markers, self-renew, and require the use of a feeder layer which is necessary for the establishment of prostate cancer cell lines from primary tumors.

The goal of **Specific Aim I** was to test the hypothesis that normal human prostate stem cells express markers of other tissue stem cells, require a defined *in vitro* growth environment for self-renewal and differentiation, generate progeny that differentiate into cells found within the prostate epithelial compartment, and form functioning prostatic glandular structures *in vivo*. **We have completed all the work set forth in Specific Aim I and have submitted the manuscript encompassing this aim for peer review.**

Specific Aim II was aimed at testing the hypothesis that growth-arrested feeder layers, along with other defined factors, will facilitate the establishment of human prostate cancer cell lines. These cells will be tested for genetic aberrations associated with prostate cancer, behave like cancer cells *in vitro* and *in vivo*, expressing AR and PSA, and form hormone-responsive tumors. We are currently acquiring such cells and culturing them using the techniques documented in Specific Aim I. In addition, we have conducted an in depth analysis of stem cell marker expression in prostate cancer cell lines and the concomitant cancer initiating cell ability.

BODY

The normal prostate is composed of a stratified epithelium which is functionally organized in stem cell units and subject to strict paracrine control via stromally-derived growth and survival factors, as shown in Figure 1 (1-3). Adult prostate epithelial stem cells reside within the basal layer at a very low frequency, possess high self-renewal capacity, proliferate infrequently to renew themselves, and simultaneously generate progeny for two distinct cell lineages (2, 4, 5). The first and much less frequent lineage commitment is to differentiate into proliferatively quiescent CD56⁺ neuroendocrine cells which secrete a series of peptide growth factors (6, 7). The second and more common lineage commitment is to differentiate into Δ Np63-positive transit-amplifying (TA) epithelial cells. TA epithelial cells undergo a limited number of proliferative replications before maturing into intermediate cells, characterized by a loss of Δ Np63 coupled with gain of expression of prostate stem cell antigen (PSCA) (8, 9). These TA cells do not express AR protein and are dependent for proliferation, but not survival, on AR signaling in the stroma (4, 5). Intermediate epithelial cells migrate upward to form the luminal-secretory layer, where they express and engage the AR and undergo terminal differentiation characterized proliferative quiescence and expression of PSA and other prostate luminal-secretory specific markers (2, 4, 10, 11). Unlike their proliferating precursors, luminal-secretory cells depend on stromally-derived andromedins for survival and, hence, androgen ablation or specific inactivation of AR function in prostate stroma induces apoptosis of the luminal-secretory cells (4, 12).

Although it is clear that prostate cancer arises within the epithelial compartment, the identification of the specific epithelial cell subtype in which the carcinogenic process is initiated has been the focus of intense study. There is a growing literature supporting that cancer lethality is the result of the hierarchical expansion of “cancer initiating cells” (CICs) which function as stem-like cells to maintain malignant growth (13). Defining characteristics of such CICs include cells which are present at low frequency, possess an unlimited proliferative capacity, undergo self-renewal, and produce phenotypically heterogeneous progeny with only a limited proliferative potential. This has raised the issue of whether these CICs are derived from a malignantly transformed normal adult stem cell or from a more differentiated progeny which has acquired stem-like abilities.

Resolving the specific cell of origin for prostate cancer is critical to appropriately define rational targets for therapeutic intervention, since there are major differences in the growth regulatory pathways, particular those involved in the AR axis for stem cells versus their more differentiated progeny. As a consequence, it is critical to develop experimental systems to isolate and characterize both the human normal prostate stem cells and the prostate CICs.

Along these lines, it has been suggested that CD133 is a marker for both of these cell types (14). CD133 (a.k.a. Prominin-1 or AC133) is a membrane glycoprotein with an N-

terminal extracellular domain, five transmembrane loops with two large extracellular loops containing eight putative N-linked glycosylation sites, and a cytoplasmic tail (14). Very little is known about the biological function of CD133 except that it is localized to membrane protrusions where it interacts with membrane cholesterol and marks cholesterol-based lipid microdomains (15). Adult stem cells often express CD133 as a surface marker (14, 16, 17), and it is thought that CD133-marked cholesterol microdomains function to maintain stem cell properties by suppressing differentiation (18). CD133 was identified as the target of two monoclonal antibodies, AC133 and AC141, and both monoclonal antibodies bind to uncharacterized glycosylated epitopes on the extracellular loops of the CD133 protein (19). However, there are discordant observations regarding the expression and modulation of CD133 binding using these carbohydrate specific antibodies among various cell types, and antibodies are now

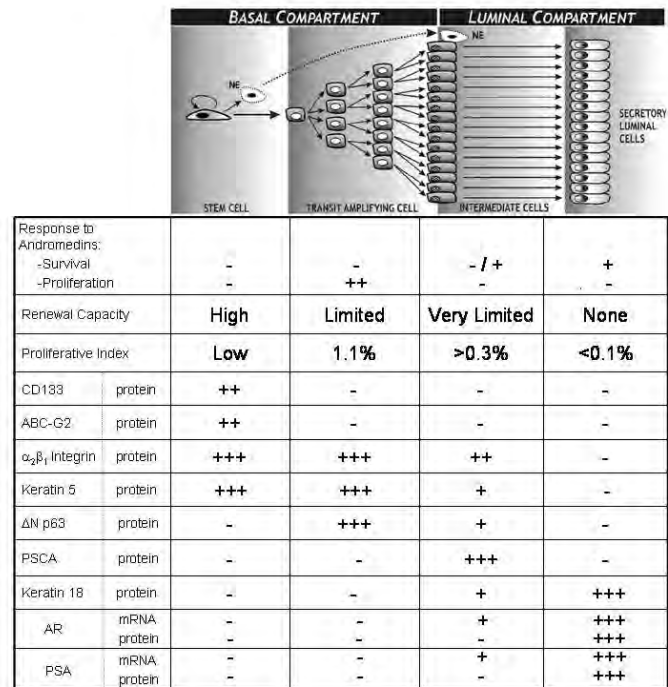


Figure 1. Stem-Cell Model of Prostate Epithelial Differentiation. Prostate stem-cells give rise to all epithelial type cells in the prostate (i.e. transit-amplifying, intermediate, luminal-secretory and neuroendocrine cells). Each stage of epithelial differentiation can be identified based on morphological changes and expression of specific molecular markers. Adapted from Uzgar AR, Xu Y, and Isaacs JT. J of Cell Biochem **91**: 196-205 (2004).

available which bind specifically to peptide-epitopes in the extracellular loops of human CD133 (19).

In the adult human prostate, CD133 expression is thought to be restricted to stem-like populations based upon their expression of $\alpha_2\beta_1$ integrins (20), rapid attachment to type I collagen (21), and high clonogenic ability when grown in low-calcium serum-free defined (SFD) medias (22, 23). Furthermore, CD133 expression has been reported to mark putative prostate CICs (24). In the present study, we document that: 1) single cell suspensions from freshly dissociated human prostate tissue contain a small population of CD133+ cells and that the unfractionated single cell suspension regenerates prostate glands when recombined with rodent embryonic urogenital sinus mesenchyme and grown as xenografts in a host mouse; 2) that from such dissociated single cell suspensions, *in vitro* epithelial (PrEC) cultures can be established which contain a sub-population of CD133+ cells which retain the stem-like ability to regenerate progeny containing neuroendocrine, TA, and intermediate cells; and 3) that human prostate cancer cell lines contain sub-populations of CD133+ cells which are clonogenic, but unlike normal CD133+ prostate stem cells, co-express AR. Using different monoclonal antibodies, we discovered that CD133 has a critical role in the attachment and subsequent growth of CD133+ normal prostate stem cells, which was not observed with prostate cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

1. Single-Cell Suspensions of Human Adult Prostate Epithelial Cells Contain Gland-Regenerating Stem Cells.

Collagenase digestion of whole human prostate tissue liberates epithelial aggregates, known as organoids, which can be separated from supporting stromal cells. Previous data documented that when these human prostatic epithelial organoids are injected sub-cutaneously with Matrigel into nude mice, a population of stem cells proliferates and give rise to progeny regenerating stratified glands in which the luminal cells terminally differentiate and secrete PSA (25, 32). In order to evaluate whether the CD133 expressing cells are the prostate stem cells responsible for this regenerative ability, the organoid must be dissociated into single cells and the CD133 subpopulation isolated and tested for its regenerative ability. In rodent prostates, stem cells are a minor

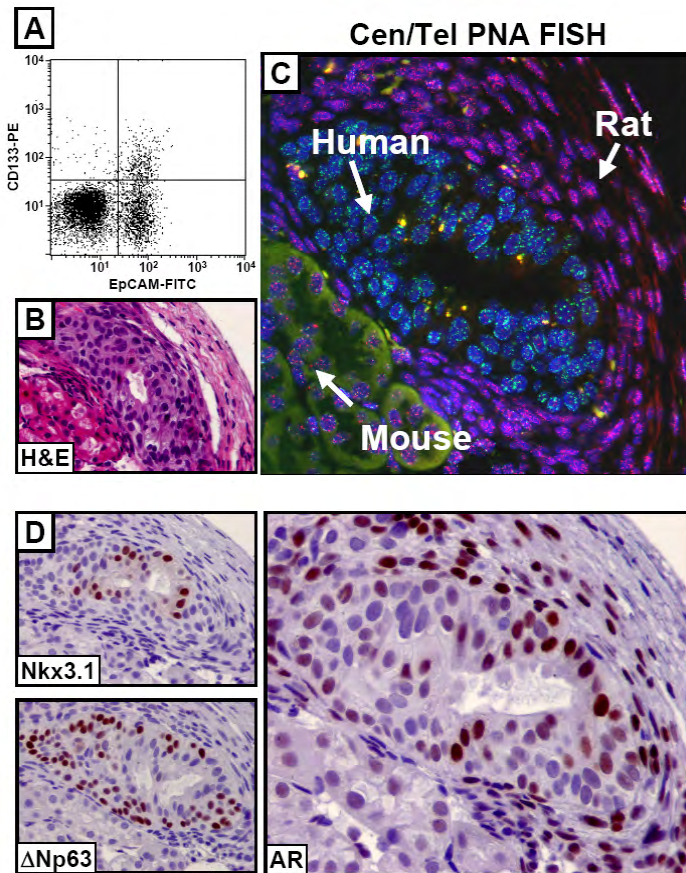


Figure 2: Glandular Regeneration *in Vivo* from Single-Cell Dissociations of Adult Human Prostatic Epithelia. A, Detection of CD133+ cells in single cell suspensions of whole prostate tissue. Fresh prostate tissue was dissociated into single cells and contained both stroma and epithelial cells. B-F, Recombination of rat urogenital sinus mesenchyme (UGSM) with dissociated human epithelial cells regenerate human glandular structures when implanted under the renal capsule of a nude mouse. Single cells from dissociated human prostate epithelial organoids (devoid of stroma cells) were combined with rat urogenital sinus mesenchyme (UGSM) cells isolated from day E17 rat embryos and placed under the renal capsule of male nude mice. B, Glandular structures were observed in as few as two weeks. C, Centromere-Telemore protein-nucleic acid (PNA) fluorescence in-situ hybridization (FISH) (Cen/Tel PNA FISH) demonstrating human cells comprising the gland, with surrounding rodent stroma. D, Luminal expression of prostate-restricted Nkx3.1 (upper left panel). Expression of Δ Np63 in the basal epithelial cells (lower left panel). Androgen-Receptor (AR) expression in the luminal epithelial cells and the surrounding rat stroma (right panel). Adjacent mouse renal parenchymal cells were AR-negative.

fraction of the adult epithelium (33). This raises the issue of whether the available antibodies are sensitive enough to detect the potentially low number of CD133+ putative prostate stem cells. To evaluate this, the proportion of CD133+ cells was analyzed in non-fractionated (i.e. containing both stroma and epithelia) dissociated cells from fresh human prostate tissue using flow cytometry (Figure 2). As a second marker, the pan-epithelial surface antigen, EpCAM, was used to discern epithelial cells from stromal cells. Such analyses reveal a minor (i.e., <10%) sub-population of CD133+ cells present of which >80% are of epithelial origin (i.e., EpCAM positive). Notably, the percentage of CD133+ cells is higher in donors under the age of 30 versus those older than 50 years (i.e. 10-15% vs. 1-5%, respectively).

