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Stem Cells and Prostate Cancer

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1. Introduction

Latest statistics based on GLOBOCAN 2008, the standard set of worldwide estimates of cancer incidence and mortality produced by the International Agency for Research on Cancer (IARC), revealed that prostate cancer (PC) is the most commonly diagnosed malignancy and the second leading cause of cancer-related mortality in male in developed countries [1]. The options in the treatment of PC are surgical tumor resection, hormonal therapy, radiotherapy, and adjuvant chemotherapy. These therapies, alone or in combination, show beneficial effects and a significant curative rate in treating patients with localized PC in the early stages. However, the development of locally advanced and/or metastatic hormone-refractory prostate cancers (HRPCs) eventually results in disease recurrence. Most patients who undergo potentially curative resection for advanced and/or metastatic HRPCs subsequently relapse due to the persistence of foci and micro-metastases. Therefore systemic chemotherapy may represent another option to eradicate the PC cells, including the highly tumorigenic stem/ progenitor cells that can drive tumor growth at primary neoplasms and distant metastatic sites.

The existence of stem cells (SCs) was firstly demonstrated by James Till and the late Ernest McCulloch in 1963 in their earlier work on the radiation sensitivity of mouse bone marrow cells by showing that limited numbers of cells could give rise to clonal colonies of erythroid and myeloid cells in the spleens of the irradiated hosts [2]. Although, much improvement has been achieved in the development of methods to kill cancer cells that form a variety of malignancies; nevertheless, relapse is an ongoing problem along with the development of metastatic tumors at sites remote from that of the original tumor. One suggestion to account for these phenomena is the existence of a stem cell with tumorigenic properties capable of regenerating all the differentiated cell types presented in the original tumor. The key paper

supporting the cancer stem cell (CSC) hypothesis from the laboratory of John Dick appeared in 1997, in which they demonstrated that an isolated cell type was capable of initiating acute myeloid leukemia (AML) [3]. With the knowledge provided by the science of stem cell biology, the Nobel Prize in Physiology or Medicine in the year 2007 was awarded jointly to Mario R. Capecchi, Sir Martin J. Evans and Oliver Smithies "for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells".

Stem cells possess some unique properties: a) they are undifferentiated and unspecialized; b) they are able to multiply for long periods while remaining undifferentiated (slowly cycling); c) they are capable of differentiating into specialized cells of a particular tissue (produce progeny in at least two lineages); and d) they can be serially transplanted. The combination of these properties is often referred to as "stemness" [4]. Stem cells can divide symmetrically or asymmetrically. A symmetrical division occurs when two daughter cells share the same stem cell features and happens when their numbers (stem cell pool) need to be expanded, such as during embryonic development or after tissue injury. An asymmetrical division occurs when one of the progeny remains undifferentiated, thereby replenishing the pool of SCs, while the other daughter cell can proliferate and differentiate into specialized cells to generate new tissue mass.

Stem cells have long been implicated in prostate gland formation. The prostate undergoes regression after androgen deprivation and regeneration after testosterone replacement. Regenerative studies suggested that those stem cells are found in the proximal ducts and basal layer of the prostate. Many characteristics of PC also indicate that it originates from stem cells. In this chapter, the biological and clinical implications of stem cells in prostatic carcinogenesis and the involvement of prostate cancer stem cells (PCSCs) in the many faces of PC are demonstrated and summarized. The theory of a stem cell origin of cancers represents a major paradigm shift that may completely revamp to diagnosis, monitoring, and therapy of PC.

