

Stem cells in genetically-engineered mouse models of prostate cancer

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Abstract

The cancer stem cell model proposes that tumors have a hierarchical organization in which tumorigenic cells give rise to non-tumorigenic cells, with only a subset of stem-like cells able to propagate the tumor. In the case of prostate cancer, recent analyses of genetically engineered mouse (GEM) models have provided evidence supporting the existence of cancer stem cells *in vivo*. These studies suggest that cancer stem cells capable of tumor propagation exist at various stages of tumor progression from prostatic intraepithelial neoplasia (PIN) to advanced metastatic and castration-resistant disease. However, studies of stem cells in prostate cancer have been limited by available approaches for evaluating their functional properties in cell culture and transplantation assays. Given the role of the tumor microenvironment and the putative cancer stem cell niche, future studies using GEM models to analyze cancer stem cells in their native tissue microenvironment are likely to be highly informative.

Key Words

- ▶ prostate
- ▶ oncology
- ▶ endocrine therapy resistance

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Introduction

For the past 70 years, androgen-deprivation therapy has remained the mainstay of treatment for prostate cancer. Although most prostate cancers initially respond to androgen deprivation, many will ultimately progress to lethal castration-resistant disease. The widespread usage of next-generation anti-androgen agents continues to highlight the clinical significance of the emergence of treatment-resistant disease. Thus, understanding the molecular mechanisms that promote tumor propagation during the progression of prostate cancer to castration-resistance is of fundamental importance for the development of reliable biomarkers and effective treatments.

Studies using genetically engineered mouse (GEM) models have revealed that the normal prostate contains castration-resistant stem/progenitor cells that retain their stem cell properties after androgen deprivation. These findings raise the possibility that similar stem cell populations that resist castration may also exist in prostate

tumors and contribute to the emergence of castration-resistant disease. Here, we review findings from studies using GEM models to identify cancer stem cells in prostate cancer.

Stem/progenitor cells in the normal prostate epithelium

In both the mouse and human adult prostate epithelium, there are three primary cell types, corresponding to luminal cells, basal cells, and neuroendocrine cells, which can be distinguished by morphology as well as marker gene expression (Shen & Abate-Shen 2010). In particular, luminal epithelial cells express cytokeratins 8 (CK8) and CK18, as well as high levels of androgen receptors (ARs), whereas basal cells express p63, CK5, CK14, and low levels of AR. Luminal cells also produce secretory proteins such as prostate-specific antigen (PSA)

in humans and probasin in mice. The rare neuroendocrine cells are epithelial cells that display neuronal-like processes and express neural markers such as synaptophysin and chromogranin A (Abrahamsson & di Sant'Agnese 1993, Terry & Beltran 2014). Finally, a population of basally localized cells that co-express luminal and basal CK are termed 'intermediate' cells (De Marzo *et al.* 1998, Xue *et al.* 1998), but whether these cells constitute a distinct functional cell type remains unclear.

Following androgen deprivation by surgical or chemical castration, the prostate epithelium regresses, with extensive apoptosis of luminal cells (Evans & Chandler 1987, Isaacs *et al.* 1992). However, on re-administration of androgens, the mouse prostate can regenerate to its original size and is histologically indistinguishable from the hormonally intact state. Importantly, the mouse prostate can undergo multiple rounds of regression and regeneration in response to androgen deprivation and restoration, indicating the existence of a castration-resistant stem cell population within the regressed prostate epithelium (Isaacs 1985, Tsujimura *et al.* 2002). Notably, an analysis during serial regression and regeneration of BrdU-label retaining cells, which are presumed to be enriched for stem/progenitor cells, showed that these label-retaining cells are highly enriched in both basal and luminal populations in the proximal region of the prostate, near the junction with the urethra (Tsujimura *et al.* 2002).

