Concise Review: Prostate Cancer Stem Cells: Current Understandin

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Originally published:

June 2018

Stem Cells 36(2018), 1457-1474

DOI: https://doi.org/10.1002/stem.2859

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Prostate cancer stem cells: current understanding.

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Abstract

Prostate cancer (PCa) is heterogeneous harboring phenotypically diverse cancer cell types. PCa cell heterogeneity is caused by genomic instability that leads to the clonal competition and evolution of the cancer genome and by epigenetic mechanisms that result in subclonal cellular differentiation. The process of tumor cell differentiation is initiated from a population of prostate cancer stem cells (PCSCs) that possess many phenotypic and functional properties of normal stem cells. Since the initial reports of PCSCs in 2005, there has been much effort to elucidate their presence and properties. In this review we discuss the current methods for PCSC enrichment and analysis, the hallmarks of PCSC metabolisms and the role of PCSCs in tumor progression.

1. Prostate cancer hierarchy, heterogeneity and plasticity

1.1. Cancer stem cells

Cellular heterogeneity represents an omnipresent feature of most human tumors. Cancer cell heterogeneity can result, in principle, from both clonal competition and evolution (i.e., selection of the 'fittest' clone) driven by genetic instability inherently high in tumor cells as well as intra‐clonal (subclonal) cellular maturation (differentiation) program driven by epigenetic mechanisms (1). The latter process of cell diversification is initiated from a subset of unique cancer cells that possess many phenotypic and functional properties of normal stem cells, hence cancer stem cells (CSCs). In the most strict sense, a single CSC should be able to regenerate a tumor that histo‐structurally recapitulates the parent (patient) tumor that consists of all different cancer cell types. No such a CSC has ever been reported in any tumor. In reality, CSCs are operationally defined as cell subpopulations that are enriched in tumor‐regenerating and (serial)

tumor‐propagating activities (2). CSCs should be distinguished from cancer cell‐of‐origin as the latter refers to the initial normal cell that became tumorigenically transformed (2).

Like most human solid tumors, prostate cancer (PCa) is heterogeneous harboring diverse cancer cell types. PCa cell heterogeneity tends to become accentuated during disease progression and, in particular, upon treatment. This is best evidenced by PCa cells that express AR and PSA – although most untreated primary tumors contain AR^+ and PSA^+ cells as the majority, AR' and PSA' PCa cells become gradually enriched in high‐grade untreated tumor and PSA' PCa cells frequently become the predominant cell population in castration‐resistant PCa (CRPC) (3). Ever since the initial reports by 3 independent groups (4‐6), in 2005, of PCa stem cells (PCSCs), there has been now substantial evidence for the presence and functions of PCSCs, which we shall further elaborate below.

1.2. Current methods for PCSC enrichment and analysis

The first proof of principle studies for prospective identification of PCSC exploited different phenotypes to isolate self‐renewing and tumorigenic cells from patient derived prostate tumors and cell lines (4‐6). These phenotypes included expression of surface markers $CD44^+/\alpha2\beta1^{hi}/CD133^+$, expression of transporter protein ATP‐binding cassette sub‐family G member 2 (ABCG2) that is involved in the cell detoxification and "side population" phenotype which is defined by Hoechst dye exclusion by tumor cells (4‐6).

Since then, a few other markers have been described for human PCSC populations, e.g., CD166 (7), aldehyde dehydrogenase (ALDH) (8), $CD44^+/CD24^-$ (9), and C‐X‐C chemokine receptor type 4 (CXCR‐4) (10). PCa cells carrying these markers have capacities to self‐renew and to generate the heterogeneous tumor cell populations.

Using antibodies to the membrane CSC markers and fluorescence activated cell sorting (FACS) or magnetic cell sorting (MACS), marker positive and marker negative cells can be isolated from cancer cell lines, xenograft tumors and patient‐derived specimens, and CSC properties of these cell populations can be analyzed and compared (Table 1).

In addition to the isolation of CSC population based on the marker expression, other strategies exploiting the CSC specific gene reporters are being developed for real‐time analysis of CSC behavior at a single‐cell level *in vitro* and in animal models. These systems are based on the stable expression of reporter gene (e.g. fluorescent protein) driven by the promoters of CSC‐ or lineage‐specific genes such as NANOG, prostate specific antigen (PSA), Sox2, and OCT4 (11‐16) (Table 1).

The methodology exploiting the marker‐based CSC isolation and enrichment markedly contributed to the progress in cancer stem cell research, however it is necessary to take into account that the majority of the above‐described markers were also identified in the malignant tumors of other origins, such as breast, colorectal, ovarian, lung carcinomas and glioblastoma (17‐19). It is also known that these markers are not specifically expressed in malignant tissues, they were also found on the normal embryonic and adult stem cells . Therefore, further investigation of additional reliable and more CSC‐specific markers is needed to improve the marker‐based CSC harvesting techniques. More specific CSC isolation can markedly contribute to the elucidation of CSC‐specific molecular and functional properties.

Since some of CSC populations are described to be treatment‐resistant, there is a strategy to enrich CSCs by the repetitive use of chemotherapeutics or radiation therapy (20). PCa cell cultivation in the presence of cytostatic drugs or after radiation exposure led to the development of the acquired treatment resistance and enrichment of

carcinoma cells positive for CSC markers and having high tumorigenic capacities (20). Some of the treatment resistant CSC populations are characterized by upregulation of ABC family members including ABCG2 transporter. This fact provides a background to use a side population technique for CSC isolation (21). For this, tumor cells either from cell cultures or from the dissociated xenograft or primary human tumors are stained with Hoechst 33342 or Rhodamine 123 dyes. Further cell analysis by flow cytometry enables detecting the cells with increased dye efflux (side population) (6, 22). It is suggested that CSCs with a high expression of ABC transporters actively release the dye out of the cells. Unfortunately, this method has a number of limitations. First, Hoechst 33342 and Rhodamine 123 are toxic for cells. Next, an efficiency of CSC isolation based on side population might be hampered by low specificity and inconsistency of the existing staining protocols. Despite the fact that carcinoma cells with acquired therapy resistance usually demonstrate upregulation of CSC biomarkers, it is important to mention that treatment‚resistant cells do not necessary represent an unified cell population, but rather a mixture of phenotypically different cell subsets with different properties (23). A number of studies indicate that therapy might act as a selective pressure on preexisting CSC populations, but also in some cases might induce tumor cell dedifferentiation (3, 20, 24).