2. Prostate epithelium and stem cells

Human prostate is an exocrine gland that consists of basal, luminal and neuroendocrine cell types embedded in a fibro-muscular stroma. The basal cells are relatively undifferentiated, not dependent on androgens and hence express low levels of androgen receptors (ARs). Additionally, basal cells generate some secretory products such as CD44 [5], p63 [6], p27^{kip} and c-Met [7], cytokeratin 5 (CK 5) and CK 14 [8]. In contrast to the basal layer of cells, luminal (or secretory) cells are terminally differentiated and specifically secrete the prostate like prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) into the glandular medulla in response to androgens. Because, survival of these luminal cells depend on androgens they express ARs on a high level; whereas, their other specific secretory products are CD57 [5], CK 8 and CK 18 [8]. The third type of cell in the cellular organization of the prostate epithelium is the neuroendocrine (NE) cell. The specific functions of NE cells have not been deduced so far. However, Bonkhoff suggested that they are post-mitotic cells derived

from luminal secretory cells [9]. NE cells are terminally differentiated, androgen insensitive and scattered throughout the epithelium. Unlike the luminal cells, NE cells do not express AR or PSA; but, they do express NE-specific markers such as chromogranin A and synaptophysin [10]. Basal and luminal cells can also be distinguished by comparing expression profiles of other genes; like basal cells do mainly express CK 5 and CK 14, whereas luminal cells express CK 8 and CK 18 [8]. Morphologically basal cells are small, flattened cells with condensed chromatin and small amounts of cytoplasm. Luminal cells instead have increased cytoplasm and their chromatin appear more opened [11]. Finally, the stroma is located under the epithelial layer of prostate. Stromal cells are androgen responsive and they do express AR. Development, maintenance and differentiation of epithelial cells are provided by these stromal cells [12].

2.1. Prostate stem cells

Prostate stem cells (PSCs) need to carry following characteristics: they must be castration-resistant, able to renew themselves and regenerate new tissue [13]. In contrast to the epithelial tissue of other adult organs, the prostate and mammary glands exert hormonal-dependence. Therefore, to account for changes in hormone levels the PSCs should be responsive to, but not dependent on, androgen for survival. This property is referred to as castration-resistance. PSCs should have tissue-regenerative capacity to replenish the gland after routine cell death. But, when compared to the hematopoietic stem cells that must generate a vast array of mature lineages, PSCs only must regenerate a relatively simple double-layered epithelium. Eventually, and most importantly, PSCs must be able to self-renew meeting the needs of the organ over the course of a man's lifetime.

2.2. Localization of stem cells within the prostate epithelium

In the 1980s, John Isaacs and colleagues performed classic androgen cycling experiments and suggested that prostate epithelium must contain a SC population. Than, when rodents are deprived of androgen by surgical or medical castration, the gland atrophies due to apoptosis of terminally differentiated cells which are dependent on androgen for their survival [14]. However, when androgen is replaced the gland regenerates and resumes its normal functions. This involution and regeneration can be repeated for many sequential cycles. The regenerative capacity has been attributed to a population of long lived SCs within adult prostate epithelium that are thought not androgen-dependent for survival, but androgen-sensitive and androgen-responsive. Apoptosis occurs mostly in androgen-dependent luminal cell epithelium, while the androgen-independent basal cells generally remain unaffected [15]. In accordance with this, the regenerative capacity is referred to the action of basal SCs, while the harbor of these self-renewing cells is confined to the basal-cell layer [14, 16]. Later, also other observations and studies have supported this hypothesis in many ways; like, that basal cells exhibit a higher proliferation rate in normal and hyperplastic acini than luminal epithelial cells [9]. Or for example, as bromodeoxyuridine (BrdU) labeling studies have suggested that prostatic tumor-initiating cells reside in the basal cell compartment and express a p63[+] signature [17]. And, that basal cells preferentially survive after androgen ablation;

whereas, 90% of luminal epithelial cells are lost through programmed cell death [18]. Androgen treatment restores the secretory glandular structure, hinting towards that the basal compartment contains SCs that undergo transit amplification to repopulate the luminal epithelium [19]. Cell types expressing an intermediate phenotype of basal and luminal cell characteristics have been identified in the developing and adult prostate [19].

On the other hand, there are also some studies that do not support the idea that SCs reside in the basal cell compartment. Experiments in mice where SCs were labeled with BrdU, suggested that stem cells are not restricted to the basal cell compartment; but, may also reside in luminal cell layer as a slow proliferating population in the proximal part of prostatic ducts [20]. Using tissue rescue experiments, Gerald R. Cunha and colleagues have demonstrated that the embryonic p63 null urogenital sinus developed into prostate when engrafted under the renal capsule of male mice [21]. Although, basal cells were absent the grafts contained luminal and NE cells, demonstrating that p63 was essential for basal but not for luminal and NE cell differentiation [21].