To date, there is substantial evidence supporting the existence of stem/progenitor activity in both the basal as well as luminal compartments, with the results potentially being assay-dependent (Tsujimura *et al.* 2002, Lawson *et al.* 2007, Goldstein *et al.* 2008, Wang *et al.* 2009, 2013, Chua *et al.* 2014, Karthaus *et al.* 2014). Lineage-tracing analyses of prostate organogenesis have shown the existence of multipotent basal progenitors that generate basal, luminal, and neuroendocrine progeny, as well as unipotent luminal progenitors that only generate luminal progeny (Ousset *et al.* 2012, Pignon *et al.* 2013). During neonatal development, the differentiation of basal progenitors into luminal cells appears to proceed through a transitional intermediate cell state (Ousset *et al.* 2012). In contrast, the adult prostate epithelium is mostly maintained by unipotent luminal and basal progenitors (Choi *et al.* 2012, Lu *et al.* 2013, Wang *et al.* 2013). Similarly, the luminal and basal compartments are also thought to be largely independent during androgen-mediated regeneration (Liu *et al.* 2011, Choi *et al.* 2012, Wang *et al.* 2013). However, luminal as well as basal bipotential populations have also been identified in regenerating

prostate epithelium. Notably, a rare Nkx3.1-expressing castration-resistant luminal population (CARN) is bipotential and can self-renew during androgen-mediated regeneration (Wang *et al.* 2009). Recent studies have also suggested the existence of bipotential basal progenitors that undergo asymmetric cell divisions to generate luminal and basal daughter cells during regeneration (Wang *et al.* 2013, 2014a, 2015, Lee *et al.* 2014). Interestingly, the fates of daughter cells from a dividing basal progenitor are correlated with mitotic spindle orientation, as asymmetric divisions occur when the spindle is oriented vertically relative to the basement membrane (Wang *et al.* 2014a).

Other approaches for identifying stem cell populations in the normal WT prostate epithelium have identified multipotent basal cells using cell culture assays as well as renal graft methods that involve the transplantation of epithelial cells together with supporting urogenital mesenchyme (Xin *et al.* 2003, Lukacs *et al.* 2010). Initial studies used flow sorting to show that cells expressing high levels of *Sca1* are enriched in the proximal prostate and display stem/progenitor properties in culture and in graft assays (Burger *et al.* 2005, Xin *et al.* 2005, Goto *et al.* 2006). Subsequent studies have further defined a population of Lin⁻Sca-1⁺CD49f^{high} (LSC^{high}) cells that displays stem-like properties and can be further enriched using the Trop2 marker (Lawson *et al.* 2007, Goldstein *et al.* 2008, Lukacs *et al.* 2010). Notably, the LSC^{high} population consists of basal cells (Mulholland *et al.* 2009, Wang *et al.* 2013), which may be consistent with the plasticity of basal cells observed in *ex vivo* assays as well as *in vivo* models of prostate cancer and inflammation (Choi *et al.* 2012, Lu *et al.* 2013, Wang *et al.* 2013, Kwon *et al.* 2014a).

Identification of putative cancer stem cells in prostate cancer

In the cancer stem cell model, tumors contain distinct cell populations that differ in their genetic/epigenetic features and thus display intratumor heterogeneity. The model proposes that these cell populations are functionally distinct, such that tumorigenic stem cells can give rise to non-tumorigenic cells, with only the stem cell population able to self-renew and thereby propagate the tumor. Thus, cancer stem cells can behave in an analogous manner to normal stem cells in an untransformed tissue, except that their proliferation and differentiation are dysregulated. In principle, this hierarchical organization of tumors has important therapeutic implications. If only cancer stem

cells possess tumor-propagating abilities, then only this population would need to be targeted for therapy. However, if most or all tumor cells possess tumor-propagating abilities, then every tumor cell would need to be eliminated.