CSCs can also be identified and isolated using the differences in sizes between non‚CSCs and CSCs. It was described that CSCs are markedly smaller than more differentiated cells (25, 26). Recent study of Li et al. showed that a population of small PC3 PCa cells (<10 Pm) has a tendency of being more tumorigenic as compared to the large (≥20 or 30 Pm) cells (26). Our unpublished studies demonstrated that prostate tumor cells with upregulation of $CD44^{+}$/CD24’ markers are smaller in their sizes as compared with marker negative cells. Further investigation is warranted to clarify the

mechanisms controlling cell size in homeostasis and cancer and whether cell size can be effectively used for CSC identification and isolation.

Growing the established cell lines or patient derived tumor cells under serum‚free and sphere forming conditions was one of the first methods to enrich CSC population *in vitro*. Most of the cells cultured under these harsh conditions undergo anoikis and die. The survived cell populations are enriched in sphere forming cells, which can sustain their proliferation independent on the cell attachment mechanisms. This assay also allows investigating the cancer cell populations with self‚renewal properties if the sphere formation is analyzed in multiple passages, because only self‚renewing cells are capable of maintaining their spherogenicity in multiple generations (3, 27). Despite an enrichment of cells expressing CSC markers under sphere forming conditions, it might not necessary correspond to their tumorigenic abilities in xenograft models (28). Kuch et al (2012) suggested that capacities of carcinoma cells to form spheres cannot be used as a reliable surrogate assay to detect the tumor‚initiating capacities in animals (28). The report of Matilainen et al. (2012) indicated that $\alpha2\beta1^{+}$ prostate tumor cells with high clonogenic and sphere forming capacities did not have high tumorigenic abilities in xenograft mice models (29).

In that respect, *in vivo* limiting dilution assay should be performed as a standard technique to analyze the frequency of tumor initiating cells in the cancer cell populations defined by putative CSC markers. For this analysis, the same numbers of marker positive and marker negative cancer cells isolated either from cancer cell lines or from dissociated xenograft or human tumor specimens are injected into immunodeficient mice by orthotopic or ectopic implantation. The cells are injected at the different dilutions: from a few or even single cell to thousands or millions cells, and tumor growth is monitored by

caliper measurements and tumor bioluminescence for a few months (3, 6, 30). Analysis of tumor incidence for the different numbers of injected cells enables to estimate the CSC frequencies in the injected cell populations (2). To validate if putative CSC populations indeed possess the long‐term self‐renewal capacities *in vivo*, the CSCs of interest are isolated from the xenograft tumors, and then serially injected into the mice. Cell populations that can maintain long‐term self‐renewal properties (CSCs) will have sustained tumor growth during multiple transplantations. Cell populations with a limited self‐renewal will lose their tumorigenicity upon serial xenografting (2, 31).

For the lineage tracing of CSC cells *in vivo*, a genetically modified mouse model can be established where CSC populations are labeled by using CSC specific gene reporters and monitored during tumor development in the living mice (32).

New approaches which facilitate genome editing such as transcription activator‐like effector nucleases (TALENs), zinc finger nucleases (ZFN) and the clustered regularly interspaced palindromic repeats/Cas9 associated (CRISPR/Cas9) technologies opened new possibilities to investigate endogenous gene functions at the genome‐wide scale and to establish a direct link between the cellular genome and cellular properties including multipotency and self‐renewal.

3. Hallmarks of PCSC metabolism

3. 1. Metabolism of normal and cancer prostate epithelial cells

In contrast to other epithelial tissues, normal prostate epithelial cells have alterations in glucose metabolism attributed to the main function of prostate gland: to produce and secrete fluid that protects and nourishes sperm. Luminal compartment of prostate gland produces and secretes a large amount of citrate into prostatic fluid. The

high level of citrate is important for the maintenance of sperm viability by supplementation with the source of energy and by calcium chelation (33). In most of normal tissues, citrate, which is produced in mitochondria or delivered into the cells by the membrane transporters, is utilized as a major substrate for energy production in tricarboxylic acid cycle (TCA) (34). High production of citrate in prostate glands is a consequence of low activity of mitochondrial aconitase (m‚ACNT) and subsequent inhibition of citrate oxidation (35). In normal luminal cells, suppression of m‚ACNT enzymatic activity is a result of a high mitochondrial concentration of zinc that is maintained by a high expression level of zinc transporter proteins (35, 36). As a result of the impaired citrate oxidation, normal prostate epithelial cells have a low level of TCA and glycolytic metabolism. In contrast to the normal luminal cells, malignant prostate cells contain a low level of mitochondrial zinc that abrogates the inhibition of ACNT activity and enables utilization of citrate in the metabolic pathways (34‚36).

