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Identification of CD166 as a Surface Marker for Enriching Prostate Stem/Progenitor and Cancer Initiating Cells

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Abstract

New therapies for late stage and castration resistant prostate cancer (CRPC) depend on defining unique properties and pathways of cell sub-populations capable of sustaining the net growth of the cancer. One of the best enrichment schemes for isolating the putative stem/progenitor cell from the murine prostate gland is Lin−/Scal−/CD49fhi (LSChi), which results in a more than 10-fold enrichment for in vitro sphere-forming activity. We have shown previously that the LSChi subpopulation is both necessary and sufficient for cancer initiation in the Pten-null prostate cancer model. To further improve this enrichment scheme, we searched for cell surface molecules upregulated upon castration of murine prostate and identified CD166 as a candidate gene. CD166 encodes a cell surface molecule that can further enrich sphere-forming activity of WT LSChi and Pten null LSChi. Importantly, CD166 could enrich sphere-forming ability of benign primary human prostate cells in vitro and induce the formation of tubule-like structures in vivo. CD166 expression is upregulated in human prostate cancers, especially CRPC samples. Although genetic deletion of murine CD166 in the Pten null prostate cancer model does not interfere with sphere formation or block prostate cancer progression and CRPC development, the presence of CD166 on prostate stem/progenitors and castration resistant sub-populations suggest that it is a cell surface molecule with the potential for targeted delivery of human prostate cancer therapeutics.

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Introduction

Despite advances in the early detection and management of prostate cancer, castration resistant prostate cancer (CRPC) remains the second most common cause of male mortality in the United States [1]. Mounting evidence suggests that a subpopulation of prostate cancer cells can initiate prostate cancer and may be responsible for the castration resistance [2,3,4,5]. Therefore, these cancer initiating cells [6] may serve as promising cellular targets for prostate cancer and identification of this subpopulation has become the necessary step toward future effective therapy.

The origins of prostate cancer initiating cells are controversial [7,8]. Normal prostate from human or mouse contains three different types of cells, namely luminal secretory, basal and neuroendocrine cells. Since human prostate cancer is characterized by loss of basal cells and expansion of luminal cells, several animal models posit that luminal-specific progenitors are the sources of prostate cancer initiation [9,10,11]. However, using the tissue regeneration approach, basal cells have proved to be more efficient oncogenic targets for both human and mouse prostate cancer initiation [12,13]. Interestingly, Xin’s group demonstrated that adult murine prostate basal and luminal cells are self-sustained lineages that can both serve as oncogenic targets for prostate cancer initiation [14].

PTEN plays an important role in human prostate cancer and CRPC development [15] and is inactivated in 20% of primary and 60% of metastatic lesions [16]. The murine Pten prostate cancer model (Pb-Cre+/Pten−/−) recapitulates the disease progression seen in humans, including CRPC [17,18,19,20], and shares many signature genetic changes with human disease [17]. Importantly, the Pb-Cre+/Pten−/− model provides a unique tool for studying tumor initiating cells as the majority of luminal cells and subpopulations of basal cells have Pten deletion [17,18]. Using this model, we demonstrated that Pten deletion causes an...
CD166 Enriches Prostate Cancer Sphere Forming Cell

Results

CD166 Expression is Upregulated in Murine Castrated Prostatic Epithelium and can be used for Enriching Stem/progenitor Cells

Rodent prostate contains stem-like cells that are enriched in the castrated prostate gland and can undergo more than 15 cycles of involution-regeneration in response to androgen withdrawal and replacement [21]. We reasoned that castration may also lead to upregulation or enrichment of those stem cell surface molecules that can potentially serve as marker for isolating stem/progenitor cells and for targeted drug delivery. We therefore mined publically available databases describing gene expression profiles of murine prostates at day 0 and day 3 post-castration [22,23]. We focused on those genes that fell in the gene ontology category of ‘plasma membrane’ and identified CD166/ALCAM as one of only two common castration-enriched cell surface molecules (Table S1). CD166 was significantly increased (1-tail t-test <0.015) 3 days after castration as compared to intact mice. While Cxcl12 is also upregulated, we chose not to focus on this gene as it is a chemokine and not amenable for FACS-mediated stem/progenitor cell enrichment.