In order to eliminate the population of CD133+ stromal cells, prostate tissue was collagenase digested and the epithelial organoids collected. The prostate epithelial organoids were dissociated into single cells and implanted *in vivo* under the renal capsule

of host nude mouse. No glandular formation was observed up to 3 months; these negative results are consistent with the known stromal requirement for prostate glandular morphogenesis (34). To provide such stromal support, co-inoculation with urogenital sinus mesenchyme (UGSM) was tested, based upon the established ability of UGSM to induce prostate epithelial organogenesis of human embryonic tissues and single cells *in vivo* (34, 35). Such single cell recombination results in the regeneration of stratified prostate glands detectable as early as 2 weeks post inoculation.

To discern the cellular contribution of human, mouse, and rat cells to the regenerated glands, a novel technique was employed which takes advantage of unique genomic differences between all three species (rat, human, and mouse). Specifically, telomeres in commonly used inbred laboratory rodent strains are significantly longer than human telomeres (50-150kb in rodent vs. 5-10kb in human), and this difference in length results in a notable difference in the intensity of telomeric FISH signals (36). Thus, rodent cells (mouse and rat) are easily distinguished from human cells by virtue of their very bright telomeres. The Centromere-specific PNA probe utilized hybridizes to DNA repeats in human and mouse centromeres but does not hybridize to rat centromeres (37, 38). Thus, simultaneous staining with these centromere and telomere PNA FISH probes (Cen/Tel FISH) allows for rapid and unequivocal identification of species origin (human vs. rat vs. mouse) at the single cell level in tissue hetero-recombinants. In these rat UGSM/ human recombinants, Cen/Tel FISH confirmed the presence of human epithelial glands surrounded by rat stroma and adjacent to renal parenchymal cells of mouse origin (Figure 1C).

Such human-derived glandular structures are positive for Nkx3.1, a prostate epithelial cell restricted protein (39), contain a Δ Np63-positive basal layer, and an AR-positive luminal layer. The stroma surrounding the human-derived glands is derived from the rat UGSM and contains AR-positive stromal cells providing androgen-dependent paracrine signaling. These data document that within single-cell suspensions of human adult epithelial cells, there are prostate stem cells which are capable of regenerating complete prostatic glandular structures, and that the recombination of human prostate tissue with rodent UGSM is a useful assay for detecting such prostate stem cell capabilities. Thus, the presence of CD133+ cells are present within such single cell

dissociates, consistent with their being putative stem cells. However, their frequency within fresh tissue is so low that a culture method is needed to obtain sufficient numbers to test their stem cell ability in such an *in vivo* assay.

2. Phenotypic Characteristics of CD133+ Cells Isolated From Human Prostate Epithelial Cultures.

From freshly dissociated tissue, human prostate epithelial cells (PrEC) can be routinely cultured and propagated for 8-10 serial passages using low calcium, serum-free defined media (i.e. either Keratinocyte Serum-Free Media with ~100 μ M Calcium or PrEGM media with ~300 μ M Calcium) (23). By the second serial passage, such cultures are devoid of prostate fibroblasts and smooth muscle cells (5, 23). Early passage cultures from a series (N=12) of different commercial and in-house donors were analyzed for the

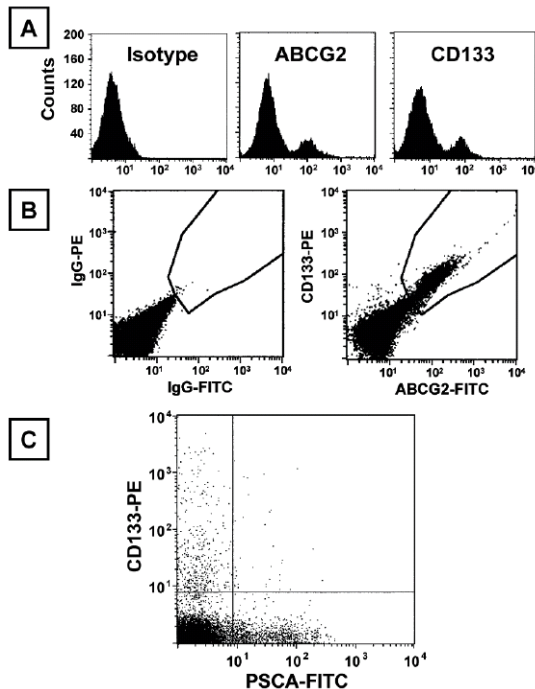


Figure 3: Expression of Stem Cell Markers in PrEC Cultures. A, ABCG2-FITC and CD133-PE (AC141) expression in commercially available PrECs. A non-specific IgG was used as an isotype control. B, Dual-label flow cytometry demonstrating that CD133+ cells are also ABCG2+. C, CD133+ PrECs do not express the intermediate cell marker PSCA.

expression of CD133, ABCG2, β_1 -integrin, Δ Np63, PSCA, AR, CD56, and Chromogranin A. These analyses consistently documented that these cultures are phenotypically heterogeneous being composed of at least 4 discernable sub-populations: 1) a minor population of small-sized putative stem cells (CD133+/ABCG2+/ β_1 -integrin+/ Δ Np63-/PSCA-/AR-/CD56-); 2) a major population (~80%) of small to intermediate sized TA cells (Δ Np63+/CD133-/PSCA-/AR-/CD56-); 3) a minor population (~10 %) of larger sized intermediate cells (PSCA+/AR+/CD133-/ Δ Np63-/CD56-); and 4) a minor population (~2-5%) of dendritic-shaped neuroendocrine cells (CD56+/ChromograninA+/CD133-/ Δ Np63-/PSCA-/AR-) (5, 23).

3. Glycosylation Specific Monoclonal Antibodies Prevent Attachment and Survival of CD133+ Cells.

Western blot analyses were unable to detect CD133 protein expression in unsorted PrECs (Figure 4). As a positive control for these CD133 western blots, the CaCo-2 human colon cancer cell line was used, since it is uniformly CD133+ (40). To increase the sensitivity of western blot analysis, CD133+ PrECs were enriched using live

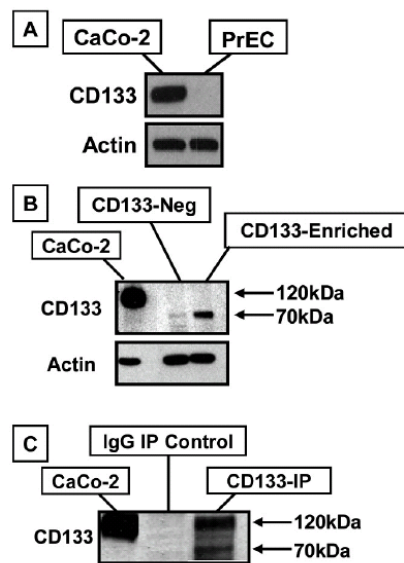


Figure 4: Molecular Weight of CD133 in PrECs. A, Western blot of non-fractionated PrECs compared to the CD133+ CaCo-2 colon cancer cell line. Actin was used as a loading control. B, Western blot of CD133-enriched PrECs and CaCo-2 cells. PrECs and CaCo-2 cells were subjected to magnetic enrichment of CD133(AC133) positive cells and the expression of CD133 compared to non-CD133 expressing PrECs (CD133-Neg). Actin was used as a loading control. Western blotting using the peptide-derived CD133 antibody (293C3) reveals a lower molecular weight form of CD133 expressed in the AC133-enriched PrECs as compared to CaCo-2 cells. C, Immunoprecipitation (IP) of AC141 from lysed PrECs and CaCo-2 cells and western blotting using the peptide-derived anti-CD133 antibody reveals the expression of CD133 at the correct MW in addition to the smaller 70kDa form.

cell Magnetic-Activated Cell Sorting (MACS) with the AC133 antibody. This monoclonal antibody binding is glycosylation-dependent, recognizing a carbohydrate specific epitope on the extracellular loop of CD133 and has been used previously to isolate stem cells from a variety of non-prostate tissues (19). The AC133-enriched cells were subsequently lysed and subjected to western blotting using an anti-CD133 rabbit monoclonal antibody (C24B9) which specifically recognizes a peptide epitope in the second extracellular loop of CD133 (19). This documented that AC133-enriched PrECs have a detectable level of a truncated and glycosylated 70kDa form of CD133 but not full length glycosylated 120-130kDa CD133 protein detected in CaCo-2 cells. Full length CD133 protein is expressed, however, by a subset of PrEC cells before binding the AC133 antibody. This is documented by the observation that lysis of unsorted PrECs followed by immunoprecipitation using a second glycosylation specific antibody (i.e. AC141) revealed a full-length form of CD133 in addition to the smaller, 70kDa form.

Binding of the glycosylation-specific AC133 antibody not only results in truncation of the glycosylated 120 kDa full length CD133 protein but also the inability of antibody-associated CD133+ PrECs to attach and grow *in vitro*. This was initially observed when CD133+ PrECs were isolated via FACS using the anti-CD133-PE conjugated AC141 mouse monoclonal antibody consistently exhibited an inability to attach and spread when re-plated in culture, resulting in their eventual death. A variety of culture conditions were used in an attempt to improve the survival and growth of AC141 isolated PrECs cells including Poly-D-Lysine or Type I Collagen coating, adding either conditioned media from unfractionated PrEC cultures, or culturing on an irradiated feeder layer (i.e. mouse STO cells). All conditions failed to increase the viability and growth of the AC141-sorted PrECs. A series of controls were used to document that specific binding of the AC141 antibody uniquely inhibits PrEC cells attachment and growth after sorting. First, sham-sorted or EpCAM-sorted PrECs attach and proliferate in a manner similar to non-manipulated PrECs; second, FACS isolation using the same carbohydrate specific AC141 monoclonal antibody to isolate CD133+ cells from the CaCo-2 human colon cancer line yielded viable cells which attached and grow equally well as unsorted CaCo-2 cells. In addition, to test whether this inhibition is unique to the AC141 monoclonal antibody or is a general property of antibodies which bind the carbohydrate portion of CD133 on PrEC cells, MACS-isolation using the AC133 antibody yielded similar results, whereby CD133+ PrECs failed to attach and grow. These combined results document that the glycosylation-specific AC141 and AC133 anti-CD133 antibodies inhibit the attachment and growth of CD133+ PrECs in a cell-context dependent manner.