Reprogramming of cellular energy metabolism plays an important role in tumor initiation, progression and therapy resistance. To meet high energetic demands, fast growing tumor cells that exhibit the Warburg effect, need to reprogram their metabolic pathways for a high level of nutrient consumption that is especially true in case of metabolically active aggressive primary tumors and castration‚resistant metastatic disease. These metabolic features of prostate tumors can be used in clinical setting for detection of metastatic disease and assessment of the therapeutic response using positron emission tomography (PET) for glucose uptake imaging with 2‚[18F]Fluoro‚2‚ deoxyglucose (FDG) (37). In addition to an increased glucose uptake, highly proliferative cancer cells require additional supplies for their biosynthesis that cannot be met by

glucose consumption. A significant proportion of the biosynthetic needs may be covered by the metabolism of glutamine that is the most abundant amino acid in human plasma.

3. 2. Glutamine metabolism in PCa

Glutamine is an important donor of nitrogen and carbon for the growth‚promoting pathways. Although most normal tissues can synthesize glutamine, it becomes conditionally essential for the fast growing tissues (38, 39). A high demand for glutamine is particularly true for tumors that acquire oncogene‚dependent glutamine addiction including prostate carcinomas (38, 39).

Glutamine contributes to both energy producing pathways in cancer cells: mitochondrial respiration and anaerobic glycolysis (40, 41). In addition, glutamine is involved in maintaining homeostasis of the reactive oxygen species (ROS) and is important for nucleotide and amino acid biosynthesis (40, 41). Beyond its role in cellular metabolism, glutaminolysis also modulates many intracellular mechanisms that play a pivotal role in cancer progression. These mechanisms include activation of the mammalian target of rapamycin (mTOR) signaling pathway (42), promoting glycosylation of the growth factor receptors required for their cell surface localization and signaling activation (43), activation of the metabotropic glutamate receptor pathways as well as inducing replicative immortality and resistance to cell death (40, 41).

The curative rate of radiation therapy that is one of the main treatment for PCa, depends on its ability to induce irreparable DNA damage in tumor cells by direct DNA ionization or through production of chemically reactive oxygen species (ROS) (44, 45). During tumor growth, some of the tumor regions can outgrow their blood supplies and therefore have a low oxygen tension, or hypoxia. Cancer cells residing in the hypoxic

areas can be more shielded from the radiation‐induced DNA damage through reduced production of ROS and activation of the pro‐survival signaling pathways such as hypoxia‐inducible factor (HIF) signaling (46). HIF1 is also associated with development of PCa metastases (47) and activates transcription of genes involved in glycolytic flux and Warburg effect (48). To defend against ROS‐mediated oxidative stress, some metabolic pathways including glutamine and pentose phosphate pathway (PPP) enable cancer cells to produce a high level of glutathione (GSH) involved in scavenging and detoxification of ROS (49). Recent work demonstrated that D‐3‐phosphoglycerate dehydrogenase (PHGDH) that is one of the key enzymes to catabolize glutamate for ATP and GSH synthesis, is required for the maintenance of breast CSC and metastasis, and PHGDH knockdown was associated with reduced tumorigenicity (49).

The essential role of glutamine metabolism in cancer cell survival and proliferation under nutrient‐poor tumor microenvironment makes it an attractive target for cancer therapy and tumor imaging, and [18F]‐labelled‐ and hyperpolarized [5‐13C] analogues of glutamine are currently under development for localization of malignant tissues including prostate tumors (50).

3.3. MYC dependent metabolic reprogramming and maintenance of PCSCs

Although many cancer cells require glutamine for survival and growth, tumor cells with enhanced expression of MYC oncogene are particularly dependent on glutamine metabolism to sustain their viability. In these cells, deprivation of glutamine results in depression of TCA cycle, decrease in ATP level, and apoptosis (38, 51). MYC is a broad transcription regulator that activates the embryonic stem cell program in human cancer and induces tumor initiating cells (52). Recent findings indicate that targeting of MYC

inhibits the maintenance of CSCs and tumorigenicity (53). MYC contributes to metabolic adaptations of tumor cells which exhibit the Warburg effect by regulation of the glucose transporter GLUT1 and the enzymes involved in the glucose metabolism such as hexokinase 2 (HK2), phosphofructokinase (PFK1), enolase 1 (ENO1) and lactate dehydrogenase A (LDHA) (54).

Together with androgen receptor (AR) and well established tumor driver mTOR, MYC regulates expression of glutamine transporters genes *SLC1A4* and *SLC1A5*, which are frequently overexpressed in PCa, and thereby increases glutamine uptake (55). Through the suppression of miR‚23a/b, MYC also regulates level of GLS1 glutaminase that converts glutamine to glutamate at the first step of glutamine utilization (51). Analysis of human prostate tissues has revealed upregulation of *MYC* mRNA in the majority of primary PCa lesions (53). This overexpression of *MYC* can be attributed to *MYC* oncogene amplification that occurs in up to 29% of hormone‚refractory recurrent prostate carcinomas (56). In addition, *MYC* expression can be regulated at the mRNA level as a result of APC hypermethylation and Wnt/β‚catenin pathway activation, deletion of FOXP3 gene as well as TMPRSS2‚ERG rearrangement that activates ERG‚ dependent transcription program (57‚60). The single‚step overexpression of *MYC* is sufficient to bypass senescence and immortalize prostate cells derived from benign prostate tissue specimens (61). Transgenic Hi‚Myc mice that express human MYC in the mouse prostate in response to androgen, first develop prostatic intraepithelial neoplasia (PIN) followed by invasive adenocarcinoma (62). This process has a MYC dose‚dependent manner, and MYC‚driven tumors share molecular signatures with human PCa such as loss of Nkx3.1 tumor suppressor and upregulation of Pim‚1 protein kinase that increases energy metabolism and protein synthesis (62, 63). The two‚step

transformation of prostate basal or luminal cells from benign prostate tissues either by MYC overexpression and AKT activation, or by overexpression of MYC and knockout of the tumor suppressor phosphatase and tensin homolog (PTEN) induces development of heterogeneous tumors from both luminal and basal cell compartments (64,67). High expression of MYC and loss of PTEN contribute to the androgen,independent growth and are associated with highly invasive and metastatic prostate tumors (66,69). Interestingly, MYC is a context,dependent regulator of the expression of glutamine transporters *SLC1A4* and *SLC1A5*, and does not regulate these transporters in PTEN wild,type tumors (55).

