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CD133 does not enrich for the stem cell activity *in vivo* in adult mouse prostates

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ABSTRACT

CD133 is widely used as a marker for stem/progenitor cells in many organ systems. Previous studies using *in vitro* stem cell assays have suggested that the CD133-expressing prostate basal cells may serve as the putative prostate stem cells. However, the precise localization of the CD133-expressing cells and their contributions to adult murine prostate homeostasis *in vivo* remain undetermined. We show that loss of function of CD133 does not impair murine prostate morphogenesis, homeostasis and regeneration, implying a dispensable role for CD133 in prostate stem cell function. Using a CD133-CreER^{T2} model in conjunction with a fluorescent report line, we show that CD133 is not only expressed in a fraction of prostate basal cells, but also in some luminal cells and stromal cells. CD133^{+} basal cells possess higher *in vitro* sphere-forming activities than CD133^{-} basal cells. However, the *in vivo* lineage tracing study reveals that the two cell populations possess the same regenerative capacity and contribute equally to the maintenance of the basal cell lineage. Similarly, CD133^{+} and CD133^{-} luminal cells are functionally equivalent in maintaining the luminal cell lineage. Collectively, our study demonstrates that CD133 does not enrich for the stem cell activity *in vivo* in adult murine prostate. This study does not contradict previous reports showing CD133^{+} cells as prostate stem cells *in vitro*. Instead, it highlights a substantial impact of biological contexts on cellular behaviors.

1. Introduction

CD133, also known as Prominin-1, is a pentaspan transmembrane glycoprotein (Miraglia et al., 1997) containing two large extracellular and two small intracellular loops. Since its discovery in 1997 (Yin et al., 1997; Weigmann et al., 1997), CD133 has been shown to be widely expressed in many tissues, including kidney (Weigmann et al., 1997), pancreas (Oshima et al., 2007), liver (Kordes et al., 2007; Karbanova et al., 2008), mammary glands (Florek et al., 2005), and prostate (Richardson et al., 2004; Missol-Kolka et al., 2011), *etc.* To this end, only very limited knowledge has been obtained regarding the physiological function of CD133 in development. For examples, loss of CD133 in mice results in defects in disk formation of photoreceptors (Zacchigna et al., 2009) and mutations in CD133 are associated with some human ocular diseases (Maw et al., 2000; Yang et al., 2008; Zhang et al., 2007). Despite a lack of understanding of CD133-mediated biology, it has been shown to serve as a marker for stem cells or progenitors in many organ systems, such as the hematopoietic system (Yin et al., 1997), brain (Weigmann et al., 1997), small intestine (Zhu et al., 2009), muscle (Alessandri et al., 2004), skin (Belicchi et al., 2004), as well as prostate (Richardson et al., 2004; Missol-Kolka et al., 2011).

However, there are some controversial reports. For example, Zhu et al. reported that CD133 is co-expressed with stem cell marker Lgr5 in cells at the base of small intestine crypts and those cells are able to generate the entire small intestinal epithelia (Zhu et al., 2009). But the other study showed that CD133 is expressed in both the Lgr5^{+} intestinal stem cells and the transit-amplifying progenitors (Snippert et al., 2009). In contrast to its role as a stem/progenitor cell marker, Shmelkov et al. reported a ubiquitous expression of CD133 in the differentiated colonic epithelia (Shmelkov et al., 2008). In the human prostate, an $\alpha_2\beta_1^{hi}$/CD133^{+} basal population has been shown to display higher *in vitro* colony-forming ability and was capable of generating prostatic tissues when transplanted subcutaneously in immunodeficient mice (Richardson et al., 2004). A small fraction of human primary prostate epithelial cells expressing CD133 can self-renew and regenerate whole cell populations (Dail et al., 2014). However, Missol-Kolka et al. reported that CD133^{+} cells can be detected in both human and rodent

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prostate luminal cells (Missol-Kolka et al., 2011), indicating that CD133 may not be exclusively expressed in the basal stem cells.