While the cancer stem cell model is conceptually well defined, there are substantial experimental challenges associated with investigating the validity of this model for a given tumor. To assay their functional differences, both cancer stem cell and non-cancer stem cell populations must be identified, and most studies to date have isolated these cell populations for *ex vivo* analyses using cell culture and graft assays. In the case of solid tumors, tumor cells are generally dissociated using mechanical and/or enzymatic methods and sorted by flow cytometry using cell surface markers that enrich for putative cancer stem cells. Following their isolation, the putative cancer stem cells can be compared with non-stem cells from the same tumor for their functional activity.

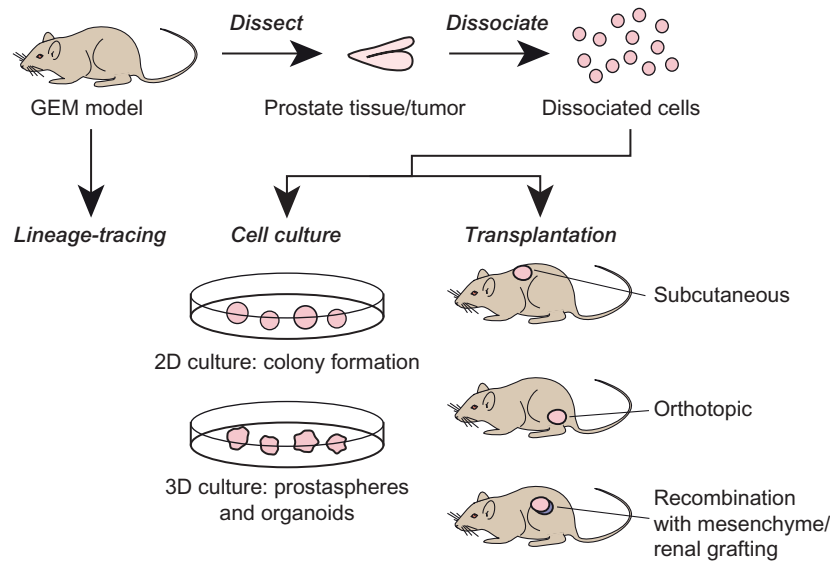
Many cancer stem-like cells that have been identified to date express similar markers as normal non-cancerous stem cells. However, cancer stem cells may or may not be related to a normal stem cell, which may depend in part on the cell of origin of a tumor, which is defined as the normal untransformed cell type from which the tumor arises. In many tumor types, the cell type of origin corresponds to a normal stem cell, but there is also substantial evidence for cells of origin that are not stem/progenitor cells (Visvader 2011, Blanpain 2013). Thus, if the cell type of origin is not a stem cell, it is conceivable that the putative cancer stem cell derived from it might not share specific markers with normal tissue stem cells. In studies of the mouse and human prostate, it is currently unresolved whether luminal cells or basal cells, or both, may serve as cells of origin (Goldstein *et al.* 2010a, Lawson *et al.* 2010, Choi *et al.* 2012, Lu *et al.* 2013, Wang *et al.* 2013), although lineage-tracing studies using multiple GEM models indicate that luminal cells are generally favored as the cell of origin (Wang *et al.* 2014b).

Whether stem-like cells that function to maintain and propagate tumors exist in prostate cancer, and whether such cells display basal-like or luminal-like properties, has been a topic of great interest (Goldstein *et al.* 2010b, Maitland *et al.* 2011, Wang & Shen 2011, Chen *et al.* 2013). Notably, most prostate tumors display a luminal epithelial phenotype, because prostate adenocarcinoma is identified histologically by an absence of basal cells (Brawer *et al.* 1985, Wojno & Epstein 1995, Weinstein *et al.* 2002). The luminal phenotype of prostate cancer is consistent with

the hypothesis that stem cells should have luminal properties but does not exclude the possibility that rare stem cells with basal features may exist. Furthermore, as may be the case for the normal prostate, it is conceivable that multiple stem-like populations may exist in prostate tumors.