3.4. Androgen signaling and prostate cancer metabolism

Dependence on androgen signaling is a hallmark of initial stages of prostate tumor progression. Because of this, androgen deprivation therapy (ADT) is one of the main treatments for PCa beside prostatectomy and radiotherapy. While most of the patients initially respond to ADT, about 90% of them eventually develop androgen refractory tumors with a fatal prognosis (70). Androgen,independent tumor progression depends on the different mechanisms including *AR* gene amplification and gene mutation, ligand,independent activation of AR signaling pathway and alterations in the expression of AR coregulators leading to an increase in AR,dependent transcription activity (71). Like MYC, AR regulates expression of many proteins involved in glutamine metabolism including GLUT1, HK1/2 and calmodulin,dependent kinase kinase 2 (CAMKK2). CAMKK2 is highly upregulated in PCa and CAMKK2,dependent activation of AMP,regulated kinase (AMPK) results in an increased 1,phosphofructokinase (PFK1) activity and promotes glucose uptake and lactate production (72). Metformin that is the

first‐line drug for treating diabetes enhances AMPK activity by inhibition of mitochondrial complex I (73). Metformin treatment was clinically associated with a significant decrease in early biochemical relapse and cancer specific mortality in PCa patients (74), and also led to an increase in tumor oxygenation and response to radiotherapy in mice xenograft models (74). Metformin combined with doxorubicin completely inhibits growth of PC3 PCa cells in mice xenografts (75). Metformin treatment of PC3‐docetaxel‐resistant (PC3‐DR) cells which have an increased oxidative metabolism, results in the inhibition of their proliferation and invasiveness without affecting parental PC3 cells (76). However, the effect of metformin on PCSCs has not been investigated so far. Interestingly, in the above described Hi‐Myc mice model of PCa progression, metformin supressed AR‐dependent expression of MYC and inhibited development of PIN and PCa lesions (77).

An increase in MYC copy number in tumor cells was observed as a result of androgen deprivation therapy in PCa patients (78). Experimental data showed that overexpression of MYC antagonizes the AR‐dependent transcription program and confers growth advantage for tumor cells grown without androgens. Therefore, increase in MYC expression might induce androgen‐independent prostate tumor progression (69, 79).

3.5. Metabolic reprogramming of prostate tumor epigenetics

Interestingly, MYC expression level inversely correlates with global level of H3K27me3 repressive histone mark in human PCa and MYC‐driven mice prostate tumors (80). Decreased level of H3K27me3 correlates with a higher Gleason score and pathological stage. The basal compartment of normal prostate epithelium has a lower level of H3K27me3 as compared to the luminal cells (80). In support to this observation,

MYC expression and MYC‐dependent transcriptional program were shown to be upregulated in basal cells (81). Recent work revealed that normal stem cells such as embryonic stem cells (ESCs) have an increased consumption of glutamine that is utilized to maintain a high level of α‐ketoglutarate (AKG). AKG is a co‐factor for the Jumonji C domain‐containing histone lysine demethylases as well as ten‐eleven translocation hydroxylases (82) that regulate DNA demethylation (83, 84). In ESC, AKG promotes histone demethylation including H3K27me3 mark and therefore suppresses differentiation (20, 85). Targeting glutamine uptake via inhibition of the major glutamine transporter in cancer cells ASCT2 (SLC1A5) supress tumor growth in PCa xenografts (86). Prostate tumor initiating populations also exhibit a high dependence on glutaminolysis. Recent studies employed two clonal populations derived from PC3 PCa cells with different orthotopic and metastatic growth in xenograft models to characterize metabolic features associated with PCSCs (87, 88). These studies showed that PC3 CSC‐like cells exhibit a high level of anaerobic glycolysis. These cells have a high level of lactate production and increased consumption of glutamine to compensate the acidification derived from the Warburg effect through release of ammonia (88). In support of this data, other studies showed that advanced PCa have an increased glycolytic phenotype and a high activity of lactate dehydrogenase (LDH) that correlates with poor clinical prognosis (25, 89).

4. The role of CSC in prostate cancer progression

4.1. A “vicious cycle” of prostate bone metastases

Majority of PCa patients with advanced disease develop bone metastases (90). Once tumor spreads to the bone, it typically cannot be cured (91, 92).This stage of

disease is associated with a high risk of morbidity caused by the various complications from bone metastases (93). Prostate bone metastases can produce factors that can enhance both osteoblastic and osteoclastic activity and disrupt normal bone homeostasis. In turn, the supportive sites which maintain survival and proliferation of cancer cells in bone marrow are defined by special cytokines and growth factors produced by osteoblast, osteoclasts and bone marrow stromal cells or released from the bone matrix upon resorption. This feedback loop between the seeds (prostate tumor cells) and soil (bone microenvironment) leads to a “vicious cycle” of tumor growth as reviewed previously (94).

The bone microenvironment produces or releases various growth factors and cytokines which bind to the receptors of prostate tumor cells and regulate their growth and survivals. Among them are Wnt proteins that bind to the Frizzled receptor and its LRP5/6 co‐receptor and activates β‐catenin dependent gene expression, C‐X‐C motif chemokine ligand 12 (CXCL12) that binds to C‐X‐C chemokine receptor type 4 (CXCR4), as well as growth factors e.g. transforming growth factor beta (TGFβ), insulin like growth factor 1 (IGF1), bone morphogenetic proteins (BMPs) and fibroblast growth factor (FGF) that promote metastatic growth (95).