CD166 is a type I transmembrane protein of the Ig superfamily that mediates cell-cell interactions via heterophilic (CD166-CD6) and/or homophilic (CD166-CD166) mechanisms [24,25]. We found that in the intact mice, CD166 is preferentially expressed in the stem/progenitor-enriched proximal region [21] but low in the stem/progenitor-poor distal region of the WT prostate (Figure 1A upper panels). CD166 protein levels are also up-regulated immediately following castration (Figure 1A lower panels; comparing day 0 and day 3 post-castration).

Prostate stem/progenitor cells are characterized by their ability to form spheres in vitro [26]. We performed the sphere-forming assay using sorted CD166hi and CD166lo cells and found that CD166hi cells have significantly higher sphere-forming activity compared to CD166lo cells (Figure 1B, left). Since we had previously developed the LSChi enrichment scheme [26], which yields 10-fold enrichment of WT sphere-forming cells, we tested whether CD166 can be used for further enriching sphere-forming activity. We gated LSChi cells according to their CD166 expression and found that LSChi;CD166hi cells have 5-fold higher sphere-forming activity as compared to their LSChi;CD166lo counterpart (Figure 1B, right). Therefore, CD166 can be used as a marker to further enrich sphere forming cells within the WT prostate. Serial passaging of the spheres generated from LSChi;CD166hi cells demonstrated that this enhanced sphere-forming activity could be maintained in vitro through at least three passages (Figure S1A). In contrast, less spheres were generated from LSClo;CD166lo cells (P0–P2) and cannot undergo continuous passage due to the limited cell number. We observed no significant difference in the sphere size distribution between LSChi;CD166hi generated spheres and LSChi;CD166lo generated spheres (Figure S1B and S1C). Similar to the LSChi subpopulation [26], castration also leads to significant enhancement of the LSChi;CD166lo subpopulation (Figure 1C).

CD166hi Human Prostate Cells Have Higher Sphere Forming and Regeneration Potential

Certain cell surface markers, such as Sca-1, are only expressed in the mouse and therefore cannot be used for isolation of human stem/progenitor cells. CD166, on the other hand, is expressed in various human organs and upregulated in humanized cancers, including prostate cancer [27]. To determine whether CD166 can be used for enriching human prostate stem/progenitors, we first examined its expression and found that CD166 is highly expressed in the developing human fetal prostate epithelium (Figure 2A, left panel) and focally expressed in the benign adult prostate, which overlaps with a subset of TROP2 and CD49f – positive cells (Figure 2, middle and right panels).

We then evaluated whether CD166 could be used as a marker for enriching human stem/progenitor cells. Benign regions of prostate tissue were collected from multiple patients who underwent radical prostatectomy and dissociated to single cells. Consistent with our previous studies [13,28], the percentages of CD166+ cells vary from patient to patient (data not shown). However, the majority of sphere forming activity was identified in the CD166hi population (Figure 2B), similar to our findings with murine prostate cells. Data are shown from 6 representative patients.

To evaluate whether CD166 can enrich human prostate tissue regeneration capacity in vivo, benign human prostate cells were dissociated and sorted according to cell surface CD166 expression levels. Equal number of viable CD166hi and CD166lo cells (2 × 10⁵) was implanted subcutaneously into NOD-SCID/IL2rg™ null mice, in combination with 2 × 10⁵ rUGSM inductive mesenchymal cells. After 8–16 weeks, grafts were harvested, fixed and embedded in paraffin for quantification and analyses. CD166lo cells have more tissue regeneration capacity as evidenced by increased number of tubule-like epithelial structures found in the grafts, which is rarely seen in the CD166hi grafts (Figure 2C). Further analyses showed that the tubule-like structures initiated by CD166lo cells contain CK5 and p63 expressing basal cells, CK8 luminal cells and AR positive cells (Figure S2).