In human prostate, there is a consistent body of evidence that the SCs reside in the basal layer. Within the basal layer, $CD133^+/\alpha_2\beta_1^{hi}$ (high expression of $\alpha_2\beta_1$ integrin) cells represent a small subpopulation of quiescent cells with SC characteristics: they have a high proliferative potential *in vitro* and can reconstruct functional prostate acinar structures *in vivo* [22]. Molecular characterization of these cells revealed that they do not express AR at mRNA level [23], indicating that they are not dependent on androgen for their survival. Using CD49f and tumor-associated calcium signal transducer-2 (TROP2) as markers, Goldstein and collaborators identified basal cells with enhanced sphere-forming and tissue regenerating abilities [24].

2.3. Characterization of prostatic stem cells

Recent studies have revealed that a very small subpopulation of multipotent and undifferentiated PSCs, comprising about 0.1–3.0% of the total prostatic epithelial cell population, principally reside within specialized areas or "niches" localized in the basal cell layer of acinar and ductal regions of the human prostate gland [5]. Anne T. Collins and colleagues isolated and characterized human adult SCs based on the identity of cell surface integrin antigens [25]. They showed that, *in vivo*, putative SCs express higher levels of the α_2-integrin subunit than other cells within the basal layer. Later, it was shown that a subpopulation of $\alpha_2\beta_1^{hi}$ basal cells express the CD133 antigen and that this expression correlates with a high proliferative potential and ability to regenerate a fully differentiated prostatic epithelium with expression of prostatic secretory products *in vivo* [22]. $CD133^+$ cells possess three important attributes of epithelial stem cells: they are rare, comprise a high *in vitro* proliferative potential, and are capable of reconstituting highly branched ductal structures. Besides, Patricia E. Burger and colleagues reported that SCs can be purified from isolated proximal duct regions by virtue of their high expression of the cell surface protein stem cell antigen 1 (SCA-1) [26]. Subsequently, it was demonstrated that the Sca-1 surface antigen can be used to enrich for murine prostate cells displaying multiple properties of primitive cells including androgen independence, replication quiescence, multi lineage differentiation, and *in vivo*

prostate regenerative capacity [27]. Combined cell surface markers such as CD45⁻CD31⁻Ter119⁻Sca-1⁺CD49f⁺ were defined by Devon A. Lawson and colleagues who found that prostate cells can self-renew to form spheres for many generations and can differentiate to produce prostatic tubule structures containing both basal and luminal cells *in vivo*. These cells also localize to the putative PSC niche in the proximal region of the prostate gland [28].

2.4. Prostate stem cell niche

In all epithelial organs, adult SCs are maintained in a tissue niche that regulates stem cell fate decisions. The niche provides structural support, as well as the biological cues that influence the SCs' decision to self-renew or divide into more differentiated progeny. Integrin and junctional proteins play a major role in regulating SC differentiation in the prostate [29]. For instance, integrin α_6 shows a wider distribution amongst SC populations in the prostate tissue [28]. It was also shown that the high surface expression of $\alpha_2\beta_1$ integrin in human prostate epithelium correlates with colony forming ability and the potential to regenerate a fully differentiated prostate epithelium *in vivo* [25]. Additionally, proteins belonging to the connexin, cadherin and catenin families were reported as key molecules mediating cell-cell and cell-extracellular matrix interaction that dictate cell differentiation decisions [30].

Prostate homeostasis is maintained as a result of androgenic regulation of stromal epithelial interactions. Mesenchyme is the key androgen target tissue during development of prostate and many androgenic effects expressed in epithelium are elucidated trough paracrine influences from the mesenchyme [31].