Finding robust and reproducible methods to assess the activity of prostate cancer stem cells has been a principal challenge in the field. Both cell culture-based assays and transplantation assays have been used to assess stem/progenitor activity (Shibata & Shen 2013) (Fig. 1). A major approach that has been utilized for assaying prostate cancer stem cell activity has been the prostasphere assay, a three-dimensional culture method in which cells are suspended in a Matrigel matrix together with prostate epithelial growth (PrEGM) media, which allows cells to self-renew and differentiate (Lawson *et al.* 2007, Xin *et al.* 2007, Lukacs *et al.* 2010). In addition, tumor propagation can be evaluated by orthotopic, intravenous, or subcutaneous transplantation into immunocompromised animals in the absence of exogenous stromal tissue or in combination with urogenital sinus mesenchyme in renal grafts (Lukacs *et al.* 2010, Lawrence *et al.* 2013, Shibata & Shen 2013). However, both methods have been severely limited for analysis of primary human patient samples because it has not been possible to culture primary tumor samples using the prostasphere assay (Garraway *et al.* 2010). Furthermore, it has been difficult to perform high efficiency xenografts using primary prostatectomy tissue (Li *et al.* 2009, Toivanen *et al.* 2013), perhaps due to the generally indolent phenotype of most prostate tumors.

Another challenge for the analysis of cancer stem cells arises from the selective pressures experienced by clones within a tumor during cancer progression, as individual clones accumulate distinct genetic and/or epigenetic changes (Greaves & Maley 2012, Kreso & Dick 2014). Such mutations can be either neutral or advantageous, resulting in the expansion of certain clones and the reduction of others, leading to clonal evolution of the tumor. In prostate cancer, androgen-deprivation therapies lead to clonal reduction and provide selective pressures such that castration-resistant clones can become dominant (Fig. 2). As tumors evolve, the cancer stem cells may themselves change genotypically and phenotypically (Anderson *et al.* 2011, Greaves & Maley 2012). Thus, clonal evolution may alter the phenotype and functional properties of cancer stem cells during tumor progression, which may create difficulties for experimental interpretation.

**Figure 1**

Assays for cancer stem cells from genetically engineered mouse (GEM) models of prostate cancer. Prostate tumor cells from GEM models can be dissociated and flow sorted to assay for tumor propagation in cell culture

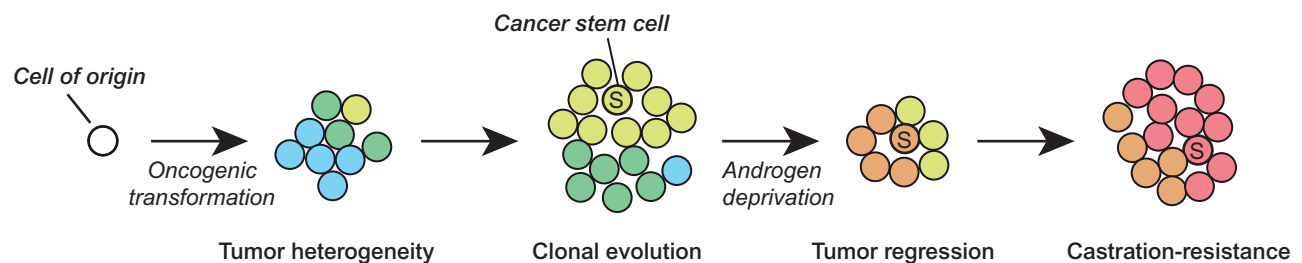
or by transplantation into immunodeficient mice. The fate of candidate cancer stem cells can be also directly assessed by *in vivo* lineage tracing.

