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The Potential Target Therapy of Prostate Cancer Stem Cells

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1. Introduction

Prostate cancer is the most commonly diagnosed cancer in men and the second leading cause of cancer related mortality (Byers et al., 2006; McDavid et al., 2004; Sedjo et al., 2007). Localized prostate cancer is treated by either radical prostatectomy or radiotherapy. For aggressive prostate cancer, hormonal therapy is the standard treatment however; approximately 30% of these tumors become hormone–independent (hormone-refractory) (HRPC). Furthermore, prostate cancer cells that survive chemotherapy or radiation treatment may be able to repair most of the radiation-induced DNA breaks (Kimura et al., 1999; Kimura & Gelmann, 2000). Therefore, a primary goal in the diagnosis and management of prostate cancer is the identification of biomarkers that can reliably predict the degree of malignancy of the tumor and can also be used as potential molecular targets to improve the response to therapy. This necessity has arisen from the fact that for American males, prostate cancer is the most diagnosed neoplasia and the second leading cause of cancer-related deaths (Kendal & Mai, 2010; Sajid et al., 2011).

Risk factors associated with increased prostate cancer incidences include both age and a sub-Saharan African ancestry, with African-American men having the highest reported incidence rates of all ethnic groups in the United States (239.8 cases/100,000) (Chu et al., 2003; Odedina et al., 2009). Furthermore, deaths from prostate cancer following surgery are more than 2-fold higher in African-American men (56.3/100,000) succumbing to the disease compared to white men (23.9/100,000) (Abbott et al., 1998; Chornokur et al., 2011; Talcott et al., 2007). Death from prostate cancer is generally due to metastatic disease that results from resistance to treatment such as anti-hormonal therapy. Since African American men are two-times more likely to die of prostate cancer, identifying the mechanisms that support indolent against aggressive disease is an important area of research.

Increasing evidence suggests that primary and metastatic tumors may be initiated and sustained by a subpopulation of low abundance cancer cells with stem-like properties (Clarke et al., 2006; Clevers, 2005). These cells, known as cancer stem cells (CSC) or tumor-initiating cells (TIC), share some characteristics with normal stem cells, such as the potential of self-renewal, the capacity to clonally expand, and the ability of multi-lineage differentiation (Dalerba et al., 2007; Lang et al., 2009; Weissman et al., 2001). In addition, through acquired genetic and epigenetic changes, these cells can exhibit abnormal behavior

like increased resistance to apoptosis, decreased senescence, and capacity to escape from immune surveillance. All these features can contribute to tumor dissemination, resistance to therapy, and disease recurrence.

There is little doubt that identification and characterization of CSCs is of enormous interest because they may provide important information related to the aggressiveness of the disease as well as be relevant for the development of targeted drugs to treat metastatic tumors and reduce recurrence (Park et al., 2009; Wang, 2007). It is important to note that several anticancer therapies are frequently used to eliminate prostate cancer cells; however; a number of these cells with malignant phenotypes can survive and may eventually lead to tumor regrowth (Dean et al., 2005; Dingli & Michor, 2006). Therefore, the recognition of markers for CSC and cell proliferation during the processes of prostate tumor initiation and progression is of vital importance to track CSCs for the development and improvement of therapies. It is also important to remark that subpopulations of prostate tumor cells with cancer progenitor properties are thought to support refractory response to a given treatment, leading to prostate tumor recurrence; therefore, new approaches to identifying and targeting these cancer subpopulations might provide an avenue to managing metastatic and recurrent disease refractive to treatment (Chaffer & Weinberg, 2011; Lang et al., 2009; Polyak & Hahn, 2006). This chapter will discuss the potential applications of CSC markers that may predict risk of clinical outcome and provide a guide for appropriate therapy of prostate neoplasias.

2. Stem cells in the prostate

One of the best-accepted models to explain the origin of the different cell types that make up a given tissue is the stem cell model (Reya et al., 2001; Weissman et al., 2001). In this model, most of the differentiated cell subtypes that give raise to the tissues can trace their origin to a few low-abundance progenitor cells and no longer term tissue maintenance is supported through normal adult stem cells that have the capacity for self-renewal and multi-lineage differentiation. These stem cells or progenitor cells are characterized by the high expression of specific embryonic markers and by a marked degree of plasticity that allows them to differentiate into the specific cell types required at a given point in time. According to the prostate stem cell model (Isaacs & Coffey, 1989), the progenitor cells reside primarily within the basal layer of the prostatic epithelium and have the capacity to give rise to all basal, luminal and neuroendocrine epithelial cells (Figure 1).

Prostate progenitor stem cells are androgen-independent and express high levels of stem cell markers (Table 1) such as prominin-1 (CD133), stem cell antigen (Sca1), cluster of differentiation 44 (CD44), integrin alpha2beta1 ($\alpha 2\beta 1$) and nestin (Collins et al., 2005; Li et al., 2008). As these progenitor cells differentiate into luminal secretory cells, they acquire the capacity to express androgen receptor (AR), prostatic specific antigen (PSA), prostatic acid phosphatase (PAP), cluster of differentiation 57 (CD57), 15-lipoxygenase 2 (15-LOX2), and cytokeratins 8 (CK8) and 18 (CK18). When the adult stem cells commit instead to the generation of non-secretory basal cells, they primarily express CD44, tumor protein 63 (p63), cytokeratin 5 (CK5) and 14 (CK14) but not AR, PSA, and PAP (Liu et al., 1997; Signoretti et al., 2000). There is a cell subtype intermediate between basal and luminal that shows expression of the CK5, CK8 and CK18 but not CK14 (Schalken, 2005). Neuroendocrine (NE) cells are found dispersed throughout the epithelium and can be identified by their expression of chromogranin A, synaptophysin, and neuron-specific enolase (NSE) (Bonkhoff

et al., 1995; di Sant' Agnese, 1996). NE cells also express some peptide hormones such as somatostatin, calcitonin and serotonin. However, they do not express AR or PSA. The prostate also contains several types of stromal cell including fibroblasts, myofibroblasts, and smooth muscle cells that guide the growth and differentiation of the epithelium.