The pathways controlling SC fate in prostate include NOTCH1 and Transforming growth factor beta-1 (TGFβ1) signaling. NOTCH signaling is critical for normal cell proliferation and differentiation in the prostate, and deregulation of this pathway may facilitate prostatic oncogenesis [32]. Increased TGFβ1 signaling has been found in the quiescent proximal region of the ducts in an androgen-replete animal and cells in this region were also overexpressing the B-cell leukemia/lymphoma-2 (Bcl-2) protein, which protects them from apoptosis [33]. This signaling seems to be responsible for a quiescent stem cell niche.

3. Cancer stem cells

The cancer stem cell (CSC) theory has started more than a century ago with the "embryonal rest hypothesis" that was relying on histological similarities between teratocarcinomas and embryonic tissue [34] and later was than accelerated by findings that leukemia could be transferred by a single cell in a mouse model system [35]. Later investigations clarified that when this single cell was transplanted to non-severe combined immunodeficiency (SCID) mice it could induce leukemia that was phenotypically identical to the parental tumor leading to the conclusion that a leukemic tumor stem cell had developed from hematopoietic stem cells [3]. The first CSCs in a solid tumor was discovered for breast cancer in the year 2003 [36]. Following that, CSCs were also found in solid tumors like liver, lung, thyroid,

skin, pancreas, colon and prostate cancer [37]. Nevertheless, through the 1960s transplant experiments had proven that cancers were composed of heterogeneous cell populations with some differences in their self-renewal capability and potential for reconstituting a tumor following transplantation [38-40]. These early investigations made the researchers think that the actual tumor cell population could be arisen from a small group of CSCs and two theories were suggested upon this idea [39]. In the stochastic theory, every cell in a tumor population is believed to be a potentially tumor initiating cell; but, each cell's possibility of entering the cell cycle is low and controlled stochastically. Whereas, the hierarchy theory assumes that the tumor is functionally heterogeneous and only a small subpopulation of cells in it have the ability to initiate tumor growth [40]. Regardless of the theories, CSC is generally accepted as the original cell of a tumor that generates an accumulation of self-sustaining cells with unlimited self-renewal capability. Meaning it is that one cell that later raises the formation of a heterogeneous bulk tumor which differentiates, comprises metastatic ability, preserves itself by activating anti-apoptotic pathways, and is responsible of tumor relapse. In this context, the self-renewal capability is very important to SCs; *i.e.* the one or both daughter cells -that result after cell division- that keep the ability to replicate and form the same differentiated cell lineage as the parental cell. CSCs have the capability of creating the generations of a constantly growing tumor and can either arise from the stem cells of a corresponding tissue or from mutation bearing tissue cells that dedifferentiate to become cancerous SCs [41].

3.1. Cell division in cancer stem cells

Stem cells can divide symmetrically or asymmetrically: while the symmetric division results in two new SCs; asymmetric division gives rise to a new stem cell and a daughter cell that undergoes a differentiation process. Stem cells alternate between these two division types. Asymmetric cell division is regulated by some intrinsic factors such as the specific arrangement of cell polarity and/or cell fate factors like Numb or PAR-aPKC, and by extrinsic mechanism like the stem cell niche. Thus, asymmetric division is not necessary for stem-cell identity but rather is a tool that stem cells can use to maintain appropriate numbers of progeny. The facultative use of symmetric or asymmetric divisions by stem cells may be a key adaptation that is crucial for adult regenerative capacity [42]. The result of each division is different; since symmetric cell division gives rise to induce new tumors, the machinery that promotes asymmetric cell divisions has an evolutionarily conserved role in tumor suppression [43, 44].