Combination of markers TROP2 and CD49f can separate lineage-negative human prostate epithelial cells into various subpopulations, with TROP2hi;CD49fhi (Lin+Trop2hi or LTC) cells possessing the highest sphere forming capability in vitro [29]. Additionally, LTC cells can develop cancer-like phenotype in vivo following oncogenic transformation [13]. We tested whether CD166 can further segregate this LTC population. FACS analysis of benign human prostate cells indicated that more than 50% of LTC;CD166lo cells varied from patient to patient (data not shown). We performed the sphere-forming assay using sorted CD166hi and CD166lo cells (2 × 10⁵ rUGSM inductive mesenchymal cells). After 8–16 weeks, grafts were harvested, fixed and embedded in paraffin for quantification and analyses. CD166lo cells have more tissue regeneration capacity as evidenced by increased number of tubule-like epithelial structures found in the grafts, which is rarely seen in the CD166hi grafts (Figure 2C). Further analyses showed that the tubule-like structures initiated by CD166lo cells contain CK5 and p63 expressing basal cells, CK8 luminal cells and AR positive cells (Figure S2).
Figure 1. CD166 expression is upregulated in castrated prostate epithelium and CD166 can be used to enrich stem/progenitor cells in WT mice prostate. (A) Top: Comparison of p63 (red) and CD166 (green) co-IF staining between prostate proximal region and distal region. Bottom: IHC for CD166 expression from intact vs. castrated mouse prostate. Scale bar: 50 μm. (B) Lin⁻;CD166hi, Lin⁻;CD166lo, LSC hi;CD166hi, and LSC hi;CD166lo cells were isolated by FACS from 8- to 12-week-old mice. Graph shows the percentage of sphere-forming cells, based on the spheres from each population per 2500 cells plated after 8 days of growth. Data shown as mean ±/− STD (**, p<0.001, n = 3). (C) Fold change of LSC hi;CD166hi content based on intact WT from FACS analysis (*, p<0.05, n = 3).

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Figure 2. CD166$^{hi}$ human prostate cells have higher sphere forming capacity in vitro and more graft outgrowth in vivo. (A) IHC staining of CD166 on human fetal prostate tissue and patient prostate cancer tissues. Scale bar: 50 μm. (B) Total dissociated prostate cells, CD166$^{hi}$ and CD166$^{lo}$ populations were isolated by FACS from 6 patient samples. Graph shows the percentage of sphere-forming cells, based on the spheres from each population per 5,000 cells plated after 7 days of culture. Data shown as mean ±/− STD (**, p<0.001). (C) CD166$^{hi}$ and CD166$^{lo}$ populations were isolated by FACS from 3 patient samples. CD166$^{hi}$ and CD166$^{lo}$ cells (2×10$^5$) were implanted subcutaneously into NOD-SCID/IL2γ null mice, in combination with 2×10$^5$ rUGSM inductive mesenchymal cells. Grafts were harvested, fixed and analyzed after 8–16 weeks. Left, graph shows that CD166$^{hi}$ human prostate cells can form more tubules in graft regeneration assay compared to CD166$^{lo}$ human prostate cells. Right, H&E staining of representative graft. Scale bar: 100 μm. (D) Left, FACS plots show gates drawn for sorting of LTC (TROP2$^{hi}$;CD49f$^{hi}$) CD166$^{hi}$ and LTC;CD166$^{lo}$ subpopulations from one patient. Right, representative graph shows that LTC;CD166$^{hi}$ human prostate cells can form more tubules in graft regeneration assay compared to LTC;CD166$^{lo}$ human prostate cells. doi:10.1371/journal.pone.0042564.g002
CD166 can be used to Enrich Tumor Sphere-forming Cells in the 
Pten Null Prostate Cancer Model

To examine whether CD166 can enrich tumor initiating cells after castration, we compared the percentage of CD166hi subpopulation between intact and castrated Pten mutant mice and observed the expansion of CD166hi subpopulation after castration (Figure 3A). Next, we compared the sphere formation capabilities of LSC;CD166hi, LSC;CD166lo, LSC;CD166hi, and LSC;CD166lo subpopulations at the pre-cancer PIN (6 weeks) and cancer stages (11 weeks). We found that the LSC;CD166hi subpopulation has much higher sphere-forming ability, and nearly all sphere-forming activity in the cancer stage resides in the LSC;CD166hi subpopulation (Figure 3B). Consistent with our previous observation that Pten mutant spheres are larger than WT control spheres [19], both LSC;CD166hi and LSC;CD166lo subpopulations form large prostate spheres (Figure S3). Our previous study suggested that Pten deletion promotes the expansion of LSC;CD166hi prostate stem/progenitor cells [18,19]. Within the LSC;CD166hi population, we observed selective expansion of LSC;CD166hi cells. Pten mutant mice have more than a 3-fold increase in the percentage of LSC;CD166hi subpopulation, compared to WT littermates (Figure 3C).