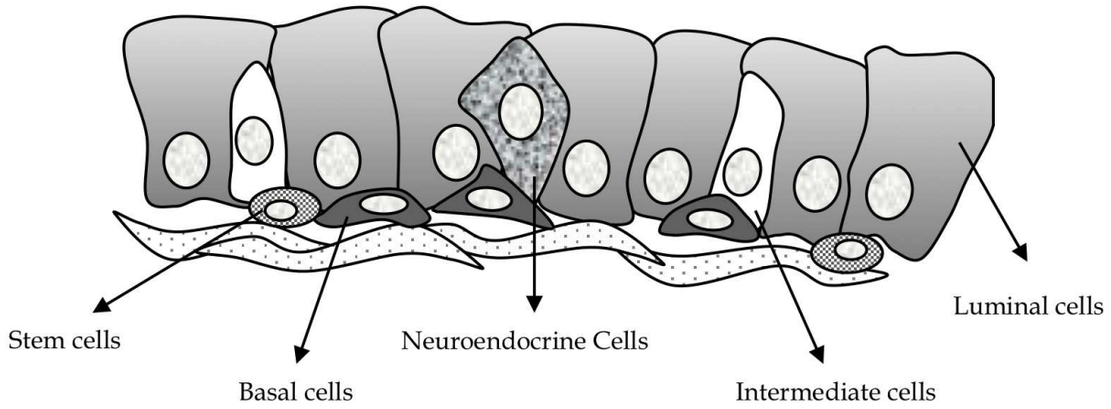

Fig. 1. The prostate epithelium niche contains a low percentage of prostate stem cells that express embryonic markers and are capable of generating the different cellular types, which compose this microenvironment. Cell types that are believed to arise from the differentiation of prostate stem cells are the basal, NE cells, and luminal secretory cells. Intermediate cells are believed to be a transitional type between basal and luminal cells.

The debate regarding the location, phenotype, number, nature, and presence of stem cells in the adult prostate still persist. However, consistent evidence supporting the existence of SC in the prostate has been reported in adult rodent prostate (English et al., 1987; Hudson, 2003; Miki, 2010). After several rounds of castration-induced regression and testosterone-induced regeneration in this animal model, a small proportion of cells (CSCs), were able to proliferate and also gave rise to differentiated and non-proliferative glandular epithelial cells. Although, the majority of luminal cells undergo apoptosis after androgen deprivation, the remaining androgen-independent epithelial cells contain a high proportion of basal cells (Montpetit et al., 1988; Webber et al., 1997a, 1997b). After regression, the remaining epithelial population appears to regenerate the prostate following androgen replacement.
The cycle of prostatic involution and regeneration can be repeated many times, indicating that the androgen-independent stem cells survive androgen deprivation and have an extensive proliferative and regenerative capacity, as well as multipotency. The identification and utilization of different types of biological markers in different *in vivo* models has led to the hypothesis that prostate stem cells reside within the basal cell layer of the gland. Indeed, mice null for p63, the progenitor gene for the tumor suppressor p53 family, are born without prostate or mammary glands (Mills et al., 2002; Signoretti et al., 2000). Both progenitors and basal cells of the prostate express p63, however this protein is lost during prostate differentiation and is not expressed in malignant prostatic lesions (Signoretti et al., 2000).
In humans prostate basal cells express B-cell lymphoma 2 (BCL2), an anti-apoptotic protein that is frequently expressed by stem cells and is inversely related to androgen stimulation (Lu et al., 1996). While administration of a pulse of bromodeoxyuridine (BrDU) identifies rapid proliferating transit-amplifying cells in the luminal compartment, a region that is close to the urethra, and also contains stem cells (Tsujimura et al., 2002). Because stem cells are

maintained in a quiescent state, the high expression of transforming growth factor beta (TGF-β) by smooth muscle cells that form the proximal region of the prostatic ducts was proposed as a mechanism that modulates proliferation of prostatic epithelial stem cells. Indeed, blocking of TGF-β expression in the prostate induced an excessive proliferation of prostatic epithelial cells in the proximal region of the prostate (Kundu et al., 2000). Additional observations have shown that stem cells are also located in different regions of the prostate, which have the ability to survive androgen ablation and can regenerate prostatic tissue after androgen is replaced (Goto et al., 2006).

Markers expressed by prostate epithelial cells				
Stem Cells	**Basal Cells**	**Neuroendocrine Cells**	**Intermediate Cells**	**Luminal Cells**
$CD133^+$	$p63^+$	AR^-	$CD133^+$	AR^+
$CD117^+$	$CD44^+$	$CK18^+$	$\alpha2\beta1^+$	$CK8^+$
$CD49f^+$	$CD117^+$	chromogranin A^+	$CK5^+$	$CK18^+$
$nestin^+$	$CK5^+$	synaptophysin $^+$	$CK8^+$	$CD57^+$
$\alpha2\beta1^+$	$CK14^+$	$serotonin^+$	$CK14^-$	PSA^+
$ALDH^+$	AR^-	$somastostatin^+$	$CK18^+$	PAP^-
$Trop\text{-}2^+$	PSA^-			$p27^+$
$CK5^+$	PAP^-			Sca1
$CK8^+$				
$CK14^+$				
$CK18^+$				
$CK19^+$				
AR^-				
$p63^-$				
PSA^-				
PAP^-				

Table 1. Complex expression of markers associated with different prostatic epithelial cells