GEM models of prostate cancer

Over the past 20 years, numerous GEM models have been developed that model various stages of prostate cancer progression, from the precursor state known as prostatic intraepithelial neoplasia (PIN) to adenocarcinoma as well as advanced metastatic and castration-resistant disease. These GEM models usually incorporate inactivation of tumor suppressor genes such as *Nkx3.1*, *Pten*, and *Trp53* and/or overexpression of oncogenes such as *c-Myc*, *Erg*, and *K-ras* (Shappell *et al.* 2004, Irshad & Abate-Shen 2013, Ittmann *et al.* 2013). Notably, alterations such as

down-regulation of *NKX3.1*, mutation of *Pten*, and increased expression of *ERG* are frequently observed in human prostate cancers (Taylor *et al.* 2010, Barbieri *et al.* 2012, Baca *et al.* 2013, Robinson *et al.* 2015), and consequently these models may be relevant for studies of prostate tumorigenesis.

Although many GEM models of prostate cancer have been developed, it is important to note that some features of the human prostate are intrinsically difficult to model in mice. In particular, the mouse prostate is comprised of distinct anterior, ventral, dorsal, and lateral lobes, whereas

**Figure 2**

Clonal evolution of cancer stem cells in prostate cancer. Intratumor heterogeneity in prostate tumors increases as tumor cells accumulate mutations. Distinct clones within the tumor are indicated by different colors. Although most prostate tumors respond to androgen-deprivation therapy, as shown by the loss of some clones (blue and green) and reduction of others (yellow), they may contain castration-resistant stem

cells (indicated by 'S') that can survive androgen deprivation and propagate castration-resistant prostate cancer. As the clonal architecture of the tumor evolves during progression, depicted by the emergence of castration-resistant clones (orange and red), the cancer stem cells that propagate the tumor may also evolve.

the human prostate lacks overt lobular structure. Instead, the human prostate displays a zonal architecture at the histological level, being composed of peripheral, transition, and central zones, with most prostate cancers originating in the peripheral zone (McNeal *et al.* 1988). In certain GEM models, the tumor phenotype may vary between prostate lobes, but overall there does not appear to be a specific lobe that more accurately recapitulates human prostate cancer (Ittmann *et al.* 2013).

Several additional limitations of GEM models of prostate cancer reflect the current state of the art and are likely to be overcome by future technical improvements. At present, many current GEM models utilize androgen-dependent promoters, frequently the *Probasin* promoter or a modified derivative such as the *ARR₂PB* promoter used in the commonly used *PB-Cre4* driver (Wu *et al.* 2001). However, the use of such androgen-dependent promoters has posed a challenge for the modeling of the emergence of castration-resistant disease, because androgen deprivation eliminates driver expression (Irshad & Abate-Shen 2013). Furthermore, it has been relatively difficult to model metastatic prostate cancer, particularly bone metastasis, which is a major metastatic site in human patients (Irshad & Abate-Shen 2013). Finally, the frequent chromosomal rearrangements and extensive intratumoral heterogeneity observed in advanced human prostate cancers are not observed in many GEM prostate cancer models (Bianchi-Frias *et al.* 2015, Wanjala *et al.* 2015). However, a recent study has reported high frequencies of genomic alterations and intratumoral heterogeneity in the *PB-Cre4; Pten^{flox/flox}; Trp53^{flox/flox}* model (Wanjala *et al.* 2015). This GEM model may therefore be useful for investigating the extensive intratumoral heterogeneity and clonal evolution typically observed in metastatic human prostate cancers (Gundem *et al.* 2015, Hong *et al.* 2015, Shen 2015).

Cancer stem cells in GEM models of prostate cancer

To date, candidate cancer stem cell populations have been identified in several prostate cancer GEM models using cell culture and/or grafting assays. One cell population of particular interest has been the $\text{Lin}^- \text{Sca-1}^+ \text{CD49}^{\text{high}}$ (LSC^{high}) population, based on the original finding that WT LSC^{high} cells display stem cell properties (Lawson *et al.* 2007). In particular, the percentage of Sca-1^+ and LSC^{high} cells increases with disease progression in the *PB-Cre4; Pten^{flox/flox}* model of high-grade PIN and prostate cancer (Wang *et al.* 2006, Mulholland *et al.* 2009), and LSC^{high}

cells from *PB-Cre4; Pten^{flox/flox}* prostates form larger prostatespheres than controls (Mulholland *et al.* 2009). In addition, LSC^{high} cells display tumor-propagating activity after sorting and recombining with WT urogenital sinus mesenchyme in renal grafts (Mulholland *et al.* 2009). Furthermore, isolation of cells that express high levels of CD166 (ALCAM) from the LSC^{high} population further enriches for tumor prostatesphere formation (Jiao *et al.* 2012).