To further study the LSC;CD166hi subpopulation, we isolated RNA from LSC;CD166hi, LSC;CD166lo subpopulations and the cell fraction depleted of LSC cells (non-LSC) and compared their gene expressions by RT-PCR analysis. LSC;CD166hi subpopulation expresses similar levels of basal cell markers Ck5 and p63 as the LSC;CD166lo subpopulation (Figure 3D, left panel). However, LSC;CD166lo subpopulation expresses much higher level of luminal marker Ck8 and Trop2, a new epithelial surface marker we recently identified for enriching stem cell activities in both murine and human prostates [13,29] (Figure 3D, right panel). Further examination of several other epithelial cell stem markers [10,30,31,32,33] showed that LSC;CD166hi cells have significantly higher CD44 and Axin3.1 expression compared to LSC;CD166lo cells. Although compared to non-LSC population, LSC;CD166hi cells express less Nkx3.1. No significant differences were found in CD17, and CD333 expressions between these two populations (Figure 3D, right panel).

CD166 Expression is Upregulated in Human Castration Resistant Prostate Tumor

Having found that CD166 can be used to enrich for human LTC cells and mouse tumor in initiating cells, we then examined the relationship between CD166 expression and human prostate cancer progression. In clinically annotated data of 218 prostate tumors [34], CD166 gene expression significantly correlates with increased prostate cancer aggressiveness, as indicated by Gleason score, with highest expression in metastasis samples (Figure 4A). We further surveyed CD166 expression on human prostate cancer tissue microarrays, which consist of 14 castration resistant (CRPC) metastasis samples and 98 hormone naive primary cancer samples from patients receiving either neoadjuvant hormone treatment (NHT) for various periods or receiving no treatment. CD166 is significantly enhanced in CRPC samples (Figure 4B for representative images). Compared to the predominant membrane localization of CD166 in hormone naive primary cancer samples, we observed intense cytoplasmic localization of CD166 in CRPC bone metastasis samples (Figure 4B, high magnification). CD166 expression levels were scored and p values are computed by Mann-Whitney test. CD166 protein expression level is significantly higher in CRPC samples as compared with primary cancers with (p<0.0001) or without (p<0.02) NHT (Figure 4C). These data suggest that CD166 is a castration-enriched marker for both murine and human prostate cancer.

Loss of CD166 does not Interfere with WT Prostate Development and Prostate Sphere Formation

While expressed in a wide variety of tissues, CD166 is usually restricted to subsets of cells involved in dynamic growth and/or migration, including neural development, branching organ development, hematopoiesis and immune response [27]. To test whether CD166 plays an intrinsic role in regulating prostate stem/progenitor cells, we analyzed CD166 knockout mice (CD166−/−). Genetic deletion of CD166 gene was achieved by replacing its first exon with a CDNA encoding EGFP [35]. CD166 null mice are phenotypically normal and fertile [35]. We examined the prostate at 8 and 20 weeks of age and found no difference in gross anatomy and histology among WT (data not shown), CD166+/− and CD166−/− mouse prostates (Figure 5A).

To further examine whether loss of CD166 has any effect on prostate stem/progenitor cells, we compared sphere formation activities of CD166−/− and CD166+/− prostate epithelium and found there is no significant difference (Figure 5B). In addition, spheres generated from CD166−/− prostate have similar size distribution compared to those from CD166+/− prostate epithelium (data not shown). Similarly, FACS analysis demonstrated that loss of CD166 does not affect LSC content of prostates isolated from the CD166−/− mice (Figure 5C), suggesting that CD166 does not play an essential role in normal prostate gland development or prostate stem/progenitor number and function.

Genetic Deletion of CD166 does not Block Prostate Cancer Progression

It has been postulated that CD166 functions as a cell surface sensor for cell density and controls the transition between local cell proliferation and tissue invasion during melanoma progression [36]. To examine whether CD166 plays an essential role in prostate cancer development, especially in the tumor initiating cells, we crossed CD166−/− mice with the Pten conditional knockout mice [17]. Histopathologic analysis indicated that loss of CD166 did not significantly change the kinetics of prostate cancer development in Pten null model and all Pb-Cre;Pten−/−;CD166−/− mice developed adenocarcinoma around 9 weeks of age (Figure 6A and data not shown). We observed similar levels of Ki67+ cells between Pb-Cre;Pten−/−;CD166−/− and Pb-Cre;Pten−/−;CD166+/− prostates (Figure 6A). SMA staining also demonstrated that loss of CD166 does not block prostate cancer cells from local invasion (Figure 6A, right panels).