The prolonged regenerative capacity of prostate progenitor stem cells may increase their susceptibility to accumulate genetic or epigenetic alterations during their life cycle, events that may help to promote increased proliferative rates, decreased cell death, and overall survival advantages over prostate progenitor stem cells, contributing thus to transformation (Al-Hajj & Clarke, 2004; Bapat et al., 2005; Beachy et al., 2004; Miller et al., 2005; Mimeault & Batra, 2006b, 2007a; Odedina et al., 2009). The tumor associated stem cell compartment may therefore, represent a self-replicating reservoir of malignant cells, which may accumulate further genetic and epigenetic aberrations that can thus result in therapy-resistant, tumor recurrence and/or metastasis (Hsieh et al., 2007; Rajan et al., 2009; Witte, 2009). It is believed that tumor stem cells maintain many of the features and capabilities of their normal counterpart, including long-term self-renewal and multipotency. Stem-like functions of CSCs inherited from their normal stem cells counterparts may be critical for survival during long periods of time in tissues (Hsieh et al., 2007; O'Brien et al., 2010; Rajan et al., 2009; Rosen & Jordan, 2009; Vermeulen et al., 2008; Witte, 2009). In this regard, the generation of cancer cells and their success in tumor growth are probably dependent on dysregulations affecting cells with stem-like characteristics and have the ability to promote tumor and support tumor development (Dalerba et al., 2007; Lang et al., 2009; Weissman et al., 2001). In

fact, malignant tumors, like normal tissue, frequently contain cells at various stages of differentiation, a mechanism that mimics dedifferentiation of mature cell types and presumes a hierarchical organization (Clarke et al., 2006; Clevers, 2005). Because there is the possibility that CSCs share various signaling characteristics with normal stem cells, several methods are frequently utilized to identify putative CSCs in human prostate cancer tissues, human and animal cell lines, and *in vivo* cancer animal models.

3. Assaying prostate cancer stem cells

The determination of cancer stem cells in primary tumors is a difficult task, with many variables and potential for confounding results. CSCs constitute a minority proportion of the constituent cells of tumors, and therefore their analysis can only be undertaken after isolation and propagation in tissue culture or *in vivo* systems. Several methods are being used to identify, enrich, and propagate these tumor cells and to study their biology as crucial players in tumorigenesis (Table 2).

3.1 Enrichment of CSCs from cancer cell lines and primary tumors

Human immortalized prostate cancer cell lines have been accessible and frequently utilized as biological models to investigate cancer biology and to test the efficacy of potential anticancer drugs. Most of cancer cell lines available have been continuously cultured under different conditions, and only a minor percentage of cells preserve their clonal growth, clonogenic potential, and the ability to form tumors when transplanted in animal hosts. Criticisms to cancer cell lines arise due to the high potential for culture contamination by other cell lines (Borrell, 2010; Li et al., 2009). These cells represent highly selected subgroup of cells that may have accumulated additional genetic abnormalities as they adapt to an artificial environment. Nevertheless, the utilization of cancer cell lines is an important tool used by researchers to study CSCs and carry out mechanistic studies.

Following isolation, analysis of CSCs is performed by culturing primary tumor cells *in vitro*, either as monolayers or as spheres, both of which are particularly difficult in the case of prostate cancer cells derived from primary tissues (Miki et al., 2007; Tokar et al., 2005). Indeed, most human prostate cancer cell lines utilized to dissect the events associated to cancer progression and metastasis has been established from metastatic lesions or from xenograft tumors (Sobel & Sadar, 2005a, 2005b). An efficient cell immortalization culture based on the inhibition of the serine/threonine kinase ROCK have been shown to reversibly immortalize primary human keratinocytes and to increase the cloning efficiency of murine prostate cells (Chapman et al., 2010). Since immortalized keratinocytes are genetically and functionally similar to the primary tissues they were derived from, this method is an advance over the traditional procedures that promote genetic changes. Strict parameters defining CSCs require that upon isolation these cells exhibit certain biological characteristics such as self-renewal and proliferative potential, as well the capacity, under the proper conditions, to differentiate into other cell types. These capabilities can be determined *in vitro* through the use of colony formation assays. However, the stem-like features of these cells is confirmed through experimental approaches in which these cancer cells in a very low number have the capacity to generate tumors in *in vivo* systems.

The most commonly used *in vivo* system used to propagate these tumorigenic cells are xenograft models, either subcutaneous in the renal capsule or orthotopic. For these isolated putative CSCs to be established as such, certain conditions must be met. Isolation of CSCs

should be capable of generating a tumor *in vivo*, and successful serial transplantation of these tumors must be possible for several generations. Importantly, a number of variables that can affect the outcome of these *in vivo* assays includes: the manner in which cells are isolated; whether these cells are initially propagated *in vitro* before transplantation, which can cause them to become more aggressive as a result of new acquired mutations; the procedure employed to inoculate the mice; the strain of mice employed, etc, that is often taken, in particular during the experimental planning phase and analysis process.

Assaying Prostate Cancer Stem Cells
Isolation through stem cell markers expression
CD44+
Integrin α1β2+
CD133+
CD44+/α1β2+
CD44+/CD24-
α1β2+/CD133+
CD44+/α1β2+/CD133+
Isolation of cancer stem cells
Established human cancer cell lines
Fresh human surgical samples
Culture in ultra-low attachment surface
Isolation of holoclones
Limiting dilution assay
Hoechst 33342 dye and exclusion
Rhodamine 123
Methods to measure proliferation, growth rate, invasion
Fluorescent activated cell sorting
Matrigel invasion
Matrigel 3-D culture
Sphere formation anchorage independent growth protasphere
Methods to identify gene signatures and/or protein expression
Microarray
Quantitative RT-PCR
Sequencing
Tissue microarray
Flow cytometry
Immunohistochemistry
Immunofluorescent analysis
Immunoblotting
Animals models (into immunocompromised mice)
Transplantation
Xenografts

Table 2. Methods utilized for the enrichment of prostatic cancer stem cells.