4. CD133+ Cells Regenerate Phenotypically Heterogeneous PrEC Cultures.

In contrast to the negative results using the glycosylation-specific AC141 and AC133 antibodies, FACS sorting and subsequent growth of CD133+ PrECs was possible using the peptide specific C24B9 rabbit monoclonal antibody (Figure 5). CD133+ PrECs were sorted by flow cytometry to generate a >98% pure population. Western analysis of flow-sorted CD133+ PrECs revealed that the expression of Δ Np63 is below the level of detection. This CD133+ population was placed back into culture and tested for its ability to regenerate all the cell populations present within heterogeneous PrEC cultures. By two

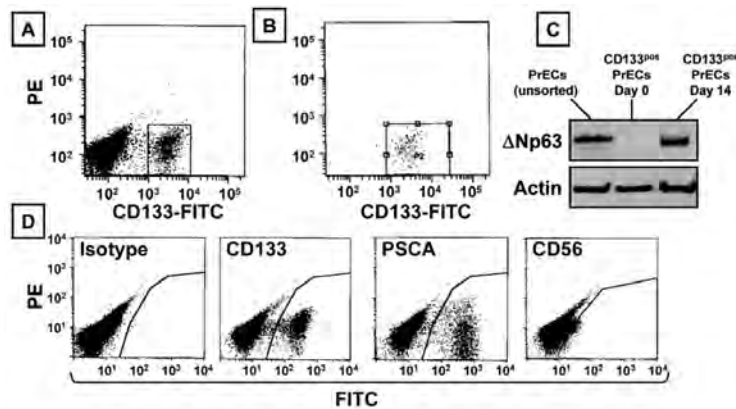


Figure 5: Pure Populations of CD133+ Cells Regenerate PrEC Cultures.

A, Flow cytometric analysis of PrECs using the peptide-derived 293C3 anti-CD133 antibody. CD133+ cells (box) were isolated via FACS. B, Fluorescence-activated cell sorting (FACS) yield a population of CD133+ cells which is >98% pure. CD133+ cells were either re-plated for growth or lysed for western blotting. C, Expression of the basal marker Δ Np63 in CD133+ PrECs is below the level of detection, but is restored after 14 days in culture. Actin was used as a loading control. D, Cultures established from pure CD133+ cells contain an expanded number of CD133+ stem cells, as well as PSCA+ intermediate cells and CD56+ neuroendocrine cells. A non-specific IgG was used as an Isotype control. Thus, cultures derived from pure populations of CD133+ cells contain the original progenitor population (CD133+ cells) and two distinct cell lineages (basal-intermediate and neuroendocrine).

cytometry documented that by two weeks, the number of CD133+ cells is approximately six times greater than the initial number of CD133+ cells plated, indicating that the CD133+ cells not only renew themselves but also give rise to progeny of two distinct cell lineages: the neuroendocrine cell lineage, and the transit-amplifying cell lineage, where a subset of Δ Np63+ TA cells mature to form PSCA+ intermediate cells. In contrast to the ability of CD133+ cells to regenerate PrEC cultures, flow sorted PSCA+ and CD56+ PrECs attached but did not grow when placed back into culture. These data document that CD133+ PrECs are both self-renewing and capable of generating progeny of two distinct cell lineages (neuroendocrine and TA) and are thus bone fide prostate stem cells.

5. CD133+ Human Prostate Cancer Cells have Cancer Initiating Ability

Similar to normal prostate epithelial cultures, the frequency of CD133+ cells within a series of human prostate cancer cell lines is so low that the protein is below detection in mass culture by western blot analysis. Using more sensitive flow cytometry,

weeks, the cultures had undergone approximately six population doublings and were morphologically identical to those of unsorted parental PrEC cultures, being composed of a heterogeneous mixture of small, medium, and large sized epithelial, as well as dendritic shaped cells. CD133-derived cultures maintain a CD133+ population and also regenerate a Δ Np63+ population of TA cells, a population of PSCA+ intermediate cells, and a population of CD56+ neuroendocrine cells. Flow

however, prostate cancer lines do contain a minor population (~1-5%) of CD133+ malignant cells (Figure 6). These results raise the issue of whether the CD133+ cancer cells have cancer initiating ability. Therefore, to test if CD133+ prostate cancer cells have these abilities, three androgen ablation refractory prostate cancer cell lines (LNCaP, LAPC-4, and CWR22Rv1) were analyzed for their clonogenic ability, percent CD133+

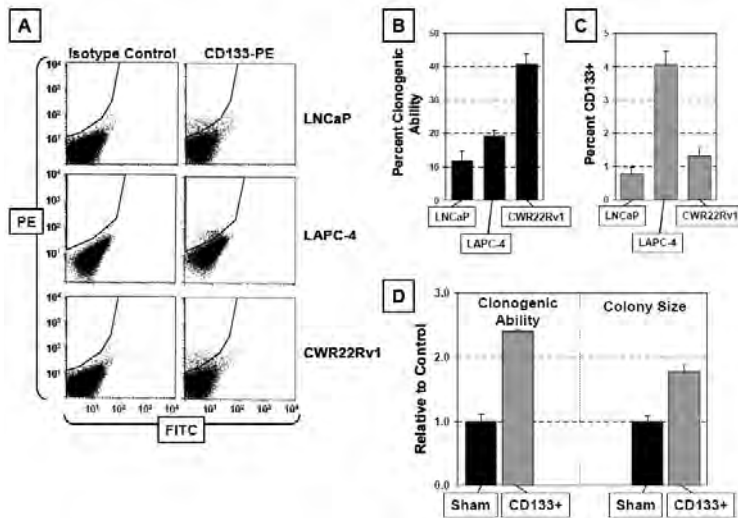


Figure 6: CD133 Marks Prostate Cancer Initiating Cells. A, Flow cytometric analyses revealed minor populations of CD133+ cells in three androgen-responsive prostate cancer cell lines, LNCaP, LAPC-4, and CWR22Rv1. A PE-conjugated CD133 antibody (CD133-PE) and a non-specific IgG was used as a control. B, Clonogenic ability of LNCaP, LAPC-4 and CWR22Rv1 cells to form microscopic clones over ten days. Clones were stained and counted at 10x magnification. C, Percentage of CD133+ cells in LNCaP, LAPC-4 and CWR22Rv1 cultures. These data represent the average of six individual analyses. D, CD133+ CWR22Rv1 prostate cancer cells are more clonogenic and form larger clones as compared to than the sham-sorted control cells.

cells, and their ability to generate progeny with a different phenotype. These lines were chosen to be representative of the range of lethal metastatic prostate cancers observed clinically since LAPC-4 expresses wild-type AR (41), LNCaP expresses point mutated AR (42), and CWR22Rv1 expresses Exon 3 duplicated AR and Exon 2 truncated isoform of AR protein (43). Initially, the clonogenic ability of unfractionated cultures for all three cell lines was determined based upon

formation of colonies (>10 cells) within ten days. These clonogenic abilities were 5- 40 fold higher than the percentage of CD133+ cells in the three lines. These results are explainable by the fact that either: 1) CD133+ cells are not CICs or 2) CD133+ cells are CICs with unlimited ability to self renew but the majority of their progeny become CD133 negative with a sufficient proliferative ability to form the vast majority of colonies in primary clonogenic assay, but not unlimited proliferative ability to form colonies in serial clonogenic assays. If the first possibility is true and CD133+ cells are present at <5%, then 5-10 individual clones derived from each of these lines should be

negative for CD133 expression. In contrast if CD133⁺ cells are the CICs, then serially passaged cultures initially derived from single cell clones should always be heterogeneous, containing a majority of CD133 negative cells and a small fraction of CD133⁺ cells. Thus, to resolve between these possibilities, multiple clones containing >200 cells were isolated from LNCaP, LAPC-4 and CWR22Rv1 lines and the clones serially propagated for more than 25 population doublings (i.e. >50 days) before being analyzed by flow cytometry. These analyses documented that all of the clones from LNCaP (N=5 clones), LAPC-4 (N=8 clones), and CWR22Rv1 (N=6 clones) contain ~1-5% CD133⁺ cells consistent with their CIC ability.

To directly test whether CD133⁺ cell give rise to CD133 negative progeny with limited proliferation ability, CWR22Rv1 cells were flow sorted using the AC141-PE antibody. In contrast to the poor survival of PrECs after sorting with the AC141 antibody, CWR22Rv1 cells exhibited no differences in viability after sorting using the AC141 antibody (seven days after inoculating 2000 cells, there were 81,500 cells in the CD133-sorted vs. 84,800 cells in the mock sorted control group). Further propagation of the CD133-derived CWR22Rv1 cultures revealed that the percentage of CD133⁺ cells was only 6.15% after two weeks in culture. Thus, even though these cultures were initiated from sorted cells which were >98% CD133⁺, the population of CD133⁺ cells is maintained at the same low level as that of the unsorted cultures, with the majority (93.85%) of the progeny in the expanded cultures no longer expressing CD133. The clonogenic ability of flow sorted CD133⁺ CWR22Rv1 cells is 2.4 times greater than the unsorted population and the average colony size was 2x larger by 10 days (Figure 5D). These combined results document that the CD133⁺ prostate cancer cells have the defining characteristics of CICs, since they are present at low frequency, self-renew, exhibit unlimited proliferative capacity, and give rise to phenotypically different progeny with a lower growth potential..

6. CD133⁺ Human Prostate Cancer Cells Express and Respond to Androgen Receptor

To determine whether the CD133⁺ CICs in human prostate cancer express AR protein, two-parameter flow cytometry was utilized. These results document that ~98% of the CD133⁺ populations in the LAPC-4, LNCaP, and CWR22Rv1 cell lines are

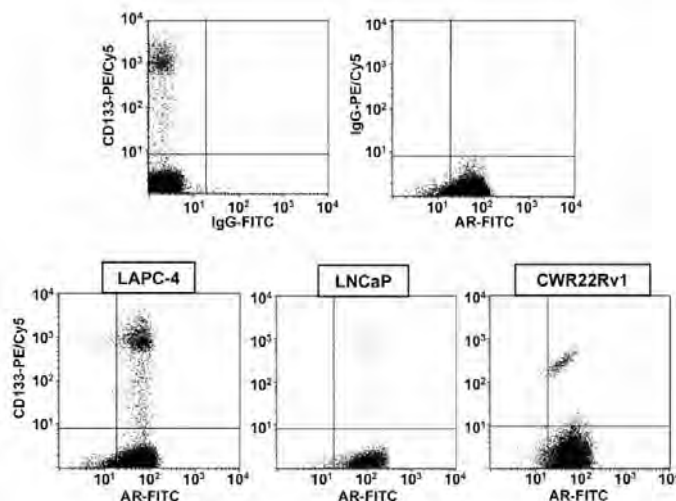


Figure 7: CD133+ Prostate Cancer Cells Express the Androgen Receptor. Dual-parameter flow cytometry of CD133(AC141) and AR in the LAPC-4, LNCaP, and CWR22Rv1 prostate cancer cell lines. *Upper Panels:* Control staining in LAPC-4 using IgG antibodies (upper left: CD133 vs. IgG; upper right: IgG vs. AR). Similar controls were documented in the LNCaP and CWR22Rv1 cell lines. *Lower Panels:* dual labeling for CD133 and AR in LAPC-4, LNCaP, and CWR22Rv1 prostate cancer cells demonstrating that CD133+ prostate cancer cells also express AR.

positive for the androgen receptor. As expected, there is a very small (>2%) population of AR-negative cells present within these exponentially growing cultures, which is consistent with our previous observation that prostate cancer cells degrade AR during mitosis (26). As a functional test to evaluate the signaling ability of the AR expressed in CD133+ cells, the clonogenic ability of LNCaP cells was tested using a growth-inhibitory dose of androgen (i.e., 10nM of the synthetic androgen, R1881) (44). This is based upon the rationale that if AR signaling is not occurring in the cancer initiating CD133+ LNCaP cells, then there will be no effect of high dose androgen on the clonogenic ability of these cells. In contrast, high dose androgen decreases by more than 95% the clonogenic ability of LNCaP cells (2.3±0.8% vs. 0.18±0.1% clonogenic ability of untreated vs. androgen-treated cells).