We then compared the sphere formation between Pb-Cre;Pten−/−;CD166−/− and Pb-Cre;Pten−/−;CD166+/− prostates and found that loss of CD166 does not interfere with sphere-forming activity of Pten null epithelium (Figure 6B). Moreover, CD166−/− prostates have similar LSC content as compared to CD166+/− Pten null prostates (Figure 6C). Since PI3K/AKT pathway activation is a driving force for cell proliferation and prostate cancer progression in Pb-Cre;Pten−/− prostate cancer [17,20], we then examined whether there is any alteration of AKT activation after genetic deletion of CD166. Western blot analysis demonstrated that Pb-Cre;Pten−/−;CD166−/− prostate has no CD166 expression, but has similar P-AKT levels compared to Pb-Cre;Pten−/−;CD166+/− and Pb-Cre;Pten−/−;CD166+/− prostate (Figure 6D). We further confirmed that there is no negative selection against Pten−/−;CD166−/− cells since equal intensity of knockin-GFP protein can be detected in all cohorts except CD166+/− mice.
Figure 3. CD166 can be used to enrich tumor initiating cells in *Pten* mutant prostate. (A) FACS blots show increased Lin\(^{-}\)CD166\(^{hi}\) population after castration of *Pten* mutant mice compared to intact *Pten* mutant mice. (B) Four subpopulations (LSC\(^{hi}\)CD166\(^{hi}\), LSC\(^{hi}\)CD166\(^{lo}\), LSC\(^{lo}\)CD166\(^{hi}\), LSC\(^{lo}\)CD166\(^{lo}\)) were isolated from *Pten* mutant prostate from either 6 weeks or 11 weeks old mice. Graph shows the percentage of sphere-forming cells. Data from several experiments were pooled. Data shown as mean +/− STD (*, p < 0.05, n = 3). (C) Left: bar graph shows fold change of LSC\(^{hi}\)CD166\(^{hi}\) between WT and Mutant.
Since we see significant overexpression of CD166 in human CRPC samples, we next investigated whether CD166 would influence the development of CRPC in the Pten null prostate cancer model. 

Discussion

Few surface markers are currently available for enriching both murine and human prostate tissue stem/progenitor cells and for identifying prostate cancer initiating cells. By searching for those cell surface molecules that are upregulated in castrated murine prostate and castration resistant prostate cancers (CRPC) of murine and human origins, we identified CD166 as a surface marker for enriching both murine and human prostate tissue stem/progenitor cells based on in vitro sphere forming and in vivo tissue regeneration analyses. Importantly, upregulated CD166 expression and expansion of CD166hi cells correlate with Pten null CRPC progression as well as human CRPC development, although genetic deletion of CD166 does not interfere with normal murine prostate development or Pten null prostate cancer progression. Together, our study suggests CD166 can be used as a potential surface marker for identifying castration resistant tumor cells for targeted drug delivery.

CD166 expression has been proposed as a prognostic marker for several cancers, including breast [37], prostate [38], ovarian [39], pancreatic [40], colon [41], oral cancers [42], melanoma [36] and gastric cancers [43]. Importantly, our microarray and TMA studies demonstrate the association of increased CD166 expression with human prostate cancer metastasis and CRPC development. Moreover, within both murine and human prostate tissue, we show that the CD166-high expressing subpopulation encompasses prostate stem/progenitor and cancer initiating cells.

To investigate human prostate tissue stem/progenitor cell properties, we evaluated adult human prostate epithelium dissociated from benign prostate, rather than cell lines and xenografts. The advantage of this approach is to maintain the original heterogeneity in human prostate samples by avoiding the effect of long-term in vitro selection. However, there appears to be greater variability among patient samples in the tissue regeneration assays. This may be due to the difference in sample variability (i.e., ischemia time prior to tissue processing and cell retrieval), individual variability in CD166 expression, and technical challenges related to the tissue regeneration assays using human prostate cells. Therefore, analysis of sufficient patient samples is essential in order to draw a valid conclusion. In the current study, 6 human samples were utilized for the in vitro sphere forming and another 6 samples were used for in vivo regeneration assays. Using this system, we have previously defined TROP2hi;CD49dhi as a cancer initiating cell (cell of origin) for human prostate cancer [13]. In the current study, CD166hi population demonstrated significantly increased sphere-forming capacity compared to the patient-matched CD166lo population. In addition, our study demonstrates that CD166 can not only enrich human sphere-forming cells, but also segregate TROP2hi;CD49dhi into two functionally different populations, with TROP2hi;CD49dhi;CD166hi having higher regeneration capacity in vivo, compared to TROP2hi;CD49dhi;CD166lo. CD166 is also highly upregulated in CRPC based on our gene expression analysis and tissue microarray study. Therefore, CD166 may enrich both human prostate tissue stem/progenitor cells and castration resistant prostate cancer cells.