3.2 Determining the frequency of CSCs based on limiting dilution assay and spherogenecity

The rarity of cancer stem cells within the tumor bulk in many cases makes the utilization of the limiting dilution assay (LDA) necessary. This type of analysis has the ability to estimate the frequency of CSCs among a population of cancer cells (Hope & Bhatia, 2011; Schroeder, 2011). LDA can also be utilized to estimate the ability of a single tumor cell to form spheroids in serum-free cultures. Importantly, to reduce possible variations and calculate the frequency of CSCs, LDA needs to be tested in both bulk and fractionated cancer cells for each individual tumor (O'Brien et al., 2010). Since CSCs are a rare population with low numbers of cells, the injection of unsorted tumor cells into mice has been used to expand these cancer cells before applying LDA. It is important to consider that even if serial dilutions of tumor cells are injected into groups of animals, the development of a tumor in a recipient animal implies that the inoculum contained at least one reproductively intact cell (Porter & Berry, 1963). The LDA can also provide important information regarding the existence of cooperation among tumor cells that may be critical for the surviving and proliferation of cells that generate a tumor, therefore validating the existence of a single CSC (Hu & Smyth, 2009).

Several experimental approaches are being utilized to study the properties of cancer stem cells *in vitro*. For example, utilizing low adherence cultures, growth in soft agar, suspension cultures, and the use of ultra low adherence plates. In general, a serum-free media containing epidermal growth factor (EGF) and fibroblast growth factor (FGF), specifically formulated for the culture of pure populations of stem cells, is utilized to enrich and grow putative CSCs (Tropepe et al., 1999). The success of these methodologies is quite relative. Because the cells grow in these stringent conditions cannot be kept in culture for long periods of time. Recent evidence has demonstrated that sphere cells with stem-like properties from prostate cancer cells can be generated and propagated without the addition of growth factors (Rybak et al., 2011). This new culturing condition also supported a long-term culture of prostate stem-like cells, adding an important tool to investigate the biology and the expression of specific cell surface markers in CSCs. Similarly, inhibition of ROCK activity in culture (Chapman et al., 2010) enabled an 8-fold increase in *in vitro* prostate colony assay, and also significantly increased the cloning efficiency of prostate stem cells from mice (Zhang et al., 2011).

3.3 Protaspheres

The utilization of spheres assay is an important tool for the in serial in vivo transplantation to verify self-renewal potential. Although, sphere cells are generated, serially passaged, and maintained in undifferentiated phenotype under appropriate cell culture conditions, they need to be inoculated into animal models to confirm their ability to generate tumor growth. In this regard, it is necessary to ensure the best animal model available in order to reproduce tumor CSCs biology as it occurs in humans. It is important to note that variation on experimental conditions would certainly influence frequency estimates. In fact, it was suggested that limiting dilution data might be dramatically affected by the duration of data analysis (Yamazaki et al., 2009) or by modification of xenotransplantation assay in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice (Quintana et al., 2008). Therefore, a main concern for the application of this methodology is that sometimes, the animal models overstate the biology of cancer formation in humans.

Prostate cancer cell lines are frequently used to investigate the mechanisms that modulate protaspheres in non-adherent cultures. Several reports have shown that most of these cells have the ability to form spheres; however, the frequency of cells that form spheres is very heterogeneous across all cell lines. It is possible that adaptation of these cells to non-adherent culture conditions may be a determinant in forming spheres (Bisson & Prowse, 2009). It has also been shown that holoclone-forming cells, cells whose progeny forms almost exclusively growing colonies, in prostate cancer specimens with the highest clonogenic potential were associated with stem cell phenotypes (Patrawala et al., 2007). More, the presence of large holoclones was also consistently observed in prostate cancer cell protaspheres (Li et al., 2008; Zhang & Waxman, 2010), suggesting that spheres with stem cell-like features have a higher proliferative potential. Indeed, protaspheres were capable of forming new generations of protaspheres and retained proliferative capacity as well as clonogenic potential after serial passages (Guzman-Ramirez et al., 2009).

3.4 Identification of cancer stem cell signatures using microarray technology

The utilization of microarray analysis has allowed for screening the expression profile of numerous genes in prostate tumors (Glinsky, 2007; Mendes et al., 2008; Witte, 2009). By using gene expression microarray technology, the expression levels of thousands of genes are analyzed in a single experiment and evidences of certain degree of relevance to cancer progression may be also obtained (Borst & Wessels, 2010; Witte, 2009; Zieger, 2008). Because cancer cells and somatic stem cells share the biological characteristics of self-renewal and proliferation, the principles of stem cell biology can be applied to improve our understanding of cancer biology (Liang et al., 2009). Although several cell surface markers have been utilized for the isolation of putative cancer stem cells, the identification of key molecules and pathways that play pivotal roles in prostate cancer progression towards an aggressive stage is crucial for a more precise prognosis in patients with prostate cancer (Ladanyi, 2008; Rubin, 2008). As a matter of fact, the identification of molecular signatures has allowed the identification of androgen subtypes of cancers that have not been distinguished by pathological criteria (Alizadeh et al., 2009; Goodison et al., 2010; Perou et al., 2010; Wegiel et al., 2010; Yang et al., 2010) and in prostate tumors, the microarray technology has identified androgen-regulated genes in prostate tumor models (Elek et al., 2000).

Laboratory animals have been used to mimic natural aspects of human prostate cancer development. The evaluation of gene expression in samples derived from these animal models has provided for the identification of gene signatures, which are represented in the altered expression in a large group of genes (Ladanyi, 2008; Mendes et al., 2008; Witte, 2009). These data can be useful to compare *in vitro* results with intact animal expression changes and the different signatures that may eventually be predictors of cancer progression. More recently, a major effort towards this end addresses global analyses of gene expression profiles. However, the large body of data sometimes does not provide clear information about the specific genes that may be associated with the aggressive growth of prostate tumors. In this regard, the identification of poor prognosis using an interspecies comparison of prostate cancer gene-expression profiles has been a valuable tool to predict the association of potential oncogenic events to an aggressive phenotype of prostate cancer (Kela et al., 2009).