In return, PCa cells in bone can produce several pro‐osteolytic factors including interleukins IL‐6, IL‐1, parathyroid hormone‐related protein (PTHrP) and prostate specific antigen (PSA) that stimulate formation of osteoclasts and promote resorption of bone matrix. Unlike bone metastases from breast and myeloma tumors, PCa metastases almost always form osteoblastic lesions (96). The factors released by PCa cells in bone include signaling molecules affecting osteoblast proliferation or differentiation such as WNT ligands, transforming growth factor‐β (TGFβ), and

osteoprotegerin (OPG) that regulate both, bone remodeling and tumorigenisis (96, 97). OPG that is produced by PCa cells and by bone marrow, is an inhibitor of a TNF-related apoptosis inducing ligand (TRAIL) and important survival factor in hormone independent PCa cells (98). Interestingly, PSA has been shown to inhibit OPG expression and increase mRNA expression of the receptor activator of nuclear factor kappa،B ligand (RANKL) that induces osteoclast differentiation (99). It worth to mention that in contrast to primary tumors that are almost always PSA positive, up to 40% of prostate metastases are negative for PSA expression (100, 101).

4.2. Metastatic spread as CSC evolution process

Metastatic spread is a multistep process that starts from invasion of cancer through the basement membrane and entering the bloodstream (intravasation). At this stage circulating tumor cells (CTCs) can be disseminated to distant organs. Some of CTCs which escaped anoikis, immune clearance and mechanical stresses can leave the bloodstream and enter the tissue in the distant organs in a process called extravasation. At this stage these disseminated tumor cells (DTCs) can remain dormant at distant site for a few years prior to metastatic progression (90, 102). Only a small subset of DTCs can become metastasis initiating cells (MICs). Selection of DTCs toward a metastatic genotype is driven by the microenvironmental conditions at distant site but also by the factors from the primary tumors, which define the ability of DTCs to overcome tumor latency and to form a metastatic tumor (90, 103،105). Metastasis formation can be considered as an evolutionary process (Figure 1). Plasticity of the genetic and epigenetic tumor makeups and selection of the most adapted tumor cells at the all stages of metastatic tumor progression results in the development of cell inherent traits

that favor tumor dissemination (1, 91). CSCs that possess self‐renewal properties and genomic instability are considered to be an engine of tumor evolution. It is becoming commonly accepted that tumor metastases are driven by the evolved populations of CSCs at their worst. In support of this CTCs and DTCs express many CSC markers, and metastasis initiating cells, MIC are sharing key CSC features including ability to self‐renew, to form tumors and to activate CSC specific signaling pathways as reviewed recently (91) (Table 2). The gene signatures that are specific for CSCs correlate with tumor progression and development of prostate tumor metastasis (88, 106). Enumeration of CTCs in blood, and DTCs in bone marrow of PCa patients have prognostic and predictive value in PCa (89, 90, 107). Metastatic tumors, CTCs and DTCs also show varying degrees of concordance with molecular signatures of primary tumor of origin (108‐112). Recent study based on the analysis of a large set of RNA expression data from more than 4000 clinical PCa specimens demonstrates that tumor subtype which exhibit a high expression of luminal markers EZH2, AR, MK167 and low expression of luminal markers NKX3‐1, PSA, ERG, KLK2 and basal markers e.g. TP63 and KRT5 is associated with the highest risk of progression to metastatic disease (113). Interestingly, this gene signature was highly expressed in 58% of CTCs from patients with antiandrogen therapy‐resistant tumors (113). This study demonstrated that molecular characterization of CTCs taken by noninvasive “liquid biopsy” can be used to identify the patients at risk of metastatic disease and treatment selection. These results are consistent with previous study showed that PSA negative ($PSA^{-/low}$) prostate tumor cells are resistant to chemotherapy, androgen deprivation and stress simulation, and exhibit long‐term tumor propagation capacity (3). Another study showed that CTCs and DTCs cells isolated from mouse xenografts of human prostate tumors exhibit increased

potential to metastasize *in vivo* and resistance to the chemotherapeutic agents mitoxantrone and doxorubicin (114).

4.3. Common mechanism for metastasis and therapy resistance

A growing body of evidence suggests mechanisms connecting the development of therapy resistance and metastatic phenotypes during tumor progression (115,117). In particular, the CXCL12/CXCR4 axis is shown to regulate extravasation and guidance of PCa cells to bone. Furthermore, activation of the CXCL12/CXCR4 and WNT/β,catenin signaling pathways which are important regulators of PCSCs can promote tumor radioresistance and contribute to the development of skeletal metastases (20, 118,120). Aldehyde dehydrogenase (ALDH) activity can be used to enrich for PCa cells with increased tumorigenic and metastatic capability *in vivo* and high radioresistance (20, 121, 122). ALDH plays an important role in the cellular response to oxidative stress and can potentially serve as one of the pro,survival mechanisms in CTCs enabling them to withstand oxidative stress in the bloodstream (123). Although several ALDH isoforms are described to be highly expressed in PCa, only ALDH1A1 gene expression was significantly correlated with ALDH activity and was increased at higher levels in advanced,stage compared to low,stage PCa and benign prostate hyperplasia (124). WNT ligands produced by tumor cells or by the bone cells are acting in an autocrine or paracrine fashion by enhancing tumor cell proliferation and inhibiting apoptosis (125). In addition, WNT ligands have a paracrine effect and induce osteoblastic activity promoting bone mineralization in PCa bone metastases, whereas WNT inhibitors promote an osteolytic environment (125, 126). Activation of canonical β,catenin dependent WNT signaling is associated with malignant transformation, metastasis and radioresistance of