3.2. Regulatory mechanisms of CSCs

Regulatory proteins and pathways establish a balance between a CSC's self-renewal ability and its death by apoptosis. The WNT, SHH, NOTCH, and PI3K/AKT/mTOR signaling pathways are especially important in this regulation and are often found be impaired in tumors. The WNT signaling pathway is mainly involved in cell proliferation and differentiation. A mutation in one of its components resulting either in an up-regulation or disruption of the signaling cascade can accelerate tumorigenesis; dysregulation of the WNT pathway compo-

nent E-cadherin can also lead to metastasis [45, 46]. Differentiation and self-renewal of adult SCs is usually controlled by the SHH pathway and disruption of it results in their aberrant differentiation and proliferation [47]. The NOTCH signaling pathway also regulates the differentiation, proliferation and self-renewal of adult SCs. Dysregulation of this pathway affects specific tissues and often leads to basal cell carcinoma, breast-, kidney- and prostate cancer [48-50]. In mouse models a significant inhibition of tumor growth could be achieved when the NOTCH signaling cascade was blocked [51]. The PTEN, a tumor suppressor protein with function in cell cycle regulation, is acting on the PI3K/AKT/mTOR signaling pathway. Inactivating mutations of PTEN can cause uncontrolled growth and cell division and are often found in tumors such as brain, bladder, prostate and kidney cancers [52-54].

3.3. Therapeutic approaches to target CSCs

Searching for powerful therapeutic approaches that specifically target CSCs is an accelerating area of research, after the discovery that CSCs significantly influence metastatic diseases and drug resistance. For instance, relapse is a result of a small CSCs population's survival which has self-renewal ability. If these CSCs are not exterminated by chemotherapy or targeted disruption of the SHH or NOTCH signaling pathways, they stay dormant in the target organs or bone marrow until triggered to regenerate the heterogeneous cell populations of a tumor [55]. But, attention should be focused on whether all solid tumors are sustained by CSCs and whether cell surface specific markers could be found that differentiate between normal SCs and CSCs. A great improvement will be achieved in cancer therapy when CSCs are selectively eliminated, while normal SCs are spared and thus left unaffected. Identification of a specific CSC marker in cancer of interest would simplify the development of anticancer drugs that eliminate the CSCs from the tumor cell population [56].

4. Prostate cancer stem cells

4.1. Origin of PCSCs

The origin of PCSCs continues to stay as a controversial issue. Different cells in origin may generate clinically relevant subtypes with different prognosis and outcome. There are two possible cell origin resources in PC: the basal and luminal cell-of-origin.

4.1.1. Basal cell-of-origin

Much stronger studies came from several independent laboratories that used different PC models to support the view that basal stem cells provide the cell-of-origin for PC. When CD49f^{hi}Trop2^{hi} cells were selected from the basal fraction, transfected with Akt/Erg vectors and transplanted to induce initiation of prostatic intraepithelial neoplasia [57]; these basal cells derived from primary benign human prostate tissue initiated PC in immunodeficient mice [24]. It was also reported that Lin$^-$Sca-1$^+$CD49f^{hi} cells isolated from the basal fraction of murine prostate produced luminal-like disease characteristics of human PC after transplan-

tation [58]. Recently, Norman J. Maitland and colleagues reported that selected cells with basal phenotypes are tumor initiating and basal SCs are the source of a luminal progeny [23]. In addition, a small population of TRA-1-60+ CD151+ CD166+ tumor initiating cells (TICs) isolated from human prostate xenograft tumors exhibited stem-like cell characteristics and recapitulated the cellular hierarchy of the original tumor in serial xenotransplantation experiments [59]. Moreover, these cells expressed basal cell markers and showed increased Nuclear factor-κB (NF-κB) signaling.

4.1.2. Luminal cell-of-origin

Luminal cells are believed to be the cells of origin for human PC, because the disease is characterized by AR+ luminal cell expansion. That is why pathologists diagnose PC based on the absence of basal cell markers. It is known, that rare luminal cells which express the homeobox gene Nkx3.1 in the absence of testicular androgens (castration-resistant Nkx3.1-expressing cells, CARNs) are bipotential with self-renewal capability *in vivo* [60]. Single-cell transplantation of CARNs can reconstitute prostate ducts in renal grafts. Besides, targeted deletion of PTEN in CARNs results in rapid formation of carcinoma following androgen-mediated regeneration. Hanneke Korsten and colleagues [61] showed that genetic alterations are first seen in a subset of luminal cells expressing the progenitor markers TROP2 and SCA-1, implying that the luminal cells are the cell-of-origin in this model.