Because gene expression is a complex process characterized by a high degree of regulation, studying which genes are active and which are inactive during tumor progression helps to

understand both how these cells may function normally and how they are affected when several genes do not perform properly. In addition, gene expression profiles can provide key information in the identification of a signature that can be used to assess corresponding biological responses in other potential candidate(s), which may be indicative of cancer progression effects and pathological endpoints (Goodison et al., 2010; Hsieh et al., 2007; Mendes et al., 2008). Therefore, there is no doubt that microarray technology is a new tool for the clinical lab and also can improve the accuracy of classical diagnostic techniques by identifying potential novel tumor specific markers. Therefore, it is important that microarray data be publicly available. The establishment of specific criteria is important to identify genes associated to diseases. This may facilitate the classification by the identification of only relevant genes, improving the tumor classification accuracy and reducing the dimensionality of the data set (Wang et al., 2005).

3.5 The side population dilemma to isolate CSCs

An innovative approach frequently utilized to isolate stem cells is the side population (SP) assay (Goodell et al., 1996). This controversial technique had generated excitement because of its enormous potential to isolate putative CSCs. Cells with and SP phenotype, which is associated with primitive and undifferentiated stem cells characteristic are enriched based on their highest efflux activity to dyes such as Hoechst 33342 or Rhodamine 123. This approach had allowed the identification of a small subset of quiescent and replicating Hoechst-staining CD34-negative in murine cells that efflux the dye, allowing defined separation of this subset from the rest of the bone marrow (Goodell et al., 1996). This subset presented a very homogeneous pattern compared to cell surface markers. Using this approach, a subset of cells with similar phenotype to mouse was also identified in human bone marrow. This conserved SP phenotype was proposed as a common molecular feature for stem cells possessing multi-organ plasticity. The assessment of the molecular basis for SP phenotype found that the ATP-binding cassette (ABC) protein breast cancer resistant protein (Bcrp1) transporter expression is highly conserved in primitive stem cells from murine bone marrow, skeletal muscle, and cultured ES cells. While Bcrp1 expression is exclusive of the primitive subset $CD34^+$ and was developmentally regulated, enforced expression of Bcrp-1 expanded the SP phenotype in bone-marrow cells (Zhou et al., 2001).

Other ABC transporters, including the multidrug resistance 1 (Mdr1a/1b, mouse; MDR1, human) and the multidrug resistant protein (MRP) are also able to efflux Hoechst 33342, which have been indicated as contributors for multidrug resistance of tumor cells (Schinkel et al., 1997). However, in the Mdr1a/b/Bcrp1 triple knockout mice model, some bone marrow cells still retained the SP phenotype. This redundancy suggested that the influence of transporters in the SP phenotype is probably not exclusive on the expression of ABC transporter proteins. Latter experiments using knock down of the Bcrp1 gene expression animal model proved that the Bcrp1 gene, and not Mdr1a/1b, drastically reduced the number of SP cells in the bone marrow and in skeletal muscle. This study also evidenced that Bcrp1 expression is crucial in protecting early hematopoietic cells against chemotherapeutic drugs such as topotecan, mitoxantrone, or 5-fluorouracil (5-FU) (Zhou et al., 2002).

It has been proposed that the lower affinity of MDR-1-encoded transporter rather than the ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2) transporter for the Hoechst dye may explain its reduced effect in the SP phenotype. Indeed, enforced expression of a MDR1 retroviral vector in murine bone marrow cells *in vitro* increased SP

cells only to 3.6% of the total population (Bunting et al., 2000), a very low percentage compared with the impressive levels provided when the ABCG2 vector (62.5% SP cells) was utilized (Zhou et al., 2001). In fact, SP cells isolated from normal prostate tissues isolated from radical prostatectomy were enriched in ABCG2 expression. The gene expression signature of the ABCG2 subpopulation showed a consistent number of markers associated with stem-like phenotype (Pascal et al., 2007), suggesting the feasibility of the SP assay to identify and isolate cells with stem-like phenotype in the prostate.

Reports associating SP cells with tumorigenicity and/or aggressiveness have also been established in many tissues including breast, colorectal, glioma, medulloblastoma, ovarian, and thyroid (Chiba et al., 2006; Hirschmann-Jax et al., 2004; Kondo et al., 2004; Mitsutake et al., 2007; Szotek et al., 2006). In prostate tumor cell lines and human tissues, the utilization of the SP assay has also allowed the enrichment of cells with stem-like characteristics, which expressed high levels of different types of transporters such as ABCG2, ATP-binding cassette, sub-family A (ABC1), member 2 (ABCA2), MDR-1 and MRP-1 (Mathew et al., 2009; Oates et al., 2009; Patrawala et al., 2005). The prostate cancer SP phenotype possessed self-renewal capacities *in vitro* and *in vivo* intrinsic properties of stem cells as evidence by the activity of Wnt and Hedgehog signaling pathways, high proliferation rate, and tumorigenic potential ((Bhatt et al., 2003; Brown et al., 2007; Patrawala et al., 2005). These findings implied that the SP subpopulation is enriched with tumorigenic stem-like cancer cells that may be resistant against therapies.