REPORTABLE OUTCOMES

1. The manuscript entitled “The Role of CD133 in Normal Human Prostate Stem Cells and Malignant Cancer Initiating Cells” is in press in Cancer Research (See Appendix 1)
2. The results reported above have been submitted as a manuscript for peer reviewed publication.
3. The Cen/Tel PNA-FISH procedure is a novel method for discerning species of origin in tissue recombinants.

CONCLUSIONS

The concept of adult prostate stem cells first emerged to explain the immense capacity of the epithelial compartment for cyclic regeneration. Previous studies document that the prostate can undergo more than 30 successive cycles of androgen deprivation and replacement without diminishing its ability for continued epithelial regeneration (45). In the present study we document that CD133 marks both normal prostate epithelial stem cells as well as malignant prostate CICs.

We identified CD133⁺ cells in PrEC cultures and demonstrated that pure populations of CD133⁺ cells are able to regenerate PrEC cultures and exhibit characteristics of stem cells by their ability to self-renew and regenerate PrEC cultures with two distinct cell lineages. A disadvantage of the low-Ca²⁺ SFD culture conditions used to establish and propagate PrEC cultures is that luminal differentiation (i.e. AR expression, terminal differentiation, and PSA expression) does not occur within these cultures. This is due to the activation of Notch signaling and the inhibition of E-cadherin signaling, both of which prevent terminal differentiation (23, 46). As such, using our current culture conditions, the prostate stem cell compartment is unable to complete its full differentiation potential and progress only to the intermediate cell stage.

The expanding use of CD133 as a human stem cell marker has yielded a variety of antibodies for the isolation and characterization of CD133⁺ stem cell populations. The glycosylation-specific AC133 and AC141 anti-CD133 antibodies were the first to be developed and aided the identification of the CD133 gene (19). However, the binding of such antibodies to normal prostate stem cells profoundly inhibited their attachment and growth after cell sorting. The same antibodies, however, did not affect the attachment and growth of CD133⁺ prostate cancer cells. These observations document that CD133 functions differently between normal and malignant prostate cells, and that the glycosylation sites of CD133 play a significant role in the function of normal prostate stem cells.

If prostate cancer were derived from a transformed normal stem cell, one would expect that prostate CICs to not express AR, give rise to Δ Np63⁺ progeny which differentiate into AR⁺ cells, and not respond to androgen-mediated growth inhibition. Prostate cancers exhibit characteristics of intermediate and luminal-secretory epithelial

cells since they express AR, PSA, and PSCA (47, 48). Furthermore, a hallmark of prostate cancer is the loss of the basal cell marker Δ Np63 (49), and normal prostate stem cells do not express AR and are thus not dependent upon androgen for their survival. We document that CD133⁺ prostate CICs express AR and are subject to AR-mediated growth inhibition. The data presented here is consistent with prostate CICs being derived from a malignantly transformed intermediate cell which has gained the expression of the stem cell marker CD133. Such an observation demonstrates that prostate CICs are valid targets for the continued development of improved anti-androgen therapies.

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The Role of CD133 in Normal Human Prostate Stem Cells and Malignant Cancer-Initiating Cells

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Abstract

Resolving the specific cell of origin for prostate cancer is critical to define rational targets for therapeutic intervention and requires the isolation and characterization of both normal human prostate stem cells and prostate cancer-initiating cells (CIC). Single epithelial cells from fresh normal human prostate tissue and prostate epithelial cell (PrEC) cultures derived from them were evaluated for the presence of subpopulations expressing stem cell markers and exhibiting stem-like growth characteristics. When epithelial cell suspensions containing cells expressing the stem cell marker CD133⁺ are inoculated *in vivo*, regeneration of stratified human prostate glands requires inductive prostate stromal cells. PrEC cultures contain a small subpopulation of CD133⁺ cells, and fluorescence-activated cell sorting-purified CD133⁺ PrECs self-renew and regenerate cell populations expressing markers of transit-amplifying cells (Δ Np63), intermediate cells (prostate stem cell antigen), and neuroendocrine cells (CD56). Using a series of CD133 monoclonal antibodies, attachment and growth of CD133⁺ PrECs requires surface expression of full-length glycosylated CD133 protein. Within a series of androgen receptor-positive (AR⁺) human prostate cancer cell lines, CD133⁺ cells are present at a low frequency, self-renew, express AR, generate phenotypically heterogeneous progeny negative for CD133, and possess an unlimited proliferative capacity, consistent with CD133⁺ cells being CICs. Unlike normal adult prostate stem cells, prostate CICs are AR⁺ and do not require functional CD133. This suggests that (a) AR-expressing prostate CICs are derived from a malignantly transformed intermediate cell that acquires “stem-like activity” and not from a malignantly transformed normal stem cell and (b) AR signaling pathways are a therapeutic target for prostate CICs. [Cancer Res 2008;68(23):9703–11]

Introduction

The normal prostate is composed of a stratified epithelium, which is functionally organized in stem cell units and subject to strict paracrine control via stromally derived growth and survival factors (1–3). Adult prostate epithelial stem cells reside within the basal layer at a very low frequency, possess high self-renewal capacity, proliferate infrequently to renew themselves, and simultaneously generate progeny for two distinct cell lineages (2, 4, 5).

The much less frequent lineage commitment is to differentiate into proliferatively quiescent CD56⁺ neuroendocrine cells, which secrete a series of peptide growth factors (6, 7). The more common lineage commitment is to differentiate into Δ Np63⁺ transit-amplifying (TA) epithelial cells. TA epithelial cells undergo a limited number of proliferative replications before maturing into intermediate cells, characterized by a loss of Δ Np63 coupled with gain of expression of prostate stem cell antigen (PSCA; refs. 8, 9). These TA cells do not express androgen receptor (AR) protein and are dependent for proliferation, but not survival, on AR signaling in the stroma (4, 5). Intermediate epithelial cells migrate upward to form the luminal-secretory layer, where they express and engage the AR and undergo terminal differentiation characterized by proliferative quiescence and expression of prostate-specific antigen (PSA) and other prostate luminal-secretory specific markers (2, 4, 10, 11). Unlike their proliferating precursors, luminal-secretory cells depend on stromally derived androgens for survival, and hence, androgen ablation or specific inactivation of AR function in prostate stroma induces apoptosis of the luminal-secretory cells (4, 12).

Although it is clear that prostate cancer arises within the epithelial compartment, the identification of the specific epithelial cell subtype in which the carcinogenic process is initiated has been the focus of intense study. There is a growing literature supporting that cancer lethality is the result of the hierarchical expansion of “cancer-initiating cells” (CIC), which function as stem-like cells to maintain malignant growth (13). Defining characteristics of such CICs include cells that are present at low frequency, possess an unlimited proliferative capacity, undergo self-renewal, and produce phenotypically heterogeneous progeny with only a limited proliferative potential. This has raised the issue of whether these CICs are derived from a malignantly transformed normal adult stem cell or from a more differentiated progeny that has acquired stem-like abilities. Resolving the specific cell of origin for prostate cancer is critical to appropriately define rational targets for therapeutic intervention because there are major differences in the growth regulatory pathways, particularly those involved in the AR axis for stem cells versus their more differentiated progeny. As a consequence, it is critical to develop experimental systems to isolate and characterize both the human normal prostate stem cells and the prostate CICs.

Along these lines, it has been suggested that CD133 is a marker for both of these cell types (14). CD133 (a.k.a. prominin-1 or AC133) is a membrane glycoprotein with an NH₂-terminal extracellular domain, five transmembrane loops with two large extracellular loops containing eight putative N-linked glycosylation sites and a cytoplasmic tail (14). Very little is known about the biological function of CD133 except that it is localized to membrane protrusions where it interacts with membrane cholesterol and marks cholesterol-based lipid microdomains (15). Adult stem cells often express CD133 as a surface marker (14, 16, 17), and it is

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thought that CD133-marked cholesterol microdomains function to maintain stem cell properties by suppressing differentiation (18). CD133 was identified as the target of two monoclonal antibodies, AC133 and AC141, and both monoclonal antibodies bind to uncharacterized glycosylated epitopes on the extracellular loops of the CD133 protein (19). However, there are discordant observations about the expression and modulation of CD133 binding using these carbohydrate-specific antibodies among various cell types, and antibodies are now available that bind specifically to peptide epitopes in the extracellular loops of human CD133 (19).

In the adult human prostate, CD133 expression is thought to be restricted to stem-like populations based on their expression of $\alpha_2\beta_1$ integrins (20), rapid attachment to type I collagen (21), and high clonogenic ability when grown in low-calcium serum-free defined (SFD) medium (22, 23). Furthermore, CD133 expression has been reported to mark putative prostate CICs (24). In the present study, we document (a) that single-cell suspensions from freshly dissociated human prostate tissue contain a small population of CD133⁺ cells and that the unfractionated single-cell suspension regenerates prostate glands when recombined with rodent embryonic urogenital sinus mesenchyme (UGSM) and grown as xenografts in a host mouse; (b) that from such dissociated single-cell suspensions, *in vitro* epithelial [prostate epithelial cell (PrEC)] cultures can be established, which contain a subpopulation of CD133⁺ cells that retain the stem-like ability to regenerate progeny containing neuroendocrine, TA, and intermediate cells; and (c) that human prostate cancer cell lines contain subpopulations of CD133⁺ cells that are clonogenic but, unlike normal CD133⁺ prostate stem cells, coexpress AR. Using different monoclonal antibodies, we discovered that CD133 has a critical role in the attachment and subsequent growth of CD133⁺ normal prostate stem cells, which was not observed with prostate cancer cells.

Materials and Methods

Cells and materials. Primary prostate cells were isolated from patients undergoing radical prostatectomy at our institution according to an Institutional Review Board-approved protocol. Dissociation of prostate tissue has been previously described (25). Briefly, 18-gauge biopsy needle cores (Bard) of prostate tissue were digested overnight at 37°C in collagenase solution [0.28% collagenase I (Sigma-Aldrich), 1% DNase I (Sigma), 10% FCS, 1× antibiotic/antimycotic (Life Technologies-Invitrogen), in RPMI 1640]. The following day, the cell suspension was washed in PBS, and epithelial organoids were isolated by density sedimentation, whereby cells in 10 mL PBS were allowed to settle for 10 min at room temperature and the top 9 mL of medium (containing fibroblasts) were removed; this was repeated two more times. Prostate epithelial organoids were further dissociated into single cells via treatment with DTT (1 mmol/L for 30 min at 37°C), a PBS wash, and trypsin/EDTA (0.25% for 30 min at 37°C). The trypsin was neutralized with RPMI 1640 + 10% FCS, and the cells were washed twice in PBS. Cells were subsequently passed through a cell strainer to ensure a single-cell suspension (BD Falcon). Ten biopsy cores yield ~100,000 single PrECs with >90% cell viability as scored by trypan blue exclusion. Nonfractionated single-cell suspensions of prostate tissue (i.e., containing both stromal and epithelial cells) were obtained by a similar enzymatic dissociation without the density sedimentation separation.