The identity of putative cancer stem cells has also been investigated in more aggressive GEM models of prostate cancer. Thus, tumor cells from *PB-Cre4; Pten^{flox/flox}; Trp53^{flox/flox}* mice, which rapidly develop lethal prostate adenocarcinomas that are non-metastatic, form prostatespheres of greater size, have increased efficiencies of colony formation, and develop tumors upon orthotopic transplantation (Abou-Kheir *et al.* 2010). Similarly, LSC^{high} cells from *PB-Cre4; Pten^{flox/flox}; Kras^{LSL-G12D/+}* prostates, which generate invasive and metastatic prostate tumors, display increased prostatesphere formation compared to LSC^{high} cells from *PB-Cre4; Pten^{flox/flox}* prostates (Mulholland *et al.* 2012). After orthotopic injection of prostatesphere cells established from *PB-Cre4; Pten^{flox/flox}; Kras^{LSL-G12D/+}* LSC^{high} cells into immunodeficient recipients, the resulting grafts form poorly differentiated carcinomas and metastases that recapitulate the original tumor phenotype (Mulholland *et al.* 2012). A recent study showed that expression of a *vimentin-GFP* reporter in *PB-Cre4; Pten^{flox/flox}; Kras^{LSL-G12D/+}* animals can be used to isolate tumor cells with mesenchymal features, as well as tumor cells harboring both epithelial and mesenchymal characteristics, which are termed 'epithelial-mesenchymal transition' (EMT) tumor cells (Ruscetti *et al.* 2015). Both mesenchymal-like tumor cells and EMT tumor cells display a tumor-initiating capability, suggesting that these populations also contain cells with cancer stem cell properties (Ruscetti *et al.* 2015). Finally, studies of *Kras^{LSL-G12D/+}* prostate cells infected with lentiviruses overexpressing *Cre* and *AR* indicate that epigenetic mechanisms, such as the increased expression of *Ezh2*, promote tumor-propagation and secondary tumor growth (Cai *et al.* 2012).

Several GEM models of prostate cancer develop castration-resistant tumors in response to androgen deprivation and have been used for studies of castration-resistant cancer stem cells. In particular, tumors in *PB-Cre4; Pten^{flox/flox}* mice initially regress in response to castration but subsequently regrow tumors that are castration resistant, which is associated with an increase in the percentage of LSC^{high} cells (Mulholland *et al.* 2009).

Moreover, the CD166^{high} population is also expanded in prostates from castrated *PB-Cre4; Pten^{flox/flox}* mice, consistent with the up-regulation of CD166 expression in human castration-resistant prostate cancer (Jiao *et al.* 2012). Finally, the existence of castration-resistant stem-like cells that have basal or intermediate phenotypes in the proximal region of *PB-Cre4; Pten^{flox/flox}* prostates is supported by analyses of *PB-Cre4; Pten^{flox/flox}; AR^{flox/Y}* animals lacking AR transcription factor activity (Mulholland *et al.* 2011).