PCa (20, 127). In different cancer models, WNT signaling pathway has been shown to regulate expression of CXCR4 and ALDH1A1 genes (20, 128, 129) whereas canonical WNT signaling pathway can be activated by CXCR4 ， dependent mechanisms (130, 131). Bone targeted therapies such as bisphosphonate drugs, exhibit inhibitory effects on osteoclast，mediated bone resorption but also inhibit the growth of PCa cells (132, 133). Recent studies showed that bisphosphonate treatment regulates the WNT signaling pathway in PCa e.g. by inhibition of *WNT5A* and *FZD5* gene expression (134). TGFβ signaling activated in bone metastases regulates the prometastatic and prosurvival properties of PCa cells including expression of CXCR4 and increase of ALDH activity (122, 135，137). PCa cells have deregulated expression of the transmembrane extracellular matrix receptors integrins (138) that play a critical role in prostate tumor cell invasion and metastatic development (139). Tumor cell population positive for α2β1 integrin expression is enriched for prostate tumor initiating cells (30, 140). Recent studies showed that integrin signaling might also contribute to the resistance to therapeutic androgen ablation and radiotherapy in PCa cells (141, 142).

Therefore, activation of the signaling pathways detected on the genomic and transcriptomic levels in DTCs, CTCs from the blood of the patients and CSCs in primary tumors can be prognostic for metastasis development and, on other hand, can be involved in the response of primary and metastatic tumors to therapy. Further studies are warranted to investigate the molecular mechanisms regulating metastasis，initiating and therapy resistant prostate tumor cells and characterize their clinical relevance.

5. Summary and future directions

PCSCs that possess self‚renewal properties and genomic instability might serve as a driving force of tumor evolution and metastatic dissemination. A number of recent studies demonstrated that analysis of CSCs in patients' tumors might serve as prognostic or predictive biomarker (143, 144). In combination with other extrinsic and intrinsic parameters that might affect the properties and complexity of CSC populations, for example heterogeneity indices and hypoxia, these markers could in the future be used for patient stratification and more personalized treatment selection. There is an increasing body of evidence that PCSCs possess a higher chemo‚ and radioresistance compared to the tumor bulk (3, 20, 145). In support to these studies, inhibition of the signaling pathways that play a role in the maintenance of PCSCs such as WNT/β‚ catenin and PI3K/AKT results in the sensitization of the experimental tumor models to the different types of therapy (121, 145, 146). Understanding the genetic relationship between PCSCs, CTCs, DTCs and MICs and better characterization of the MIC phenotypes and properties might become pivotal in the future for prevention of tumor cell dissemination and treatment of metastatic disease that is not curable with current standard therapies.

Acknowledgement

Work in Tang lab was supported, in part, by grants from the US National Institutes of Health (NIH) (R01‚CA155693), Department of Defense (W81XWH‚13‚1‚0352, W81XWH‚14‚1‚0575, and W81XWH‚16‚1‚0575), and the Chinese Ministry of Science and Technology (MOST) grant 2016YFA0101203, and by RPCI and NCI center grant P30CA016056. Work in A.D. lab is partially supported by grants from Deutsche

Forschungsgemeinschaft (DFG) (273676790), from Wilhelm Sander‚Stiftung (2017.106.1) and BMBF (Grant‚No. 03Z1NN11).

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Figures and Tables

Table 1. Methods for detection and isolation of CSC populations.

Table 2. The features of prostate cancer stem cells (CSCs), circulating tumor cells (CTCs), disseminated tumor cells (DTCs) and metastasis initiating cells (MICs). **Figure 1.** Phenotypes of CSCs, DTCs, MICs and prostate tumor progression. Prostate cancer development is an evolutionary process that reflects an evolving of cancer stem cells. Luminal or basal cells in normal prostate can act as a cell of tumor origin after oncogenic transformation. The link between the tumor initiating cells, or tumor cell of origin and cancer stem cells (CSCs) that maintain tumor growth is not yet understood. Tumor metastases are driven by the evolved populations of CSCs at their worst. Some tumor cells with malignant potential enter the blood stream (circulating tumor cells, CTCs) and can be disseminated to the distant organs. Single prostate tumor cells that can be found in bone marrow and called disseminated tumor cells (DTCs). Single tumor cells disseminated to lymph nodes called isolated tumor cells (ITCs, not shown). A small subset of DTCs or ITCs becomes metastasis initiating cells (MICs). Metastatic spread and formation is a long，time process that might take a few years. Once formed, metastases can form secondary metastasis to distant organs. Prostate cancer progression is associated with development of substantial intra，tumor heterogeneity and genomic instability that can be induced by MYC activation, loss of PTEN and mutations in DNA repair genes including BRCA2, ATM and CHEK2. Acronyms: AR – androgen receptor; CK – cytokeratin; PSA – prostate specific antigen; ALDH – aldehyde dehydrogenase; ABCG2 ，ATP binding cassette subfamily G member 2; α2β1， α2β1 Integrin; ERG1，early growth response protein 1; PTEN，phosphatase and tensin homolog; EpCAM，epithelial cell adhesion molecule; HER2，human epidermal growth

factor receptor 2; FGF，fibroblast growth factor; MAPK，mitogen‚activated protein kinase; PI3K， phosphatidylinositide 3‚kinase; NF‚κB，nuclear factor 'kappa‚light‚chain，enhancer' of activated B‚cells; TGFβ，transforming growth factor β; EGFR，epidermal growth factor receptor; CXCR4，C‚X‚C chemokine receptor type 4; EZH2，enhancer of zeste homolog 2; BRCA2，breast cancer type 2 susceptibility protein; ATM，ataxia telangiectasia mutated; CHEK2，checkpoint kinase 2.