PrEC cultures were established and grown in serum-free defined PrEGM growth medium (Lonza/Cambrex) as previously described (5). Commercially available PrEC cultures, obtained from young men, were additionally used (Lonza/Cambrex). All prostate cancer cell lines were grown as previously described (23). The human colon cancer cell line CaCo-2 was a generous gift from Dr. Fred Bunz (Johns Hopkins University) and was cultured in DMEM + 10% FCS. All chemicals were purchased from JT Baker or Sigma-Aldrich.

Flow cytometry and cell sorting. All antibody incubations, washes, and flow cytometric analyses were performed in cell sorting buffer [1× PBS, 0.5% bovine serum albumin (BSA), 2 mmol/L EDTA]. Analysis was conducted on a Becton Dickinson LSR, and a minimum of 10,000 counts was acquired for each experimental condition. Fluorescence-activated cell sorting (FACS) was performed on a BD FACSaria, and cells were sorted into HBSS (without calcium or magnesium). Primary antibody labeling for flow cytometry and cell sorting was conducted using a 20-min cold incubation with a 1:10 dilution of antibody in a volume of 100 μ L per 1 million cells in cell sorting buffer. The cells were washed in 1 mL cold cell sorting buffer, resuspended in 0.5 to 1.0 cell sorting buffer, and analyzed. For secondary antibody labeling, cells were incubated for 20 min with a 1:1,000 dilution of Alexa Fluor 488 F(ab')₂ fragment of goat anti-rabbit IgG (Invitrogen) in cell sorting buffer and similarly washed before analysis. The AC141 (293C3)-PE-conjugated mouse monoclonal antibody (Miltenyi Biotec) was used for all flow cytometric analyses and the peptide-derived CD133 rabbit monoclonal antibody (C24B9; Cell Signaling Technology) for Western blotting and FACS. Additional antibodies used for flow cytometry and sorting were PSCA (H83; Santa Cruz Biotechnology), FITC-conjugated mouse monoclonal CD56 (NCAM16.2; BD Biosciences), FITC-conjugated mouse monoclonal EpCAM (CD326; Miltenyi Biotec), FITC-conjugated mouse monoclonal ABCG2 (5D3; Chemicon-Millipore), and isotype control antibodies (Miltenyi Biotec). Enrichment of CD133⁺ PrECs was performed using the CD133 Cell Isolation kit according to the manufacturer's specifications (Miltenyi Biotec).

Dual-variable flow cytometric analysis of AR and CD133 was conducted on fixed cells. Ice-cold methanol (1 mL) was added to 1.5×10^6 cells in 350 μ L PBS, and the cells were incubated on ice for 15 min, washed with 10 mL cold PBS, and passed through a cell strainer (BD Falcon). Cells were incubated in blocking buffer (PBS, 0.5% FCS, 2 mmol/L EDTA) for 30 min on ice, and all subsequent antibody incubations were carried out in blocking buffer for 30 min followed by three washes in blocking buffer. The antibodies used were the rabbit polyclonal anti-AR (PG-21; Upstate Biotechnology) followed by a goat anti-rabbit IgG-FITC secondary antibody (Santa Cruz Biotechnology), and mouse monoclonal anti-CD133/2 (AC141, clone 293C3; Miltenyi Biotec) followed by an anti-mouse IgG-F(ab')₂-PE/Cy5 secondary antibody (Santa Cruz Biotechnology). Rabbit and mouse IgG antibodies (Santa Cruz Biotechnology) were used as isotype controls.

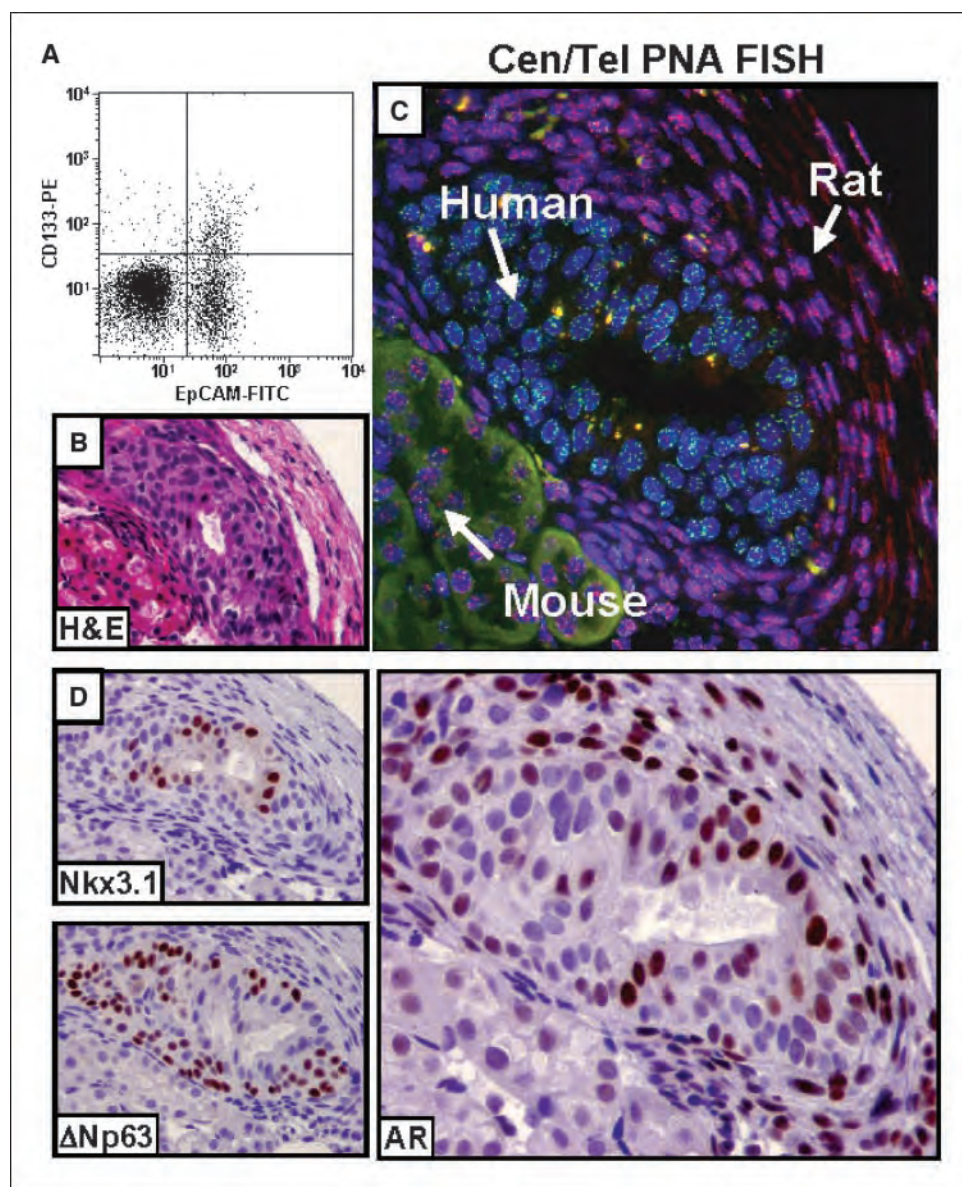
Immunoblotting, immunoprecipitation, and immunohistochemistry. Western blotting was performed as previously described (5). Whole-cell lysates collected from 100,000 cells were used per lane. Antibodies used were anti- β -actin (Cell Signaling Technology), anti- Δ Np63 (4A4; Santa Cruz Biotechnology), and anti-CD133 (Cell Signaling Technology). All secondary horseradish peroxidase-conjugated antibodies and chemiluminescent detection reagents (ECL) were purchased from Amersham Biosciences.

For CD133 immunoprecipitation, cells were lysed in immunoprecipitation lysis buffer [20 mmol/L Tris (pH 7.5), 140 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1× protease inhibitor, 1× phosphatase inhibitor (Sigma)]. Protein lysate (1.0 mg) was incubated overnight with rocking at 4°C using either 4 μ g of the AC141 antibody or a mouse IgG control antibody. Subsequently, 40 μ L of protein A-conjugated Dynabeads (Invitrogen) were washed in immunoprecipitation lysis buffer and added to protein/antibody mixture and incubated with rocking for 3 h at 4°C. Immunoprecipitation was performed using a magnetic stand (Millipore). The beads were washed with immunoprecipitation lysis buffer, resuspended in 40 μ L gel loading buffer, and incubated for 5 min at 80°C to release the CD133 protein from the protein A Dynabeads. The Dynabeads were removed using the magnetic stand, and the supernatant was collected and analyzed according to the Western blotting protocol.

Immunostaining for Δ Np63 (LabVision/NeoMarkers) and AR (N-20; Santa Cruz Biotechnology) was done as previously described (23, 26). Immunostaining for human Nkx3.1 was conducted as described previously (27).

UGSM isolation and tissue recombination. All animal studies were performed under the guidance of Institutional Animal Care and Use Committee-approved protocols. UGSM was isolated from E17 embryos of timed pregnant Sprague-Dawley rats (Harlan) according to previously reported protocols (28, 29). UGSM was dissociated into single cells using

Figure 1. Glandular regeneration *in vivo* from single-cell dissociations of adult human prostatic epithelia. **A**, detection of CD133⁺ cells in single-cell suspensions of whole prostate tissue. Fresh prostate tissue was dissociated into single cells and contained both stroma and epithelial cells. A FITC-conjugated pan-epithelial antibody (EpCAM) marked epithelial cells and a PE-conjugated anti-CD133 antibody (AC141, CD133-PE) was used to detect CD133⁺ cells. The majority of CD133⁺ cells are EpCAM⁺. **B** to **D**, recombination of rat UGSM with dissociated human epithelial cells regenerates human glandular structures when implanted under the renal capsule of a nude mouse. Single cells from dissociated human prostate epithelial organoids (devoid of stroma cells) were combined with rat UGSM cells isolated from day E17 rat embryos and placed under the renal capsule of male nude mice. **B**, glandular structures were observed in as few as 2 wk. **C**, centromere-telomere PNA FISH (Cen/Tel PNA FISH) showing human cells comprising the gland, with surrounding rodent stroma. A human- and mouse-specific FITC-labeled centromeric FISH probe (green) was combined with a Cy3-labeled telomere FISH probe (red), which highlights the longer rodent telomeres. Such a method allowed unambiguous discernment between cells of human (Cen-positive; Tel-dim), mouse (Cen-positive; Tel-bright), and rat (Cen-negative; Tel-bright) origin. **D**, *top left*, luminal expression of prostate-restricted Nkx3.1; *bottom left*, expression of Δ Np63 in the basal epithelial cells. *Right*, AR expression in the luminal epithelial cells and the surrounding rat stroma. Adjacent mouse renal parenchymal cells were AR⁻.



0.1% collagenase B (Boehringer Mannheim) in DMEM + 10% FCS at 37°C for 2 h. The single-cell suspensions of UGSM cells were washed thrice in DMEM + 10% FCS and counted. UGSM cells were recombined with human epithelial cells at a ratio of 2:1 in accordance with previous reports (40,000 UGSM cells to 20,000 prostate cells; ref. 30). Tissue recombinants were embedded in 10 μ L of type I collagen (BD Biosciences) that consisted of 88% collagen solution, 2% of 1 N NaOH, and 10% of 10 $\times$ PBS solution, which hardened on warming to room temperature. The recombinant implants were overlaid with DMEM + 10% FCS and 1 nmol/L R1881 and incubated at 37°C overnight and then implanted under the renal capsule of 4- to 6-wk-old male athymic nude mice. At various times up to 3 mo, the renal tissue was harvested, fixed in formalin, and serially sectioned for histologic analysis.