The reason why the origin of PC and the cell type of origin remains a controversial issue is in part of the distinct functional assays that were employed. Furthermore, since PC is a very heterogeneous disease it is plausible that different PCs are derived from different originating cell types.

4.2. Characterization and markers of PCSCs

Every stem cell does not express the defined markers that are used to isolate SCs from various cancerous or normal tissues. Although the CD133, CD44, SCA1 and THY1 cell surface markers are commonly used to enrich CSCs; they are also expressed in normal stem cells as well as in many non-stem cells in various tumors and tissues. Eventually, the majority of cells expressing these markers are not SCs. Apart from that, a marker that is found to be functional in identifying a SC from one tissue may not be useful for identifying the SC in another tissue. Another feasible way of identifying SCs, besides searching for specific cell surface markers, is by label retention (BrdU incorporation) assays [62]. This DNA labeling assay depends on the label retaining characteristics of the seldom dividing SCs [63]. Finally, CSCs can be isolated by the detection of a "side population (SP)" of cells that actively transport lipophilic dyes out of the cells by drug-transporting proteins [64]. Margaret A. Goodell and colleagues first observed that a small population of bone marrow-derived cells that were incubated with the lipophilic dye Hoechst 33342 failed to accumulate an appreciable amount of this dye [65]. This subpopulation was identified by dual-wavelength flow cytometry analysis as the Hoechst^low^SP. Remarkably, the SP was highly enriched for hematopoietic stem cells. Subsequently, the SP technique was widely employed to enrich stem-like cells from solid cancers. This technique was also used for PC cells and the SP of cells derivable from this primary pros-

tate tumors was ~1% [66]. Since the gold standard to confirm CSCs is *in vivo* tumor development, analyzed and sorted SP cells were inoculated into immune-deficient mice and tested for tumor producing ability. By this, it was found out that cell surface markers combined with SP analysis are a more accurate way in identifying the real SC population.

The density of CSCs in a tumor is probably less than 0.1% [37]. Therefore, to obtain a good yield after isolation these cells certainly should be specified first. So far, identification can be achieved *via* characteristic cell surface markers, DNA labeling, and the cells' ability to expel dyes. Table 1 presents the expression profiles of cell surface proteins that are specific for SCs or tumors. But, it should kept in mind, that many cell surface proteins are not too specific to CSCs, because they are also expressed on physiological stem cells; and thus, using antibodies to detect them can lead to false-positive results due to non-specific cross reactivity.

Tumor type	Cancer stem cell marker
Acute myeloid leukemia	CD34/CD38
Breast carcinoma	CD44/CD24-/ALDH
Bladder carcinoma	Side Population [67]
Colorectal carcinoma	CD133, CD44, EPCM, ALDH
Ewing's sarcoma	CD133
Gastric carcinoma	CD44
Medulloblastoma, Glioma	CD133
Pancreatic carcinoma	CD133, CD44, CD24, ALDH, EPCM
Prostate carcinoma	CD133, CD44, ALDH
Hepatocellular carcinoma	CD133, CD44, ALDH
Lung carcinomas (non-small cell and small cell)	CD133, Side Population [67], ALDH
Head and neck carcinoma	CD44, Side Population [67], ALDH
Endometrial carcinoma	CD133, Side Population [67]

Table 1. Established CSC markers expressed in tumors of different tissues in human [*56*]

4.3. Methods for assaying PCSCs

Although, a SC in any type of adult tissue has the common self-renewal and differentiation abilities, it will be wrong to generalize the results obtained from one tissue while defining a SC in another tissue. SCs in different tissues can differ significantly from one another. The actual assay to identify a CSC that has self-renewal and tumor progression capability is an *in vivo* model known as the serial transplantation in animal models. Other assays are usually generated in an *in vitro* environment and to be ideal, they have to full-fill the following criteria: they should be quantitative, highly specific in measuring only the cells of interest, sufficiently sensitive to measure candidate stem cells even at low frequencies, and fast [37].