Cells with stem-like characteristics have also been enriched in SP subsets but were not exclusive for breast, thyroid, and breast cancer cell lines in which some of the non-SP cells had the ability to generate cells with SP phenotypes (Mitsutake et al., 2007; Patrawala et al., 2005). Interestingly, non-SP cells also preserved their tumorigenic potential. Although contamination might be responsible for these unexpected results, a critical concern in this endeavor is that SP assay does not isolate cells by a definitive cell surface profile; because of that, the population being isolated is very heterogeneous (Challen & Little, 2006). It was also reported that both SP and non-SP fractions from malignant mesothelioma and lung adenocarcinomas cell lines were also equally able to form spheres, have a high tumorigenic potential, and are resistant to chemotherapeutic agents (Pan et al., 2010). More intriguing was the fact that no evidence of stem-like characteristics was found in gastrointestinal cancer cell lines in which lifespan in tissue culture and tumorigenesis potential are not exclusively associated with the SP phenotype (Haraguchi et al., 2006). This leads to the next question: are other types of cells also involved in key events during cancer evolution? These results also indicate that the SP assay is not appropriate for the identification and enrichment of putative CSCs in lung tumors (Kai et al., 2010).

Together, these results raise the inevitable question: is it correct to affirm that SP enriches a subpopulation with potential stem-like characteristics? One potential explanation for these conflicting results was provided by the kinetic analysis of Hoechst 33342 on bone marrow over a dye incubation period (Ibrahim et al., 2007). This approach demonstrated that bone marrow nuclear cells evidenced an identical staining pattern at varying rates, even under conditions where SP fraction was depleted, suggesting that the SP phenotype is not unique to stem cells. These data may be indicative of a transient feature of marrow cells when exposed to Hoechst 33342 for varying amounts of time. It is also possible that cells not exhibiting SP phenotype may have their membrane pumps overwhelmed or perhaps the dye efflux mechanism is inactivated.

All the biological assays discussed above may have important clinical implications. Indeed, proliferation assays could be included in the routine assays of pathologists to provide the most accurate diagnosis to patients with prostate cancer. As a matter of fact, the grading of prostate tumors (low versus high) is analyzed based on tubule formation, nuclear grade, and mitotic count. The mitosis cell evaluation is carried out basically by scoring the number of mitotic cells present within a giving sample. Considering that the worst histologic grade dictates the biologic behavior of prostate cancer (Chan et al., 2000; Harnden et al., 2007; Hattab et al., 2006; Pan et al., 2000; Sim et al., 2008), the inclusion of appropriate assays to identify proliferative signatures may improve the clinical diagnosis/prognosis of prostate cancer that may help to reduce misdiagnosis, missed diagnosis, or decrease the rates of overdiagnosis.

4. Prostatic cancer stem cells

A challenge in studying solid neoplasias is the heterogeneity observed in the tumor bulk where multiple cell types coexist, each exhibiting unique proliferation rates, invasion, and metastatic potential. This is the result of diverse combinations of genetic and epigenetic aberrations that arise from stressors such as inflammation, oxidative stress, aging and environmental influence.

Although several commonly mutated genes involved in the tumorigenesis process have been identified, there is no clear evidence that the transformed cells specifically responsible for tumor initiation and maintenance of solid malignant neoplasias have been clearly identified or isolated thus far. Therefore, it is crucial for the understanding of cancer evolution to provide new insights into the molecular mechanisms by which CSCs modulate cancer development. In this respect, efforts are being made toward the identification of reliable biomarkers (Keysar & Jimeno, 2010; Marhaba et al., 2008; Zoller, 2011) for the accurate identification of malignant progenitor tumor cells and also to use them as possible molecular targets to improve response to therapy. These potential markers of stem cells or CSC markers obviously preserve specific biological functions, but these have yet to be discovered as only limited research has been conducted to explore the process of their roles in tumor initiation and progression. Moreover, the limited number of cancer stem cells within the tumor, the heterogeneity of androgen receptors, the phenotypic characteristics of the tumor bulk and different genetic signatures are also important aspects that restrict the identification of this unique subset of cancer cells in prostatic cancer.

Numerous reports have described several types of tumors including prostate cancers, biological markers that can recognize a small population of cancer cells in the tumor bulk with stem-like characteristics (Miki et al., 2007; Patrawala et al., 2006; Patrawala et al., 2007; Vander Griend et al., 2008). Many of these markers associated with stem cells are cell surface proteins (i.e., CD44, CD133, CD166, etc), which is facilitating the identification of tumor subpopulations with stem-like phenotypes. In addition, signaling pathways activated by the core pluripotent transcription factors Nanog, Oct4, and Sox2, which are very important components of the machinery that modulates cell-renewal and maintains pluripotency in both embryos and stem cells, are also frequently overexpressed in poorly differentiated tumor cells (Jeter et al., 2009; Jeter et al., 2011). However, the nuclear localization of these transcription factors precludes their utilization for the enrichment of CSC.

Cancer stem cells have been enriched from human cancer prostate based on the expression of $\alpha 2\beta 1$ integrin or CD133 (Collins et al., 2005). In addition, holoclones from the prostatic cell line PC3 that were tumorigenic in xenograft assays showed increased expression of $\alpha 2\beta 1$, β-

catenin, and CD44 (Li et al., 2008). Although CD44 expression was also associated with enrichment of cancer stem cells in other prostatic epithelial cell lines, this subset of cells displayed low efficiency in formation of xenograft tumors (Hurt et al., 2008; Patrawala et al., 2007). Prostate tumor cells expressing the CD133 isolated from $\alpha2\beta1^{hi}$ cells were capable of expanding in culture. These prostatic putative stem cells were able to drive the reconstitution of prostatic-like acini in immunocompromised male nude mice (Richardson et al., 2004). Even when controversial reports have challenged the role of CD133 as a cancer stem cell marker, several evidences have supported that CD133 expression is generally detected in prostate cancers (Guzman-Ramirez et al., 2009). In fact, this subset of cells had the ability to form protaspheres from single cells. However, protaspheres generated from freshly isolated cancer prostate cells that were either $CD133^+$ or $CD133^-$, suggested that neoplastic transformation can confer self-renewal potential also to $CD133^-$ progenitor cells (Tang et al., 2007).