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Table 1. Methods for detection and isolation of CSC populations

Marker-based methods					
Technique	**Method**	**Markers**	**Ref**	**Advantages**	**Limitations**
Staining cell surface targets / fluorescence detection of enzymatic activity	Fluorescence based cell sorting, IF microscopy, IHC	CD44	(147)	Analysis of the experimental (xenograft animal models) and clinical (patient(derived tissues, TMA) samples	These markers are not specific for PCSC and express on other cells including normal tissues; PCSC can be heterogeneous for the marker expression, and marker positive cells can be functionally heterogeneous (e.g. by their self(renewal properties)
		CD133/ CD44	(17, 147, 148)		
		ALDH/CD44/α2β1	(27)		
		CD166	(7)		
		Trop2	(149)		
Gene reporter systems	Fluorescence based cell sorting, fluorescence microscopy, *in vitro* and *in vivo* cell tracking	PSA	(27)	*In vitro* and *in vivo* tracking of CSC populations, in vivo CSC imaging, performance of phenotypic assays	
		26S proteasome activity	(12)		
		NANOG	(11, 16)		
		CD44	(146)		

Marker-free methods				
Technique	**Method**	**Ref**	**Advantages**	**Limitations**
Sphere forming assay	Cell cultivation under non(adherent conditions accompanied by FACS analysis to confirm CSC enichment in spheres	(150, 151)	Elucidation of molecular and physiological properties of CSCs; CSC characteristics associated with treatment(resistance	This methods cannot be used to obtain a highly enriched CSC populations; Sphere forming properties *in vitro* are not indicative for tumorigenicity *in vivo*
Therapy(induced CSC enrichment	Cancer cell cultivation in the presence of chemotherapeutic drugs or repetitive cell exposure to ionizing radiation	(20, 152)		
Side population	Fluorescence sorting based on the Hoechst 33342 or Rhodamine 123 efflux by CSCs	(6, 22)	Various samples can be analyzed e.g. cell lines, xenograft tumors and tumor specimens	Hoechst 33342 and Rhodamine 123 are toxic; absence of the standard dye concentration; low specificity
CSC isolation based on the cell sizes	Microfluidic techniques, FACS	(26, 153, 154)	Cells with different morphology, sizes and metabolic activities can be separated	Low specificity

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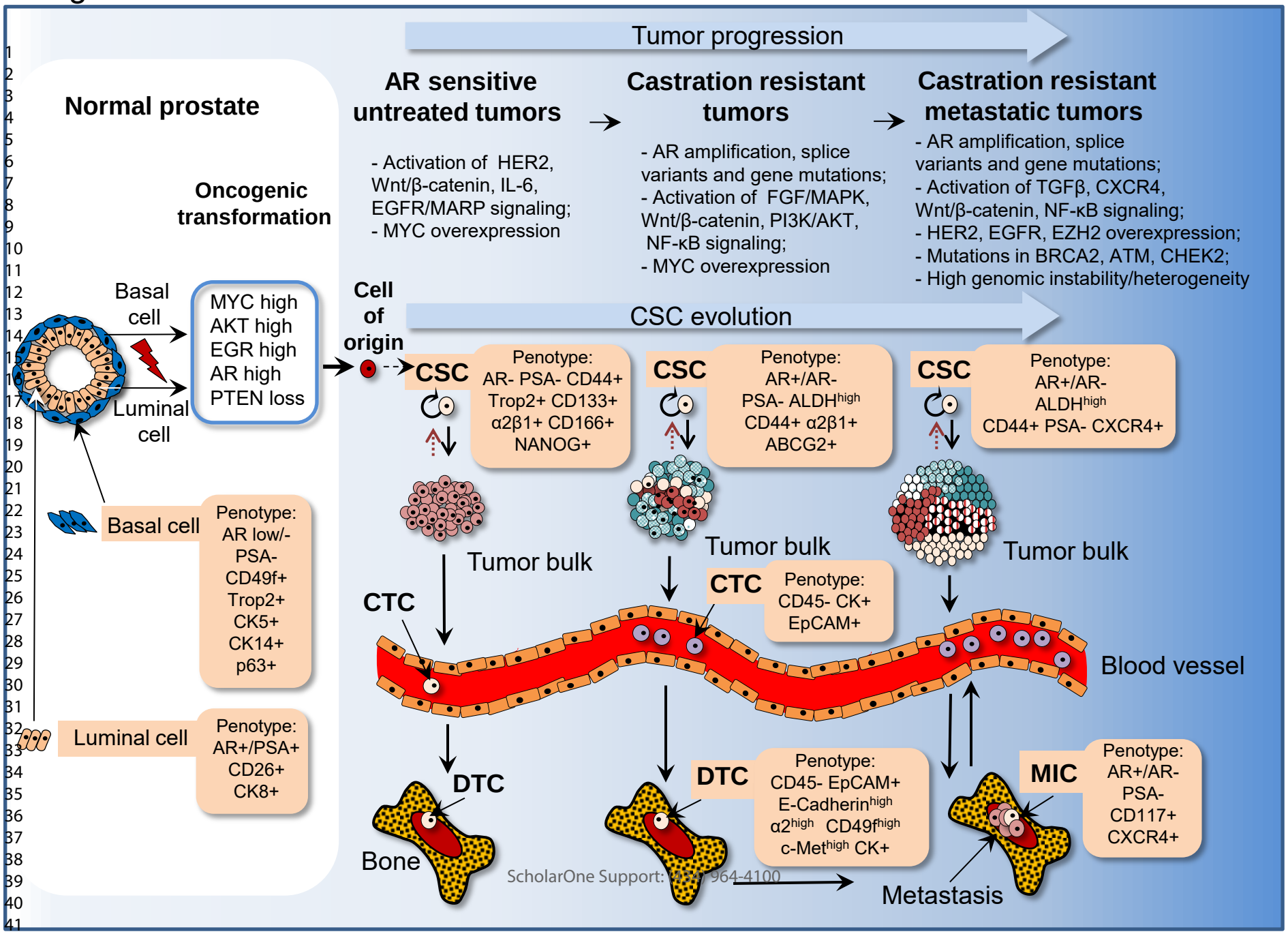
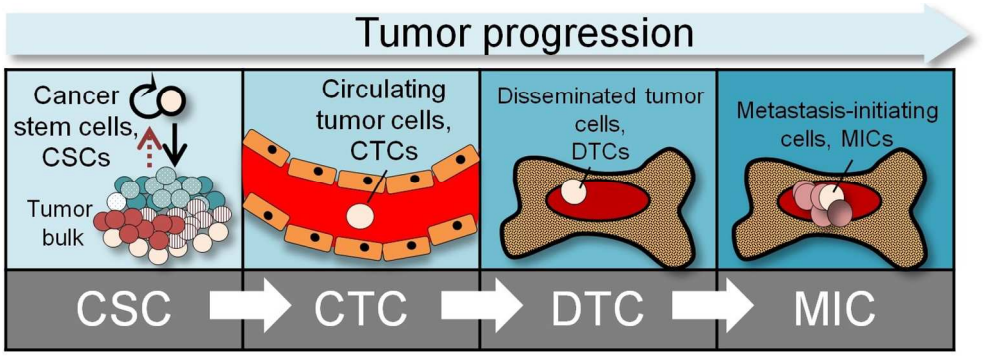
Table 2. The features of prostate cancer stem cells (CSC), circulating tumor cells (CTC), disseminated tumor cells (DTC) and metastasis initiating cells (MIC)