Taken together, these studies suggest the frequency of tumor-propagating cells increases with tumor progression and with tumor severity and supports the cancer stem cell properties of the LSC^{high} population. However, the basal phenotype of LSC^{high} cells in the normal prostate epithelium raises questions about the specific properties of this population in prostate tumors, which should have a luminal phenotype. One possible explanation is that there is a significant increase in the percentage of intermediate cells that co-express basal and luminal markers in GEM models with *Pten* inactivation (Mulholland *et al.* 2011, Wang *et al.* 2013) and may harbor LSC^{high} cells. Furthermore, the strong bias of the prostasphere assay for growth of basal cells (Wang *et al.* 2013, Huang *et al.* 2015) would also be consistent with the identification of LSC^{high} cells from GEM models. Thus, the identification of additional candidate markers for cancer stem cells is important for clarifying the role and identity of putative cancer stem cells in GEM models of prostate cancer.

Notably, several other genes that are candidate markers for stem/progenitor cells in the normal prostate epithelium have also been proposed to serve as markers for castration-resistant cancer stem cells. For example, increased expression of Sca1, CD133, and c-kit has been observed after the castration of TRAMP transgenic mice, which have a minimal probasin promoter driving expression of SV40 large and small T-antigens and develop adenocarcinoma and neuroendocrine tumors, as well as castration-resistant disease (Gingrich *et al.* 1997, Chiaverotti *et al.* 2008). In addition, expression of Sox2 appears to mark a luminal castration-resistant stem cell population in human prostate tumors and is increased in *PB-Cre4; Pten^{flox/flox}* mouse tumors after castration (Bae *et al.* 2010, Kregel *et al.* 2013, Wang *et al.* 2014a). Another candidate cancer stem marker for a luminal castration-resistant population is *Nkx3.1*, which is expressed in rare castration-resistant *Nkx3.1* expressing cells (CARNs) (Wang *et al.* 2009), while human CARN-like cells that survive castration have been identified in BM18 human prostate cancer xenografts

(Germann *et al.* 2012). However, functional studies evaluating tumor-propagating abilities have not yet been conducted on all of these populations that have been identified using these markers.

Role of tumor microenvironment and the cancer stem cell niche

In a normal untransformed tissue, stem cell activity is regulated by the surrounding microenvironment, or niche. In the case of the prostate, the stem cell niche has not been identified, although there is evidence that a stem/progenitor population resides in the proximal prostate (Tsujimura *et al.* 2002, Burger *et al.* 2005, Xin *et al.* 2005, Goto *et al.* 2006). In principle, however, the functional properties of cancer stem cells may be influenced by the tissue microenvironment and the presence or absence of the endogenous niche (Medema 2013).

For example, evidence for a role of the microenvironment is provided by the *PB-Cre4; Trp53^{flox/flox}; Rb^{flox/flox}* model, which develops aggressive tumors with both luminal and neuroendocrine features in the proximal but not distal prostate (Zhou *et al.* 2007). Interestingly, both proximal and distal cells gave rise to tumors in assays in which dissociated epithelial cells were injected subcutaneously together with Matrigel into SCID mice, suggesting a possible effect of the tissue microenvironment in suppressing distal tumorigenesis in this GEM model (Zhou *et al.* 2007). Conversely, the tissue microenvironment may also be disrupted during tumor progression, which may affect the localization of stem/progenitor cells. Thus, LSC^{high} cells have a basal location in nonmutant prostates, but tumor LSC^{high} cells from *PB-Cre4; Pten^{flox/flox}* prostates are in both basal and luminal locations (Mulholland *et al.* 2009).

An additional study has provided evidence that functional effects of the tissue microenvironment on tumor-propagating cells can be exerted by stromal components. In particular, prostasphere formation by LSC^{high} cells from *PB-Cre4; Pten^{flox/flox}* tumors is increased by co-culture with normal primary stromal cells or urogenital sinus mesenchyme and further enhanced by co-culture with cancer associated fibroblasts (CAFs) (Liao *et al.* 2010). In addition, the *PB-Cre4; Pten^{flox/flox}* LSC^{high} cells form highly proliferative tumor-like glandular structures more efficiently in renal grafts after recombination with CAFs than with normal prostate fibroblasts (Liao *et al.* 2010). To date, several distinct stromal subtypes, including a Gli1-expressing stromal stem cell population, have been defined in the normal mouse prostate (Peng

et al. 2013), but more studies are required for functional characterization of stromal populations in both the normal and the transformed prostate and to identify putative stromal signals that influence tumor propagation. As a cautionary note, these findings also suggest that results from cell culture and transplantation assays in which putative cancer stem cells are isolated from their native microenvironment should be interpreted with caution.