These controversies could be accounted for by the specificity of the CD133 antibodies utilized in different studies. The mature form of CD133 on the plasma membrane is highly glycosylated in the extracellular loops (Miraglia et al., 1997). AC133, which is a glycosylation-dependent epitope of CD133, has been utilized as a cell surface marker to isolate human hematopoietic stem cells (Yin et al., 1997). In the prostate, a rare population of $\alpha_2\beta_1^{hi}$/CD133$^+$ cells was detected in the basal layer of human prostate by the AC133 antibody (Richardson et al., 2004). In contrast, some luminal cells were also shown positive when stained with other antibodies against synthetic CD133 peptides (Missol-Kolka et al., 2011).

In this study, we took advantage of a CD133-CreER^{T2} mouse model that enables the expression of the CreER^{T2} transgene driven by the endogenous CD133 promoter. We examined the localization of CD133-expressing cells in different prostate lineages by breeding the CD133-CreER^{T2} mice with a fluorescent report line. We also employed the model to determine whether CD133-expressing cells function as stem cells *in vivo* by lineage tracing.

2. Materials and methods

2.1. Mice

The CD133-CreER^{T2} mice were purchased from Jackson Laboratory (Bar Harbor, ME). The Rosa26-eYFP mice were obtained from Dr. Andrew Groves at Baylor College of Medicine. Mice were genotyped by polymerase chain reaction (PCR) using mouse genomic DNA from tail biopsy specimens. The sequences of genotyping primers for CreER^{T2} are (forward) 5′-CCTGACAGTGACGGTCCAAAG-3′, and (reverse) 5′-CATGACTCTTCAACTCAAACT-3′. The expected band size for CreER^{T2} PCR is 700 bp. The sequences of genotyping primers for Rosa26-eYFP are (forward) 5′-CTCTGCTGCCTCCTGGCTTCT-3′, (wild-type reverse) 5′-CGAGGCGGATCACAAGCAATA-3′, and (transgene reverse) 5′-TCAA TGGGCGGGGGTCGTT-3′. The expected sizes of PCR products for homozygous, heterozygous, and wild type mice are 250 bp (single band), 250 bp and 330 bp (two bands), and 330 bp (single band), respectively. PCR products were separated electrophoretically on 1% agarose gels and visualized *via* ethidium bromide under UV light.

2.2. Tamoxifen treatment

Tamoxifen (Sigma-Aldrich, St Louis, MO) was dissolved into vegetable oil and was administrated intraperitoneally into experimental mice at the age of 5 weeks (4 mg per 40 g per day for 4 consecutive days).

2.3. Castration and androgen replacement

Experimental mice were castrated at the age of 8 weeks using standard techniques (Valdez et al., 2012). Two weeks after castration, androgen pellets (15 mg/pellet, Sigma-Aldrich, St Louis, MO) were placed subcutaneously to restore serum testosterone level and stimulate prostate regeneration for two weeks. Subsequently, androgen pellets were removed to re-induce prostate regression for two weeks and were replaced to re-induce regeneration for another two weeks.

2.4. RNA in situ hybridization

Adult mice were treated with tamoxifen for 4 consecutive days and sacrificed on the fifth day. Freshly dissected kidney tissues were embedded in O.C.T. Compound (Sakura, Alphen aan den Rijn, Netherlands), and frozen on liquid nitrogen. CD133 and eYFP transcripts were detected in frozen kidney sections by the DesignReady eGFP-Quasar 570 probe set (VSMF-1014-5, Biosearch Technologies, Petaluma, CA), and custom CD133-Quasar 670 probe set (Biosearch Technologies, Petaluma, CA). Sequences for the CD133-Quasar 670 probe set are listed in Supplementary Table 1. The RNA *in situ* hybridization assay was performed as described previously (Facciponte et al., 2014).