LSChi subpopulation has been defined as the murine prostate tissue stem/progenitor cells and expands significantly following castration [12,19,26]. LSChi cells express basal markers and demonstrated robust sphere-forming activity in vitro and prostate regeneration capability in vivo [26]. In contrast to luminal cells, LSChi cells respond efficiently to multiple oncogenic insults for prostate cancer initiation using a transplantation-based prostate regeneration assay [12]. We and others have demonstrated that the LSChi population, isolated from Pten null prostate tissue, is sufficient to regenerate cancercous morphology upon transplantation that closely mimics that of primary cancers [19,44]. In this study, we further separated LSChi subpopulation into CD166hi and CD166lo subsets and found that most of sphere-forming activities are associated with the LSChi;CD166hi cells. Importantly, this LSChi;CD166hi population was demonstrated to have self-renewal activity as spheres from this population could be passaged at least 3 generations with a high rate of sphere formation. Moreover, LSChi;CD166hi cells are expanded upon castration as well as Pten deletion in comparison to LSChi;CD166lo cells. Therefore, CD166 can further enrich murine prostate cancer initiating cells and castration resistant cells.

The relationship of LSChi;CD166hi cancer initiating cells described here to other cell populations is of obvious interest [45]. Using lineage tracing and cell type-specific Cre lines, a recent report demonstrates that both luminal cells and basal cells can initiate prostate cancer upon Pten deletion [14]. This new observation is not in conflict with our previous studies: we showed that Pten deletion mediated by PtenCre happens in both basal and luminal cells [18]. In addition, we observed significant expansion of a subset of prostate cancer cells positive for basal cell markers CK5 and p63 and luminal cell marker CK8, suggestive of transient amplifying/intermediate cells [18,46]. Compared to LSChi;CD166lo cells, one of the distinguishing features of LSChi;CD166hi cells is the higher Trop2 expression, a cell surface marker we have used for enriching both murine and human tissue stem cells [13,29]. TROP2 can functionally segregate mouse LSChi prostate population but there is no cytokeratin phenotypic difference between LSChi;Trop2hi and LSChi;Trop2lo population [29]. CD166, on the other hand, can enrich Pten null LSChi population with CK5+/p63+/CK8+ AR+ TROP2hi characteristics, suggesting that CD166 may preferentially enrich for CK5+/CK8+ transient amplifying/intermediate cells, which currently cannot be prospectively purified. Increased CK5+/CK8+ cells have been observed in the Pten conditional knockout model [18,47] as well as Pten−/−;TP53−/− prostate cancer model [48]. A recent study also identified a subset of tumor-initiating stem-like cells in human prostate cancer cell lines and xenografts based on co-expression of the human pluripotent stem cell marker TRA-1-60, CD151 and
Figure 4. Gene expression profiling and tissue microarray (TMA) demonstrates that increased CD166 expression is correlated with high Gleason score and human castration resistant prostate cancer. (A) CD166 gene expression from 147 human prostate tumors was analyzed by comparing different Gleason score groups to normal/benign (NL/BN) prostate. (B) Representative IHC staining of CD166 expression from human prostate TMA. Top: hormone naive primary prostate cancer; Low: castration resistant prostate cancer showing highly intensive immunostaining. Scale bar: 100 μm (left); 10 μm (right). (C) Data from 112 samples were calculated and statistical analysis of CD166 expression of human TMA conducted. NHT: neoadjuvant hormone therapy; CRPC: castrate resistant prostate cancer. Column, mean CD166 staining in NHT and CR tissues. Samples were graded from 0 to +3 representing the range from no staining to heavy staining by visual scoring. Error bar: standard error. Immunoreactivity of CD166 is significantly higher in CRPC group compared with untreated group (p<0.021) or NHT with different treatment times (p<0.0001).

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Interestingly, this subtype of human prostate tumor initiating cells also have the AR^+;CK5^+;CK8^+ phenotype [49]. Another characteristic of LSC^hi;CD166^hi cells is relatively higher CD44 expression. Since knockdown of CD44 was very effective to suppress cancer stem cell regeneration and metastasis [30], it will be interesting to examine whether there is any functional role for CD44 in LSC^hi;CD166^hi tumor initiating cells.