Centromere/telomere fluorescence *in situ* hybridization. Specimens underwent routine neutral-buffered formalin fixation followed by paraffin embedding. Slide preparation was performed without protease digestion as previously described (31). Briefly, 5- μ m tissue sections were deparaffinized, hydrated through a graded ethanol series, and underwent heat-induced antigen retrieval for 14 min in citrate buffer (target unmasking solution, Vector Laboratories, Inc.) using a vegetable steamer and placed into PBS + 0.1% Tween (Sigma) for 5 min. Fluorescence *in situ* hybridization (FISH)

was performed in the dark on sections by cohybridization of a custom-made NH₂-terminal Cy3-labeled peptide nucleic acid (PNA) probe that recognizes all mammalian telomeres (N-CCCTAACCTAACCTAA-C) and a NH₂-terminal FITC-labeled PNA probe that recognized both human and mouse centromeres (N-ATTCGTTGGAAACGGGA-C) but does not recognize rat centromeres (Applied Biosystems). PNA probes were incubated at 300 ng/mL in diluent [70% formamide, 10 mmol/L Tris (pH 7.5), 0.5% B/M blocking reagent (Boehringer-Mannheim)] at 83°C for 4 min followed by 2 h at room temperature. Sections were washed twice for 15 min with PNA wash buffer [70% formamide, 10 mmol/L Tris (pH 7.5), 0.1% BSA] followed by three 5-min washes in PBS + 0.1% Tween. Nuclei were counterstained with 0.05% 4',6-diamidino-2-phenylindole (Sigma). Sections were mounted using Prolong Anti-Fade Mounting Media solution (Invitrogen) and imaged with a Nikon 50i epifluorescence microscope equipped with an X-Cite series 120 illuminator and an attached Photometrics CoolsnapEZ digital camera.

Results

Single-cell suspensions of human adult PrECs contain gland-regenerating stem cells. Collagenase digestion of whole

human prostate tissue liberates epithelial aggregates, known as organoids, which can be separated from supporting stromal cells. Previous data documented that when these human prostatic epithelial organoids are injected s.c. with Matrigel into nude mice, a population of stem cells proliferates and gives rise to progeny regenerating stratified glands in which the luminal cells terminally differentiate and secrete PSA (25, 32). To evaluate whether the CD133-expressing cells are the prostate stem cells responsible for this regenerative ability, the organoid must be dissociated into single cells and the CD133 subpopulation was isolated and tested for its regenerative ability. In rodent prostates, stem cells are a minor fraction of the adult epithelium (33). This raises the issue of whether the available antibodies are sensitive enough to detect the potentially low number of CD133⁺ putative prostate stem cells. To evaluate this, the proportion of CD133⁺ cells was analyzed in nonfractionated (i.e., containing both stroma and epithelia) dissociated cells from fresh human prostate tissue using flow cytometry. As a second marker, the pan-epithelial surface antigen EpCAM was used to discern epithelial cells from stromal cells. Such analyses reveal a minor (i.e., <10%) subpopulation of CD133⁺ cells present of which >80% are of epithelial origin (i.e., EpCAM⁺; Fig. 1A). Notably, the percentage of CD133⁺ cells is higher in donors under the age of 30 versus those older than 50 years (i.e., 10–15% versus 1–5%, respectively).

To eliminate the population of CD133⁺ stromal cells, prostate tissue was collagenase digested and the epithelial organoids were collected. The prostate epithelial organoids were dissociated into single cells and implanted *in vivo* under the renal capsule of host nude mouse. No glandular formation was observed up to 3 months; these negative results are consistent with the known stromal requirement for prostate glandular morphogenesis (34). To provide such stromal support, coinoculation with UGSM was tested based on the established ability of UGSM to induce prostate epithelial organogenesis of human embryonic tissues and single cells *in vivo* (34, 35). Such single-cell recombination results in the regeneration of stratified prostate glands detectable as early as 2 weeks after inoculation (Fig. 1B).

To discern the cellular contribution of human, mouse, and rat cells to the regenerated glands, a novel technique was used, which takes advantage of unique genomic differences between all three species (rat, human, and mouse). Specifically, telomeres in commonly used inbred laboratory rodent strains are significantly longer than human telomeres (50–150 kb in rodent versus 5–10 kb in human), and this difference in length results in a notable difference in the intensity of telomeric FISH signals (36). Thus, rodent cells (mouse and rat) are easily distinguished from human cells by virtue of their very bright telomeres. The centromere-specific PNA probe used hybridizes to DNA repeats in human and mouse centromeres but does not hybridize to rat centromeres (37, 38). Thus, simultaneous staining with these centromere and telomere PNA FISH probes (Cen/Tel FISH) allows for rapid and unequivocal identification of species origin (human versus rat versus mouse) at the single-cell level in tissue hetero-recombinants. In these rat UGSM/human recombinants, Cen/Tel FISH confirmed the presence of human epithelial glands surrounded by rat stroma and adjacent to renal parenchymal cells of mouse origin (Fig. 1C).

Such human-derived glandular structures are positive for Nkx3.1, a PrEC-restricted protein (39), and contain a Δ Np63⁺ basal layer and an AR⁺ luminal layer (Fig. 1D). The stroma surrounding the human-derived glands is derived from the rat UGSM (Fig. 1C) and contains AR⁺ stromal cells, providing androgen-dependent

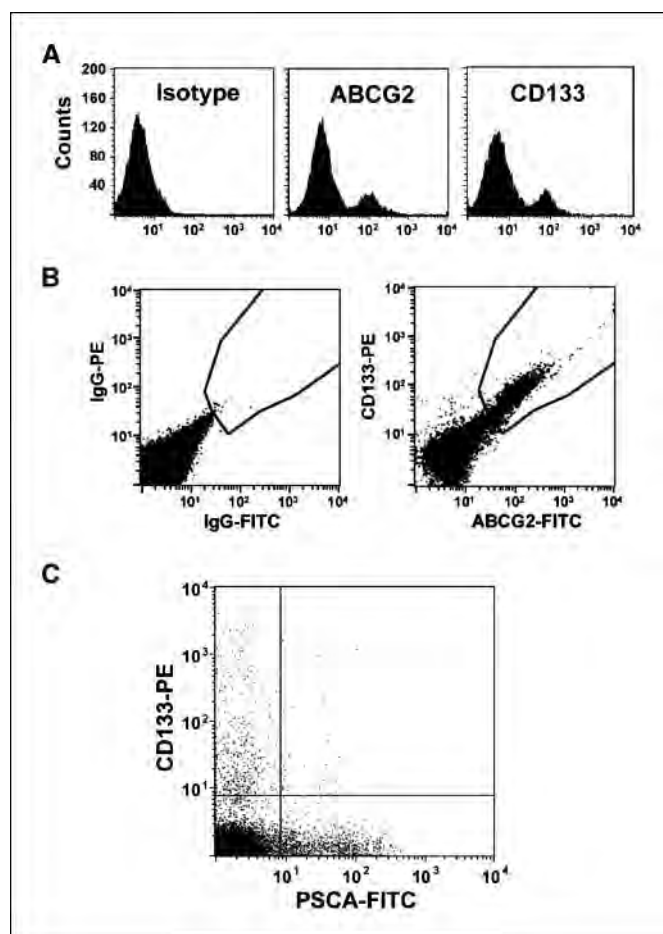


Figure 2. Expression of stem cell markers in PrEC cultures. A, ABCG2-FITC and CD133-PE (AC141) expression in commercially available PrECs. A nonspecific IgG was used as an isotype control. B, dual-label flow cytometry showing that CD133⁺ cells are also ABCG2⁺. C, CD133⁺ PrECs do not express the intermediate cell marker PSCA.

paracrine signaling (Fig. 1D). These data document that within single-cell suspensions of human adult epithelial cells, there are prostate stem cells that are capable of regenerating complete prostatic glandular structures and that the recombination of human prostate tissue with rodent UGSM is a useful assay for detecting such prostate stem cell capabilities. Thus, the presence of CD133⁺ cells is present within such single-cell dissociates, consistent with their being putative stem cells. However, their frequency within fresh tissue is so low that a culture method is needed to obtain sufficient numbers to test their stem cell ability in such an *in vivo* assay.

Phenotypic characteristics of CD133⁺ cells isolated from human prostate epithelial cultures. From freshly dissociated tissue, human PrECs can be routinely cultured and propagated for 8 to 10 serial passages using low-calcium, serum-free defined medium (i.e., either keratinocyte serum-free medium with ~100 μ mol/L calcium or PrEGM medium with ~300 μ mol/L calcium; ref. 23). By the second serial passage, such cultures are devoid of prostate fibroblasts and smooth muscle cells (5, 23). Early-passage cultures from a series ($n = 12$) of different commercial and in-house donors were analyzed for the expression of CD133, ABCG2, β_1 -integrin, Δ Np63, PSCA, AR, CD56, and chromogranin A. These analyses consistently documented that these cultures are

phenotypically heterogeneous being composed of at least four discernable subpopulations: (a) a minor population of small-sized putative stem cells ($CD133^+/ABCG2^+/\beta_1\text{-integrin}^+/\Delta Np63^-/PSCA^-/AR^-/CD56^-$; Fig. 2A–C), (b) a major population ($\sim 80\%$) of small- to intermediate-sized TA cells ($\Delta Np63^+/CD133^-/PSCA^-/AR^-/CD56^-$), (c) a minor population ($\sim 10\%$) of larger-sized intermediate cells ($PSCA^+/AR^+/CD133^-/\Delta Np63^-/CD56^-$), and (d) a minor population ($\sim 2\text{--}5\%$) of dendritic-shaped neuroendocrine cells ($CD56^+/\text{chromogranin A}^+/CD133^-/\Delta Np63^-/PSCA^-/AR^-$; refs. 5, 23).

Glycosylation-specific monoclonal antibodies prevent attachment and survival of $CD133^+$ cells. Western blot analyses were unable to detect CD133 protein expression in unsorted PrECs (Fig. 3A). As a positive control for these CD133 Western blots, the CaCo-2 human colon cancer cell line was used because it is uniformly $CD133^+$ (40). To increase the sensitivity of Western blot analysis, $CD133^+$ PrECs were enriched using live cell magnetic-activated cell sorting (MACS) with the AC133 antibody. This monoclonal antibody binding is glycosylation dependent, recognizing a carbohydrate-specific epitope on the extracellular loop of CD133 and has been used previously to isolate stem cells from a variety of nonprostate tissues (19). The AC133-enriched cells were subsequently lysed and subjected to Western blotting using an anti-CD133 rabbit monoclonal antibody (C24B9), which specifically recognizes a peptide epitope in the second extracellular loop of CD133 (19). This documented that AC133-enriched PrECs have a detectable level of a truncated and glycosylated 70-kDa form of CD133 but not full-length glycosylated 120- to 130-kDa CD133 protein detected in CaCo-2 cells (Fig. 3B). Full-length CD133 protein is expressed, however, by a subset of PrECs before binding

the AC133 antibody. This is documented by the observation that lysis of unsorted PrECs followed by immunoprecipitation using a second glycosylation-specific antibody (i.e., AC141) revealed a full-length form of CD133 in addition to the smaller 70-kDa form (Fig. 3C).