2.5. Prostate sphere and prostate organoid assays

Dissociated single prostate cells were prepared as described previously (Valdez et al., 2012). Briefly, prostate tissues were digested in Dulbecco's modified eagle medium (DMEM)/F12/collagenase/hyaluronidase/FBS (StemCell Technologies, Vancouver, Canada) for 3 h at 37 °C, followed by a one-hour digestion in 0.25% Trypsin–EDTA (Invitrogen, Carlsbad, CA) on ice. Subsequently, digested cells were suspended in Dispase (Invitrogen; 5 mg/ml) and DNase I (Roche Applied Science, Indianapolis, IN; 1 mg/ml), and pipetted vigorously to dissociate cell clumps. Dissociated cells were then passed through 70 μm cell strainers (BD Biosciences, San Jose, CA) to get single cells.

The prostate sphere assay was performed as described previously (Xin et al., 2007). Briefly, 1–2 × 10^4 dissociated prostate cells were cultured in 1:1 Matrigel/PrEGM (Matrigel (BD Biosciences, San Jose, CA)/PrEGM (Lonza, Walkersville, MD)). Prostate spheres were defined as spheroids with a diameter >30 μm after a 6-day culture.

The organoid culture was performed following the previous study (Karthaus et al., 2014; Kwon et al., 2015). Briefly, murine prostate epithelial cells were cultured in DMEM/F12 supplemented with B27 (Life technologies, Grand Island, NY), 10 mM of HEPES, Glutamax (Life technologies, Grand Island, NY), Penicillin/Streptomycin, and the following growth factors: EGF 50 ng/ml (Peprotech, Rocky Hill, NJ), 500 ng/ml of recombinant R-spondin1 (Peprotech, Rocky Hill, NJ), 100 ng/ml of recombinant Noggin (Peprotech, Rocky Hill, NJ), 200 μM of TGF-β/Alk inhibitor A83-01 (Tocris, Ellisville, MO), and 10 μM Y-27632 (Tocris, Ellisville, MO). Dihydrotestosterone (Sigma, St. Louis, MO) was added at 1 nM final concentration. 1–2 × 10^3 dissociated prostate cells were mixed with growth factor reduced matrigel (Corning, Corning, NY) by 1:1 ratio and plated in 96-well plates.

2.6. Cloning and generation of CD133 lentivirus

The cDNA encoding human CD133 was obtained from Dr. Donald Vander Griend at the University of Chicago. The cDNA was PCR amplified, verified by sequencing and cloned into the FU-CRW lentiviral vector using NheI (Xin et al., 2006). The primer sequences for human CD133 are (forward) 5′-CTAGCTAGCGCCACCATGGCCCTCGTACTCGGCT CC-3′ and (reverse) 5′-TGGGCTAGCTCACTTGTCGTCATCGTCTTTGTAG TCTCAATGTTGTGATGGGCTTG-3′. Lentivirus preparation and tittering were performed as described previously (Xin et al., 2003).

2.7. FACS

For the separation of prostate cell lineages, dissociated murine prostate cells were stained with Pacific blue-anti CD31, CD45 and Ter119 antibodies (eBioscience, San Diego, CA), PE-anti Sca-1 antibody (eBioscience, San Diego, CA), Alexa 647-anti CD49f antibody (Biolegend, San Diego, CA). PE-anti CD133 clone 13A4 (eBioscience, San Diego, CA) and clone 315-2C11 (Biolegend, San Diego, CA) were used to detect CD133. FACS analyses and sorting were performed by using the BD LSR II and Aria I, respectively (BD Biosciences, San Jose, CA).

2.8. RNA isolation and qRT-PCR

Total RNA was isolated from cells using the RNeasy Plus mini kit (Qiagen, Valencia, CA). Reverse transcription was performed using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using the SYBR Premix Ex Taq (Perfect Real Time; Takara Bio Inc., Otsu, Shiga, Japan) on a StepOne plus Real-Time PCR system (Applied Biosystems,