As an adhesion molecule, CD166 can initiate homophilic (CD166-CD166) or heterophilic interaction (CD166-CD6), and play important roles in neural guidance and the immune system [27]. CD166 has also been suggested to play a critical role in various human cancers and as a potential therapeutic target for cancer initiating cells, similar to CD44 [30] and CD47 [50]. A truncated CD166 variant has been shown to block melanoma metastasis by interfering with the CD166-CD166 homophilic interaction [51]. Similarly, novel human recombinant single-chain anti-CD166 antibodies have been shown to inhibit colorectal carcinoma growth as well as breast cancer cell invasion [52].

**Figure 5. Loss of CD166 does not block WT prostate development and stem/progenitor cell function.** (A) Top: The gross anatomy of the prostate of WT and CD166^−/− mice at 8 weeks of age, scale bar: 2 mm. Bottom: HE staining of DLP section from WT and CD166^−/− mice at 8 weeks of age, scale bar: 200 μm. (B) Comparison of sphere formation from total unsorted prostate cells (5000 per 12-well) between CD166^+/− and CD166^−/− prostates. Data represented as mean ±/− STD (p>0.05, n = 3). (C) Comparison of LSC^hi content between CD166^+/− and CD166^−/− prostates at 8–12 weeks age (p>0.05, n = 5).

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CD166 Enriches Prostate Cancer Sphere Forming Cell

Unlike subcutaneous allograft or xenograft models used in above studies, we defined the functions of CD166 in prostate cancer initiating cells and prostate cancer development in immune competent mice within the natural prostate environment. By generating the Pb-Cre; Pten+/-;CD166+/- line, our study demonstrates that loss of CD166 within LSC-positive population does not change their ability to form spheres in vitro and block prostate cancer initiation and progression in vivo. As it is possible that other members of the Cell Adhesion Molecule (CAM) family can compensate for the role of CD166 in murine prostate cancer development, we cannot conclude that CD166 has no in vivo function on prostate cancer initiation. Nevertheless, since cancer initiating cell surface markers can be used for molecular imaging [53] and/or for internalizing a death-inducing compound for initiating cell surface markers can be used for molecular imaging [53] and/or for internalizing a death-inducing compound for targeted therapies [34], our work suggests that CD166 may be for a suitable surface marker for future targeted drug delivery [55].

Recently, a promising study showed substantial cytotoxic effects of a suitable surface marker for future targeted drug delivery [55].

In vitro Prostate Sphere-forming Assays

Prostate spheres were cultured and passaged as described previously [56,57]. FACS-isolated prostate cells or unsorted prostate cells were counted and suspended into a 100 μL mixture of 1:1 Matrigel (BD Biosciences, San Jose, CA) and PrEGM (Lonza, Walkersville, MD). Plates were placed around the rims of wells in a 12-well plate and allowed to solidify at 37°C for 45 minutes, before 1 ml of PrEGM was added. Sphere media was changed every three days.

RNA Isolation and qRT-PCR

Sorted cells were collected and spun down. RNAs from sorted cells were extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA). RNAs were reverse transcribed into cDNA with SuperScript III First-Strand Synthesis System for qRT-PCR (Invitrogen, Carlsbad, CA). RNAs were reverse transcribed into cDNA with SuperScript III First-Strand Synthesis System for qRT-PCR (Invitrogen, Carlsbad, CA). Quantitative PCR was done in the iQ™ thermal cycler (Bio-Rad) using the iQSYBR Green Supermix (Bio-Rad) in triplicate. Primers used for study are C5k (F:5'-ACCTTCGAGAACCAAGCAAGC-3', R:5'-TTGGCAGAAGTGTCTTTTGAC-3'), C6k (F:5’-ATCGAGATCACCACCTCCCG-3'; R:5’TGAAGCCAGGCGCTAGTGAGT-3'), P63 (F:5’-CCCACAGACTGCGGACGAGC-3'), T65 (R:5’-GAGATGAGAAGTGAGGAGAA-3'), AR (F:5’-ACCAACCAAGATTCTCTTTCG-3'; R:5’TATAGTT-GAAGAAGCAGCCAC-3'), CD166 (F 5’-CTTACAGAGGAGGCGATTG-3'; R:5’-CAGGCATCTCCAGAACAAGG-3'), Trop2 (F:5’-AGACCAAGACCTGCGTGGC-3'; R:5’-ACCTGGGTGGTGACGCTTGAG-3'), Gadph (F:5’-ACTTGCCATGGCTCTCCCG-3'; R:5’-CAGGGCGCAGTGCAATC-3'), CD117 (F:5’-AGAACGCGATTCGCGAGCAGC-3'; R:5’TAGCTTGGTTTTCGTCTCAGG-3'), CD133 (F:5’-ACCAACCAAGAACGACG-3'; R:5’-GGAGCTGCTAGTTGAGG-3'), CD44 (F:5’-GTCACCCGGTATGGTACTCG-3'; R:5’TCTCGGTTTTTGGGCTCTCAGT-3'), Nkx3.1 (F:5’-TCCGCTTTTTGGTCTGTGAGT-3'; R:5’TGTAAAGTGCACCGCTGAAA-3').