Features \ Cells	Non-CSC (tumor bulk)	CSC	CTC	DTC	MIC
Androgen receptor expression	AR^{+} (luminal cells); $AR^{low/-}$ (basal cells) (64, 81, 155)	AR- (primary PC) or AR-/+ (CRPC) (3, 7, 156, 157)	AR- or AR+ (158-162)	AR- or AR+ (163)	AR- or AR+ (164, 165)
Anrogen dependence	Primary PC: Yes CRPC: No (70, 166)	No (3, 27, 59, 157)	Yes / No (158, 167, 168)	Yes / No (165)	Yes / No (165)
PSA expression	Yes (luminal cells) (3, 64)	No (3) or low (169)	PSA+ or PSA- (167, 168, 170)	PSA+ or PSA- (171)	No data
Phenotype	Luminal or basal epithelial cells (166)	Luminal or basal epithelial cells (3, 32, 65, 66, 73, 155, 172-174)	Epithelial or Mesenchymal (EMT) (114, 159, 162, 170, 175, 176)	Epithelial (171), Mesenchymal (EMT) (114), Hematopoietic (171, 177)	No data
Tumorigenicity	Upon de-differentiation (12, 169)	Yes (3, 32, 65, 66, 73, 155, 172-174)	Probably (178)	Probably (179, 180)	Yes (181, 182)
Metabolism	Primary PC: OXPHOS CRPC: glycolysis (Warburg effect) (25, 37)	Glycolysis (88) or/and OXPHOS (74, 183)	No data	No data	No data
Heterogeneity	Yes (82)	Yes (3, 32, 65, 66, 73, 155, 172-174)	Yes (159, 161, 162, 167, 170, 184)	Yes (163, 165, 171)	Yes (181, 182)
Cellular state	Proliferative (166)	Quiescent (3)	Proliferative or quiescent (185, 186)	Dormant or quiescent (102, 187)	No data
Genetic evolving	Yes (182, 188)	Yes (188)	Yes, reflection of tumor evolution (111, 112, 189)	Yes (110) (90, 103-105)	Yes (181, 182, 190)


Tumor progression
Normal prostate
AR sensitive untreated tumors
- Activation of HER2, Wnt/β-catenin, IL-6, EGFR/MARP signaling;
- MYC overexpression
Castration resistant tumors
- AR amplification, splice variants and gene mutations;
- Activation of FGF/MAPK, Wnt/β-catenin, PI3K/AKT, NF-κB signaling;
- MYC overexpression
Castration resistant metastatic tumors
- AR amplification, splice variants and gene mutations;
- Activation of TGFβ, CXCR4, Wnt/β-catenin, NF-κB signaling;
- HER2, EGFR, EZH2 overexpression;
- Mutations in BRCA2, ATM, CHEK2;
- High genomic instability/heterogeneity
Oncogenic transformation
Basal cell
Luminal cell
MYC high
AKT high
EGR high
AR high
PTEN loss
Cell of origin
CSC evolution
CSC
Penotype: AR- PSA- CD44+ Trop2+ CD133+ α2β1+ CD166+ NANOG+
CSC
Penotype: AR+/AR- PSA- ALDH^high CD44+ α2β1+ ABCG2+
CSC
Penotype: AR+/AR- ALDH^high CD44+ PSA- CXCR4+
Basal cell
Penotype: AR low/- PSA- CD49f+ Trop2+ CK5+ CK14+ p63+
Luminal cell
Penotype: AR+/PSA+ CD26+ CK8+
Tumor bulk
Tumor bulk
Tumor bulk
CTC
CTC
Penotype: CD45- CK+ EpCAM+
Blood vessel
DTC
DTC
Penotype: CD45- EpCAM+ E-Cadherin^high α2^high CD49f^high c-Met^high CK+
MIC
Penotype: AR+/AR- PSA- CD117+ CXCR4+
Bone
Metastasis
ScholarOne Support: 964-4100



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