Binding of the glycosylation-specific AC133 antibody results not only in truncation of the glycosylated 120-kDa full-length CD133 protein but also in the inability of antibody-associated $CD133^+$ PrECs to attach and grow *in vitro*. This was initially observed when $CD133^+$ PrECs were isolated via FACS using the anti-CD133-PE-conjugated AC141 mouse monoclonal antibody and consistently exhibited an inability to attach and spread when replated in culture, resulting in their eventual death. A variety of culture conditions were used in an attempt to improve the survival and growth of AC141-isolated PrECs cells, including poly-D-lysine or type I collagen coating, adding either conditioned medium from unfractionated PrEC cultures or culturing on an irradiated feeder layer (i.e., mouse STO cells). All conditions failed to increase the viability and growth of the AC141-sorted PrECs. A series of controls were used to document that specific binding of the AC141 antibody uniquely inhibits PrECs attachment and growth after sorting. First, sham-sorted or EpCAM-sorted PrECs attach and proliferate in a manner similar to nonmanipulated PrECs; second, FACS isolation using the same carbohydrate-specific AC141 monoclonal antibody to isolate $CD133^+$ cells from the CaCo-2 human colon cancer line yielded viable cells, which attach and grow equally well as unsorted CaCo-2 cells. In addition, to test whether this inhibition is unique to the AC141 monoclonal antibody or is a general property of antibodies that bind the carbohydrate portion of CD133 on PrECs, MACS isolation using the AC133 antibody yielded similar results, whereby $CD133^+$ PrECs failed to attach and grow. These combined results document that the glycosylation-specific AC141 and AC133 anti-CD133 antibodies inhibit the attachment and growth of $CD133^+$ PrECs in a cell context-dependent manner.

$CD133^+$ cells regenerate phenotypically heterogeneous PrEC cultures. In contrast to the negative results using the glycosylation-specific AC141 and AC133 antibodies, FACS sorting and subsequent growth of $CD133^+$ PrECs was possible using the peptide-specific C24B9 rabbit monoclonal antibody. $CD133^+$ PrECs were sorted by flow cytometry to generate a $>98\%$ pure population (Fig. 4A and B). Western blot analysis of flow-sorted $CD133^+$ PrECs revealed that the expression of $\Delta Np63$ is below the level of detection (Fig. 4C). This $CD133^+$ population was placed back into culture and tested for its ability to regenerate all the cell populations present within heterogeneous PrEC cultures. By 2 weeks, the cultures had undergone approximately six population doublings and were morphologically identical to those of unsorted parental PrEC cultures, being composed of a heterogeneous mixture of small-, medium-, and large-sized epithelial as well as dendritic-shaped cells. $CD133$ -derived cultures maintain a $CD133^+$ population (Fig. 4D) and also regenerate a $\Delta Np63^+$ population of TA cells (Fig. 4C), a population of $PSCA^+$ intermediate cells, and a population of $CD56^+$ neuroendocrine cells (Fig. 4D). Flow cytometry documented that by 2 weeks, the number of $CD133^+$ cells is approximately six times greater than the initial number of $CD133^+$ cells plated, indicating that the $CD133^+$ cells not only renew themselves but also give rise to progeny of two distinct cell lineages, the neuroendocrine cell lineage and the TA cell lineage, where a subset of $\Delta Np63^+$ TA cells matures to form $PSCA^+$ intermediate cells. In contrast to the ability of $CD133^+$ cells to regenerate PrEC cultures, flow-sorted $PSCA^+$ and $CD56^+$ PrECs

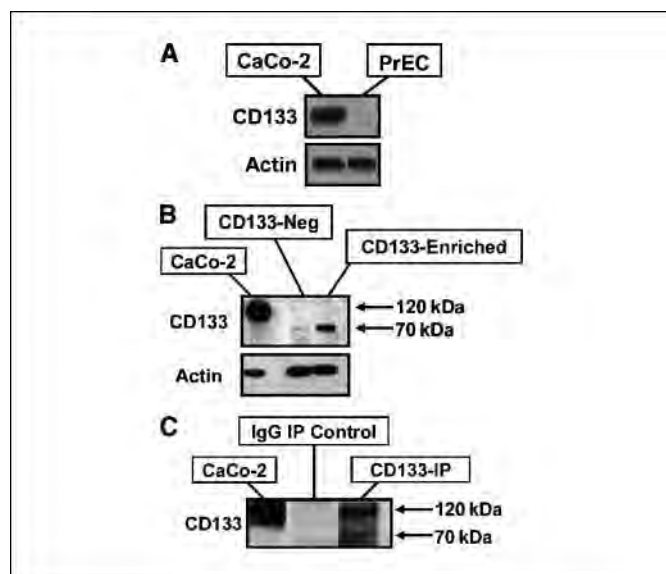


Figure 3. Molecular weight of CD133 in PrECs. A, Western blot of nonfractionated PrECs compared with the $CD133^+$ CaCo-2 colon cancer cell line. Actin was used as a loading control. B, Western blot of $CD133$ -enriched PrECs and CaCo-2 cells. PrECs and CaCo-2 cells were subjected to magnetic enrichment of CD133 (AC133)-positive cells and the expression of CD133 compared with non- $CD133$ -expressing PrECs ($CD133\text{-Neg}$). Actin was used as a loading control. Western blotting using the peptide-derived CD133 antibody (293C3) reveals a lower molecular weight form of CD133 expressed in the AC133-enriched PrECs compared with CaCo-2 cells. C, immunoprecipitation (IP) of AC141 from lysed PrECs and CaCo-2 cells and Western blotting using the peptide-derived anti-CD133 antibody reveal the expression of CD133 at the correct molecular weight in addition to the smaller 70-kDa form.

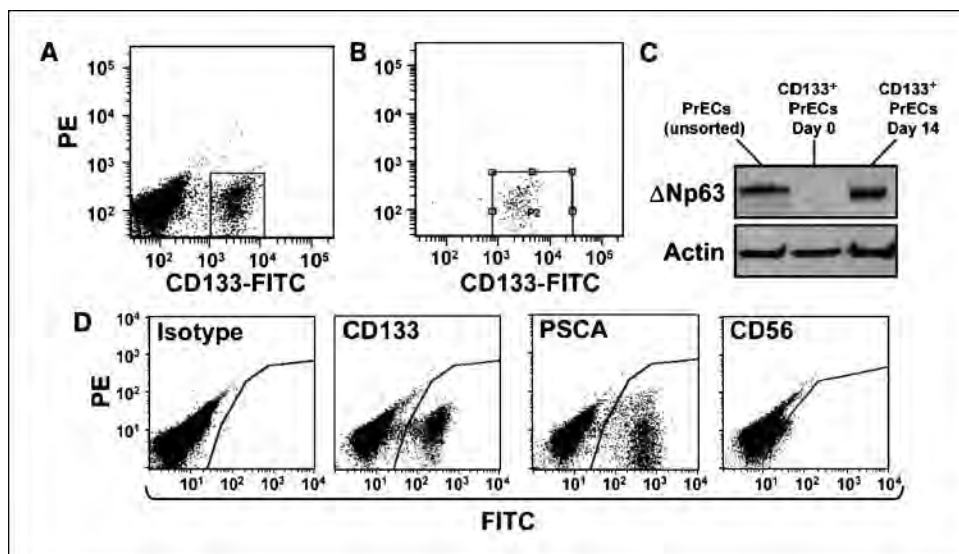


Figure 4. Pure populations of CD133⁺ cells regenerate PrEC cultures. **A**, flow cytometric analysis of PrECs using the peptide-derived 293C3 anti-CD133 antibody. CD133⁺ cells (box) were isolated via FACS. **B**, FACS yields a population of CD133⁺ cells, which is >98% pure. CD133⁺ cells were either replated for growth or lysed for Western blotting. **C**, expression of the basal marker Δ Np63 in CD133⁺ PrECs is below the level of detection but is restored after 14 d in culture. Actin was used as a loading control. **D**, cultures established from pure CD133⁺ cells contain an expanded number of CD133⁺ stem cells as well as PSCA⁺ intermediate cells and CD56⁺ neuroendocrine cells. A nonspecific IgG was used as an isotype control. Thus, cultures derived from pure populations of CD133⁺ cells contain the original progenitor population (CD133⁺ cells) and two distinct cell lineages (basal-intermediate and neuroendocrine).

attached but did not grow when placed back into culture. These data document that CD133⁺ PrECs are both self-renewing and capable of generating progeny of two distinct cell lineages (neuroendocrine and TA) and are thus bona fide prostate stem cells.

CD133⁺ human prostate cancer cells have cancer-initiating ability. Similar to normal prostate epithelial cultures, the frequency of CD133⁺ cells within a series of human prostate cancer cell lines is so low that the protein is below detection in mass culture by Western blot analysis. Using more sensitive flow cytometry, however, prostate cancer lines do contain a minor population (~1–5%) of CD133⁺ malignant cells (Fig. 5A). These results raise the issue of whether the CD133⁺ cancer cells have cancer-initiating ability. Therefore, to test if CD133⁺ prostate cancer cells have these abilities, three androgen ablation refractory prostate cancer cell lines (LNCaP, LAPC-4, and CWR22Rv1) were analyzed for their clonogenic ability, percent CD133⁺ cells, and their ability to generate progeny with a different phenotype. These lines were chosen to be representative of the range of lethal metastatic prostate cancers observed clinically because LAPC-4 expresses wild-type AR (41), LNCaP expresses point mutated AR (42), and CWR22Rv1 expresses exon 3 duplicated AR and exon 2 truncated isoform of AR protein (43). Initially, the clonogenic ability of unfractionated cultures for all three cell lines was determined based on formation of colonies (>10 cells) within 10 days (Fig. 5B). These clonogenic abilities were 5- to 40-fold higher than the percentage of CD133⁺ cells in the three lines (Fig. 5C). These results are explainable by the fact that either (a) CD133⁺ cells are not CICs or (2) CD133⁺ cells are CICs with unlimited ability to self-renew but the majority of their progenies become CD133⁻ with a sufficient proliferative ability to form the vast majority of colonies in primary clonogenic assay but not unlimited proliferative ability to form colonies in serial clonogenic assays. If the first possibility is true and CD133⁺ cells are present at <5% (Fig. 5C), then 5 to 10 individual clones derived from each of these lines should be negative for CD133 expression. In contrast, if CD133⁺ cells are the CICs, then serially passaged cultures initially derived from single-cell clones should always be heterogeneous, containing mostly CD133⁻ cells and a small fraction of CD133⁺ cells. Thus, to resolve between these possibilities, multiple clones containing >200 cells were

isolated from LNCaP, LAPC-4, and CWR22Rv1 lines and the clones were serially propagated for >25 population doublings (i.e., >50 days) before being analyzed by flow cytometry. These analyses documented that all of the clones from LNCaP ($n = 5$ clones), LAPC-4 ($n = 8$ clones), and CWR22Rv1 ($n = 6$ clones) contain about 1% to 5% CD133⁺ cells consistent with their CIC ability.