Current challenges and future directions

It is important to emphasize that the identification of cancer stem cells is limited by the functional assays available for their detection. However, the methods used for the isolation and analysis of cancer stem cells can generate inherent biases. In particular, the dissociation of tumor cells for flow cytometry and further analysis can result in a bias for cells that are robust enough to survive the dissociation methods (Kreso & Dick 2014). Furthermore, if the dissociated tumor cells are cultured *in vitro* or transplanted to assay tumor propagation, they will also undergo selection for cells able to grow in the specific conditions utilized.

Notably, a major obstacle in the analysis of prostate cancer stem cells has been the severe difficulties encountered in growing prostate cells with a luminal phenotype (Peehl 2005). Although constitutive expression of the Notch1 intracellular domain can suppress anoikis and promote survival of some prostate luminal cells in prostatesphere assays (Kwon *et al.* 2014b), prostasphere assays are highly biased toward the growth of basal cells (Wang *et al.* 2013, Huang *et al.* 2015). Thus, a significant recent technological advance has been the development of two independent culture systems that can promote growth of luminal cells into three-dimensional organoids (Chua *et al.* 2014, Gao *et al.* 2014, Karthaus *et al.* 2014). Both of these culture conditions support the long-term growth of mouse and human prostate tumor cells and should allow the identification and functional analysis of luminal cancer stem cells. Nonetheless, experimental findings using organoid systems will require validation using *in vivo* approaches and should be viewed as complementary to results obtained using GEM models.

In addition, although transplantation/grafting assays represent highly important approaches for functional analyses of candidate cancer stem cells, these assays test the potential of cells to propagate tumors, and not their actual fate (Magee *et al.* 2012). With respect to the normal prostate, stem cell properties can be highly assay-

dependent and may not reflect *in vivo* activities (Choi *et al.* 2012, Wang *et al.* 2013). Consequently, it is essential to pursue *in vivo* studies of prostate cancer stem cells in GEM models to establish their endogenous role in tumor propagation (Fig. 1). In particular, lineage-tracing, clonal analyses, and cell ablation studies have provided evidence for cancer stem cells in solid tumors of the brain, skin, and intestine (Chen *et al.* 2012, Driessens *et al.* 2012, Schepers *et al.* 2012, Oshimori *et al.* 2015). Lineage-tracing studies of metastasis in suitable GEM models, such the *Nkx3.1^{CreERT2/+}; Pten^{fllox/fllox}; Kras^{LSL-G12D/+}* model (Aytes *et al.* 2013), would also be useful to determine whether cancer stem cells have the potential to serve as metastasis-initiating cells. Interestingly, however, recent studies provide evidence for polyclonal metastases in prostate cancer patients, suggesting that metastases might be seeded by multiple cells (Gundem *et al.* 2015, Shen 2015) and raising the possibility that tumor-propagating activity may itself require cooperative interactions between different cell types (Marusyk *et al.* 2014).

Finally, it is evident that additional markers for cancer stem cells will need to be identified, particularly those that are conserved between mouse and human. For example, candidate markers may emerge from next-generation sequencing efforts of mouse and human prostate tumors, as well as from molecular analyses of candidate cancer stem cell populations identified in GEM models. Such studies could lead to the identification of new cell surface markers that will enable functional analyses of intratumor heterogeneity, as well as biomarkers with potential clinical utility. In particular, biomarkers for castration-resistant stem cells could potentially distinguish indolent from aggressive disease and thereby decrease the overtreatment of indolent prostate tumors, which represents a major clinical challenge.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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