A comparison of clonogenic cells isolated from primary prostate cancer populations identified only tumor-derived $\alpha2\beta1^{high}/CD133^+/CD44^+$. This cellular subpopulation also presented a phenotype similar to normal prostate stem cells and was also capable of self-renewal and extensive proliferation compared to more differentiated cells did not form *in vitro* secondary colonies (Collins et al., 2005). The differentiation of this subpopulation with serum and dihydrotestosterone to an androgen receptor-positive phenotype promoted in these cells the expression of $AR^+/PAP^+/CK18^+$, which suggests that they were derived from a more primitive multipotent population of CSCs. In support for these findings, Patrawala et al., (Patrawala et al., 2006) reported that a highly purified $CD44^+$ prostate cancer subset from xenografts of a human tumor, as well as from multiple cultured cell lines are enriched in tumorigenic and metastatic progenitor cells. The $CD44^+$ subpopulation was more invasive than $CD44^-$ cells and also expressed high levels of several stem-like genes such as Oct3/4, Bmi, and β-catenin.

In addition, clonally derived human prostate cancer specimens from epithelial cell lines expressing the embryonic stem markers Nanog, Oct4, and Sox2 as well as other early progenitor cell markers such as CD44, CD133, c-kit, and Nestin were able to recapitulate human prostate tumors in SCID mice and were also categorized according to Gleason score (Gu et al., 2007). In fact, these cell lines formed tumors that contained basal, luminal, and NE epithelial cell lineage of the prostate, and retained their capacity of proliferation through serial transplantations. This is consistent with studies showing that high levels of Nanog is expressed in human primary prostate cancers cells and it also have a functional role for tumor growth (Jeter et al., 2009; Jeter et al., 2011). Interestingly, the putative prostate cancer stem cells do not express AR and p63, similar to that reported for prostate stem cells (Barbieri & Pietenpol, 2006; Rizzo et al., 2005). In support of these findings, increased expression of CD133 and the stromal cell-derived factor-1 receptor (CXCR4) were detected in a subpopulation of clinical prostate specimens that do not express AR (Miki et al., 2007). It is therefore conceivable to predict that the heterogeneous nature of prostate cancer, a common characteristics of this disease may have a stem cell compartment. Therefore, it is unlikely that a single marker can be used for the identification of cancer stem cells' subpopulations within the tumor mass.

5. Therapy targeting prostate cancer stem cells

The dysregulation of the cell cycle regulatory machinery that impact tumor cell proliferation also participates in the accelerated growth observed in most malignant tumors. Although

the exact genes that comprise the proliferation status may differ in different type of tumors, evidence has demonstrated that the cell cycle regulation is frequently altered in prostate cancers, in part, by the interplay of activation of oncogenic cascades with diverse hormones, growth factors, and cytokines. These events may eventually lead to a more poorly differentiated and aggressive tumor behavior, leading to overall higher rates of progression and worse prognosis, irrespective of the size of the lesion (Flavin et al., 2010, 2011; Niu et al., 2010; Saeki et al., 2010). Therefore, inhibitors of cell cycle regulatory proteins has become an area of increased interest in targeting both cancer cells per se and CSCs (Malumbres & Barbacid, 2009). For example, we have recently demonstrated the efficacy of a novel dansylated VMY-1-103, a CDK1, CDK2 inhibitor, based on purvalanol B (Ringer et al., 2010), at very low concentrations in inhibiting Erb-2/Erb-3/heregulin-induced cell proliferation in LNCaP prostate cancer cells. It was also observed that VMY-1-103 induced apoptosis via decreased mitochondrial membrane polarity, induced p53 phosphorylation, caspase-3 activation, and PARP cleavage in these prostatic tumor cells, which express p53 wild type. More, VMY-1-103 was also effective inducing cell cycle arrest in prostate cancer cell lines compromised for p53 function, however, VMY-1-103 failed to induce apoptosis in p53-null prostate cell lines PC3 (Ringer et al., 2010). These results, strongly suggest that VMY-1-103 may be an effective therapeutic, either alone or in combinations with other drugs, in treating prostate cancer. Importantly, we have also found that VMY-1-103 is also effective in inducing apoptosis in spheroid cultures (Ringer et al., unpublished data). Given the critical role of CDK1 in proper timing of mitosis in all cells, VMY-1-103 may also be able to efficiently target CSC's, and this exciting possibility is being addressed both *in vitro* and *in vivo*.

Cancer usually treated using chemotherapy, radiotherapy, or surgery had limited effect on most primary tumors, which have already spread to other organs, leading to recurrent disease in the majority of patients. The reduced number of individuals that benefit of standard therapies may be improved with targeted clinical trials (Simon & Maitournam, 2004). In this respect, the identification of potential biological markers by microarray or sequencing technologies would help to restrict the number of patients that might response to a specific drug. However, the limited knowledge in tumor cell biology, in which multiples abnormalities need to be targeted simultaneously make difficult to predict which patients are most likely to respond to a given regimen (Sparreboom & Verweij, 2009; Woodward & Sulman, 2008). For instance, the significant therapeutic advances in patients with diverse types of localized cancers have been limited by relapse due to the persistence of cancer cells in primary tumors and micrometastases that may have intrinsic or acquisition of a resistant phenotype to current therapies available (Gray-Schopfer et al., 2007; Mimeault & Batra, 2006a, 2007b; Mimeault et al., 2007; van Leenders et al., 2001; Yang et al., 2010).