To directly test whether CD133⁺ cells give rise to CD133⁻ progeny with limited proliferation ability, CWR22Rv1 cells were flow sorted using the AC141-PE antibody. In contrast to the poor survival of PrECs after sorting with the AC141 antibody, CWR22Rv1 cells exhibited no differences in viability after sorting using the AC141 antibody (7 days after inoculating 2,000 cells, there were 81,500 cells in the CD133-sorted versus 84,800 cells in the mock-sorted control group). Further propagation of the CD133-derived CWR22Rv1 cultures revealed that the percentage of CD133⁺ cells was only 6.15% after 2 weeks in culture. Thus, although these cultures were initiated from sorted cells, which were >98% CD133⁺, the population of CD133⁺ cells is maintained at the same low level as that of the unsorted cultures, with the majority (93.85%) of the progeny in the expanded cultures no longer expressing CD133. The clonogenic ability of flow-sorted CD133⁺ CWR22Rv1 cells is 2.4 times greater than the unsorted population and the average colony size was two times larger by 10 days (Fig. 5D). These combined results document that the CD133⁺ prostate cancer cells have the defining characteristics of CICs because they are present at low frequency, self-renew, exhibit unlimited proliferative capacity, and give rise to phenotypically different progeny with a lower growth potential.

CD133⁺ human prostate cancer cells express and respond to AR. To determine whether the CD133⁺ CICs in human prostate cancer express AR protein, two-variable flow cytometry was used. These results document that ~98% of the CD133⁺ populations in the LAPC-4, LNCaP, and CWR22Rv1 cell lines are positive for the AR (Fig. 6). As expected, there is a very small (>2%) population of AR⁻ cells present within these exponentially growing cultures, which is consistent with our previous observation that prostate cancer cells degrade AR during mitosis (26). As a functional test to evaluate the signaling ability of the AR expressed in CD133⁺ cells, the clonogenic ability of LNCaP cells was tested using a growth-inhibitory dose of androgen (i.e., 10 nmol/L of the synthetic androgen R1881; ref. 44). This is based on the rationale that if

AR signaling is not occurring in the cancer-initiating CD133⁺ LNCaP cells, then there will be no effect of high-dose androgen on the clonogenic ability of these cells. In contrast, high-dose androgen decreases by >95% the clonogenic ability of LNCaP cells ($2.3 \pm 0.8\%$ versus $0.18 \pm 0.1\%$ clonogenic ability of untreated versus androgen-treated cells).

Discussion

The concept of adult prostate stem cells first emerged to explain the immense capacity of the epithelial compartment for cyclic regeneration. Previous studies document that the prostate can undergo >30 successive cycles of androgen deprivation and replacement without diminishing its ability for continued epithelial regeneration (45). In the present study, we document that CD133 marks both normal prostate epithelial stem cells as well as malignant prostate CICs.

We identified CD133⁺ cells in PrEC cultures and showed that pure populations of CD133⁺ cells are able to regenerate PrEC cultures and exhibit characteristics of stem cells by their ability to self-renew and regenerate PrEC cultures with two distinct cell lineages. A disadvantage of the low-Ca²⁺ SFD culture conditions used to establish and propagate PrEC cultures is that luminal differentiation (i.e., AR expression, terminal differentiation, and PSA expression) does not occur within these cultures. This is due to the activation of Notch signaling and the inhibition of E-

cadherin signaling, both of which prevent terminal differentiation (23, 46). As such, using our current culture conditions, the prostate stem cell compartment is unable to complete its full differentiation potential and progress only to the intermediate cell stage.

The expanding use of CD133 as a human stem cell marker has yielded a variety of antibodies for the isolation and characterization of CD133⁺ stem cell populations. The glycosylation-specific AC133 and AC141 anti-CD133 antibodies were the first to be developed and aided the identification of the *CD133* gene (19). However, the binding of such antibodies to normal prostate stem cells profoundly inhibited their attachment and growth after cell sorting. The same antibodies, however, did not affect the attachment and growth of CD133⁺ prostate cancer cells. These observations document that CD133 functions differently between normal and malignant prostate cells and that the glycosylation sites of CD133 play a significant role in the function of normal prostate stem cells.

If prostate cancer were derived from a transformed normal stem cell, one would expect that prostate CICs do not express AR, give rise to Δ Np63⁺ progenies that differentiate into AR⁺ cells, and do not respond to androgen-mediated growth inhibition. Prostate cancers exhibit characteristics of intermediate and luminal-secretory epithelial cells because they express AR, PSA, and PSCA (47, 48). Furthermore, a hallmark of prostate cancer is the loss of the basal cell marker Δ Np63 (49), and normal prostate stem cells do not express AR and are thus not dependent on androgen for their survival. We document that CD133⁺ prostate CICs express AR

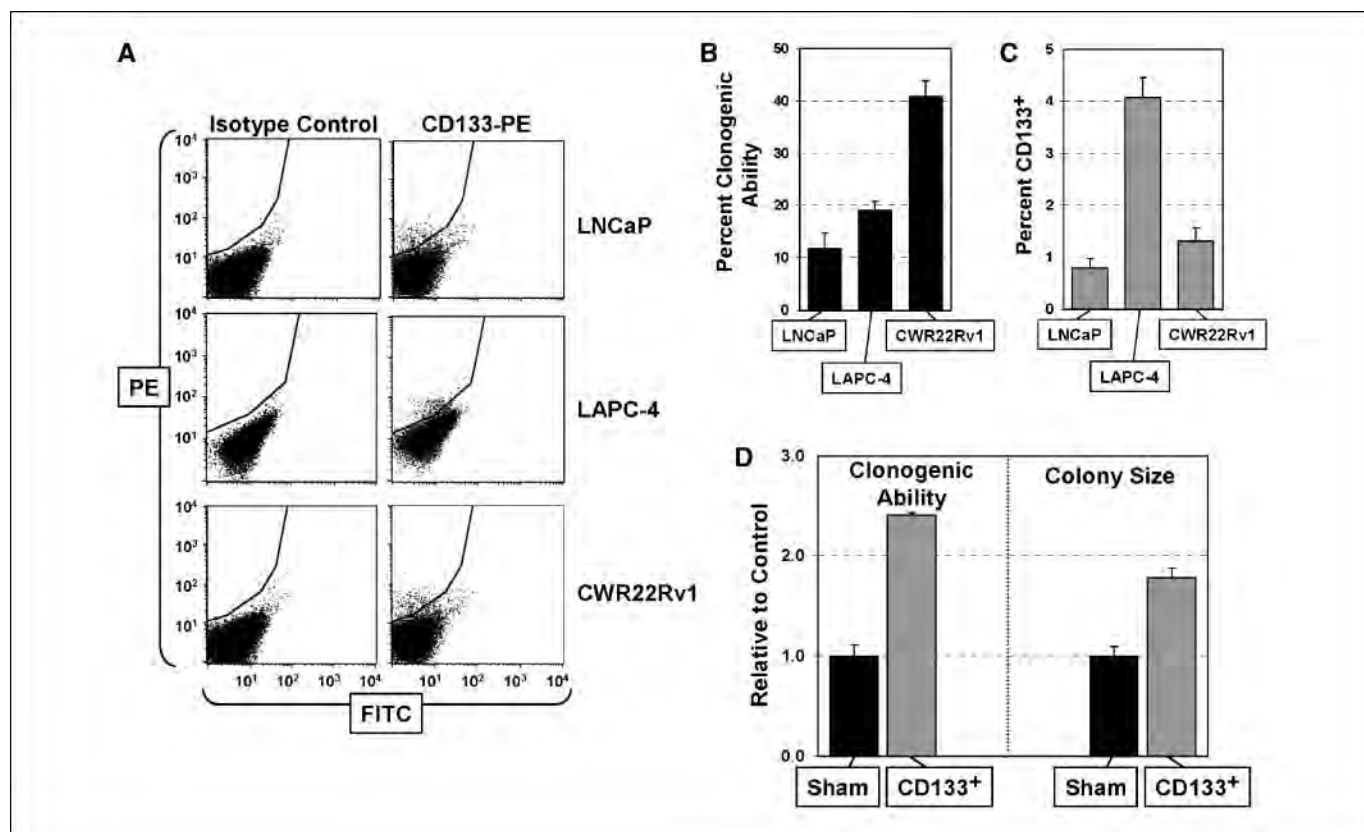


Figure 5. CD133 marks prostate CICs. *A*, flow cytometric analyses revealed minor populations of CD133⁺ cells in three androgen-responsive prostate cancer cell lines: LNCaP, LAPC-4, and CWR22Rv1. A PE-conjugated CD133 antibody (CD133-PE) and a nonspecific IgG were used as controls. *B*, clonogenic ability of LNCaP, LAPC-4, and CWR22Rv1 cells to form microscopic clones over 10 d. Clones were stained and counted at $\times 10$ magnification. *C*, percentage of CD133⁺ cells in LNCaP, LAPC-4, and CWR22Rv1 cultures. These data represent the average of six individual analyses. *D*, CD133⁺ CWR22Rv1 prostate cancer cells are more clonogenic and form larger clones compared with the sham-sorted control cells.

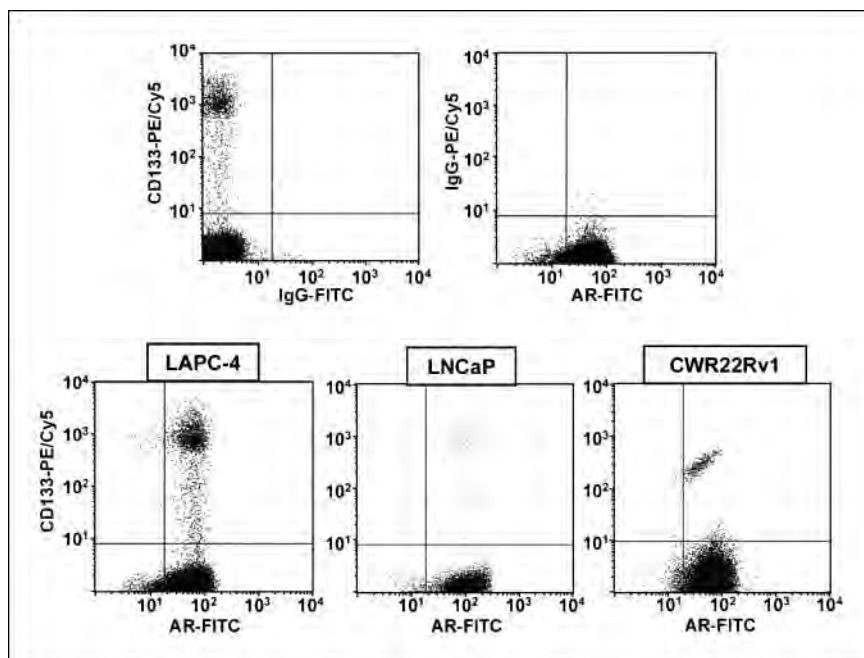


Figure 6. CD133⁺ prostate cancer cells express the AR. Dual-variable flow cytometry of CD133 (AC141) and AR in the LAPC-4, LNCaP, and CWR22Rv1 prostate cancer cell lines. *Top*, control staining in LAPC-4 using IgG antibodies (*top left*, CD133 versus IgG; *top right*, IgG versus AR). Similar controls were documented in the LNCaP and CWR22Rv1 cell lines. *Bottom*, dual labeling for CD133 and AR in LAPC-4, LNCaP, and CWR22Rv1 prostate cancer cells showing that CD133⁺ prostate cancer cells also express AR.

and are subject to AR-mediated growth inhibition. The data presented here are consistent with prostate CICs being derived from a malignantly transformed intermediate cell, which has gained the expression of the stem cell marker CD133. Such an observation shows that prostate CICs are valid targets for the continued development of improved antiandrogen therapies.

Disclosure of Potential Conflicts of Interest

J.T. Isaacs: commercial research grant, Active Biotech; ownership interest, Genspera and Protox; consultant/advisory board, Wiley Publishing. The other authors disclosed no potential conflicts of interest.

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