Immunofluorescence and Immunohistochemistry Analyses

Tissue analysis was carried out using standard techniques as described previously [17]. Sections (4 μm) were stained with hematoxylin and eosin (H&E) or with specific antibodies (Table S2).

Western Blot Analysis

Total protein was extracted with RIPA buffer (20 mM Tris- HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate) with fresh added phosphatase inhibitors (Sigma, St. Louis, MO) and protease inhibitors.
Figure 7. Loss of CD166 does not block castration resistant prostate tumor progression in Pb-Cre\(^{+}\);Pten\(^{L/L};\)CD166\(^{2/2}\) mice. Pb-Cre\(^{+}\), Pten\(^{L/L}, \)CD166\(^{+}/^{+}\) and Pb-Cre\(^{+}\), Pten\(^{L/L}, \)CD166\(^{2/2}\) mice were castrated at the age of 12 weeks using standard techniques. At 8 weeks post-castration, mice were intraperitoneal injected with a single dose of 100 \(\mu\)l (1 mg) of BrdU solution and sacrificed 4 hour later for analysis. Evaluation of the effects of CD166 deletion on (A) castration resistant prostate cancer progression (HE), and (B) cell lineage composition (CK5/CK8), cell proliferation (BrdU) and prostate tumor invasion (SMA) were performed. Scale bar: 50 \(\mu\)m.

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Human Prostate Cancer Tissue Acquisition and Dissociation

Human prostate tissue was obtained via a research protocol that was approved by the Office for the Protection of Research Subjects at UCLA and the Greater Los Angeles VA Medical Center. Informed written consent was obtained on all participants where identifying information was included. A frozen section was prepared from an adjacent slice of prostate tissue in order to determine the location of tumor nodules. Tumor areas were encircled and dissected away from benign regions within the fresh tissue slice. Benign tissue specimens were placed on ice and brought immediately to the laboratory for mechanical and enzymatic digestion [28]. Prostate tissue was minced into small fragments (1 mm³) in RPMI-1640 medium supplemented with 10% FBS and went through through enzymatic digestion (12 h in 0.25% type I collagenase followed by TripLE (Invitrogen) for 5 min at 37°C). Cell suspensions were passed through a 23-gauge needle and were filtered through 40-μm filters. Cells were plated overnight in PrEGM as described above for sphere formation assay or tissue regeneration assay.

Tissue Regeneration Assay

In vivo tissue experiments were performed in male NOD-SCID/IL2rγ null mice in accordance with protocol number 2007-189-11A, approved by the Animal Research Committee within the Office for the Protection of Research Subjects at UCLA. Cells of interest were collected from FACS sorting and subsequently incubated with primary antibodies against CD166 and GADPH (Santa Cruz Biotechnology, Santa Cruz, CA), GFP (Abcam, Cambridge, MA), phospho-AKT Ser473 (Cell Signaling Technology, Beverly, MA) and anti-mouse IgG or goat anti-rabbit IgG (Jackson Immunoresearch, Inc., West Grove, PA), and developed with Pierce ECL reagent (Thermal Scientific, Rockford, IL).

Human Prostate Cancer Tissue Microarray (TMA)

TMA used to survey CD166 expression is composed of 112 patient samples. Written consent was obtained from the patient as well as ethics approval from University of British Columbia-British Columbia Cancer Agency Research Ethics Board (UBC BCCA REB), Vancouver, Canada. The 112 patient specimens were spotted in triplicate to create a tissue microarray with 336 cores as described previously in [58]. Scoring method was based on the intensity of the staining in each core on a 4-point scale from none (0) to high (3). Mann-Whitney test was used to compare CD166 protein expression difference between different groups. p values <0.05 were considered significant.

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