Despite recent therapeutic approaches that have significantly increased survival, most prostate aggressive tumors become resistant to current treatment protocols and the proportion of cancers that progress is significantly higher in the African American population (Chornokur et al., 2011; Gurel et al., 2008). Prostate cancers that initially respond to standard chemotherapy often recur with selective outgrowth of tumor cell subpopulations that are resistant not only to the original chemotherapeutic agents but also to other therapeutics. Thus, for example, most patients that relapses with castration-resistant cancer metastatic tumors for which there are no curative treatment. In this regard, it has been suggested that the cancer stem cell model may be responsible for the degree of sensitivity to anti-androgen therapy (Schalken, 2005). Although different cellular events involving pathways may effectively activate a different path to androgen independence

probably through a paracrine androgen-independent pathway, the multifocality and heterogeneity of prostate cancer may also account for hormone therapy resistance. In a human prostate cancer progression xenograft model, most of androgen-responsive genes that were initially downregulated under conditions of androgen deprivation were later re-expressed in recurrence tumors, indicating failure of androgen-derivation therapy as well as acceleration of tumor progression (Mousses et al., 2001). Another microarray-based profiling found that increase in AR transcript, as well as protein levels are essential for accruing resistance to anti-androgen therapy (Chen et al., 2004). Indeed, multiple cellular signaling pathways including AR, Akt, mitogen-activated protein kinase (MAPK), the nuclear factor kappa B (NF-κB), TGF-β, vascular endothelial growth factor (VEGF), and Wnt have been shown to enhance AR signaling and confer development of hormone-independent/castration-resistance in preclinical models (Mellado et al., 2009; Wegiel et al., 2010). More recently, it has been reported that Nanog induction promoted castration-resistant tumor phenotype and tumor regeneration in the human tumor cell line LNCaP. These findings support the notion that AR expression in prostate tumors is modulated by CSCs (Maitland & Collins, 2008).

The most challenging problem in prostate cancer is the identification of which cell or cells are transformed and initiate carcinogenesis. In addition, tumor heterogeneity appears to mask a minor tumor population (CSCs) that have the ability to self-renew and form a tumor but are typically in a quiescent state. This has been suggested as a critical mechanism by which CSCs are resistant to conventional chemotherapy or radiation treatments. Thus, prostate CSCs are likely to modulate resistant to androgen deprivation therapy and promote recurrence as hormone-refractory tumors and metastatic lesions. Even though prostatic CSCs do not express AR or PSA (Lawson & Witte, 2007; Tang et al., 2007), it appears contradictive to propose that these cells are the modulators of tumor progression in castrate resistance. Nevertheless, three potential mechanisms have been proposed to explain poor outcome in prostate cancer patients (Sharifi et al., 2006): 1) Clonal selection to anti-androgen therapy may occur in transit-amplifying cells, which arise from the stem cells and divide a finite number of times until they become differentiated. Because of the limited growth of transit-amplifying cells, it is possible that CSCs may produce them in response to castration therapy; 2) Growth factor released by surrounding AR responsive cells may provide the resistance response to this type of therapy; and 3) CSCs produce cells that have a more differentiated morphology and express AR.

The diagnosis and treatment of prostate cancer are currently based on clinical stage, biopsy Gleason grade, and serum PSA levels, which do not provide accurate information about the status of the tumor. Several studies have identified by microarray, genetic signatures of prostate cancer that appear to provide a more accurate pathological distinction based on different degrees of severity (Lapointe et al., 2004; Singh et al., 2002). The identification of relevant cell signaling pathways signatures that are overexpressed in subpopulation with stem-like characteristics may also provide mechanistic information regarding their roles in tumor progression and metastasis (Birnie et al., 2008; Sharad et al., 2011). Therefore, it has been suggested that sophisticated therapy approaches involving specific DNA damaging agents in combination with DNA repair inhibitors may also improve available therapies against prostate cancer (Berry et al., 2008; Cano et al., 2007). In addition, the combination of targeting cancer stem cells and androgen ablation may kill tumor cells and disrupt those cells that support tumor growth and survival (Berry et al., 2008).

6. Conclusions

CSCs are believed to be a subpopulation of cancer cells that modulate malignancy and resistance to current anticancer treatments and, therefore, indicators of inferior prognosis. Although some of the mutated target genes have been identified, there is no clear evidence regarding the identification and isolation of cells that initiate tumor formation in solid malignant neoplasias. Therefore, even the role of CSC in tumorigenesis has been very well accepted, thought it remains controversial whether cancer mass arises specifically from stem cells. It is believed that in tumor bulk, there are particular subsets of cells (CSC) that may have the potential to promote tumor progression and resistance to conventional therapies. After completion of a treatment, the surviving tumor cells may have acquired even more mutations during the drug treatment. The additional genetic changes in this particular tumor subpopulation may explain why the conventional therapies fail in eradicating the cancer, a frequent concern in the diagnosis of prostate cancer and the rapid regrowth of the tumor but this time with a more aggressive phenotype. Since standard therapies applied to cancer are usually based on radiologic documentation of tumor shrinkage, the presence of CSCs may explain failure of tumor eradication. Thus, there is still an immediate priority for the identification of robust prognostic biomarkers to optimize cancer therapies to effectively target CSCs and their environment to improve prostate cancer treatment.

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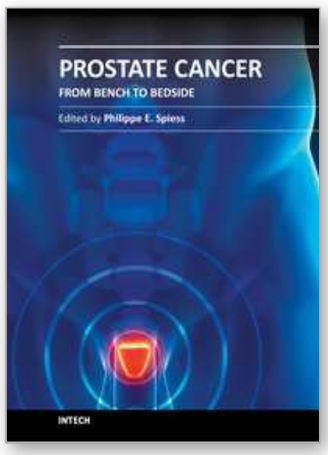

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The present textbook highlights many of the exciting discoveries made in the diagnosis and treatment of prostate cancer over the past decade. International thought leaders have contributed to this effort providing a comprehensive and state-of-the art review of the signaling pathways and genetic alterations essential in prostate cancer. This work provides an essential resource for healthcare professionals and scientists dedicated to this field. This textbook is dedicated to the efforts and advances made by our scientific community, realizing we have much to learn in striving to some day in the not too distant future cure this disease particularly among those with an aggressive tumor biology.

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