For SC studies, human primary cells are the optimal tools to mimic and represent the original characteristics of tissues; however, it is quite difficult to get primary cell cultures from PC tissues due to limited access. Furthermore, cell lines can serve as a resource for CSC studies, but there are several disadvantages in utilization of this *in vitro* model: it cannot replicate exact *in vivo* conditions during the long-term culture process and some cell property changes might take place like gene alterations; the *in vitro* cultured cells often lose their original differentiated function; and it cannot stably maintain the exact properties of the original organ. Nevertheless, primary PC cells, established PC cell lines, xenograft and animal models have all been utilized to identify PCSCs with different surface markers.

4.3.1. In vivo systems

Gerald R. Cunha and Ben Lung have developed tissue recombination of a rodent model for the growth of normal epithelial cells in 1978 [68]. In this system, tissue fragments of fetal urogenital sinus mesenchyme were used to support the growth of normal prostate epithelial tissue fragments when implanted in collagen under the renal capsule of immunodeficient mice. This system was later modified to evaluate the growth activities of different prostate cell subpopulations using mechanical and enzymatic digestion to dissociate both, the urogenital sinus mesenchyme and adult murine prostate tissue into single cell suspensions [69]. Dissociated prostate epithelia regenerate ductal structures that histologically resemble normal murine prostate. Matrigel transplantation method was described that provides a reconstitution assay of prostatic cells. It was shown that the prostate contains stem cells capable of reconstituting the whole prostate and this method can be used to analyze prostate stem cells, epithelial mesenchymal interactions, and prostate cancer stem cells [70]. Ken Goto and colleagues performed serial transplantation that was analogous to the serial reconstitution method to investigate PSCs self-renewal [71]. They showed that regenerated prostate tissue could be dissociated and transplanted to regenerate prostate tissue at least three times.

4.3.2. In vitro culture systems and assays

There are two types of culture system to study CSCs: primary cell cultures and cell lines. Primary cell cultures are directly established from human tissues and have the advantage that their cells represent the original features of the tissue. However, difficulties including the limited access to biopsy materials, the need for the exclusion of contamination by cancer or normal cells, their limited lifespan, and the small population of the putative SCs are its disadvantages [72]. Cell lines are permanent cell cultures with unlimited proliferation capacity. They are widely used in many aspects of research as the most common *in vitro* culture model, because they have a big advantage in being easy to handle for their infinite reproducible quantities. So far, most of the human PC cell lines have been established from metastatic lesions or from xenograft tumors.

Prostate colony assay: The clonal and population analyses of mammalian stem cells was first accomplished by using two dimensional culture conditions [73]. Co-culture with irradiated fibroblast feeder layer is now also used to cultivate human prostate epithelial cells. In this assay, the feeder layer contains serum free medium (but, growth factors added) and low cal-

cium [74]. Under these conditions, murine prostate epithelial cells form colonies of cells that express epithelial cytokeratins when cultured with irradiated 3T3 feeder cells [28].

Prostate sphere assay: Colonies that are derived from primitive cells cannot be passaged efficiently, since culture conditions promote cell differentiation. The three dimensional sphere is a non-adherent culture system that has been used as a useful model to elucidate stem cell characteristics [75]. A suspension culture system like this is thought to keep cancer stem cells in their undifferentiated state facilitating their enrichment; like for AR-negative and AR-positive PC cell lines that both can form prostaspheres [76]. Actually, all PC cell lines can form prostaspheres; but, because heterogeneity exists only a subpopulation of cells in each cell line can form these prostaspheres. The expression of stem cell markers, such as CD133 and CD44, is also significantly enhanced in a prostasphere.

In contrast to the suspension sphere culture systems 3-D culture in Matrigel, which is a widely used commercially available basement membrane, has been demonstrated to promote the differentiation of PSCs. It was possible to induce morphological and phenotypical differentiation in normal and malignant prostate epithelial cell lines with Matrigel [72].

4.4. Alterations in signaling pathways of PCSCs

Alterations in the signaling pathways are probably one of the reasons why cancer stem cells gain a tumorigenic potential. Thus, disclosing the signaling pathways' expressional regulations might provide potential therapeutic targets. The WNT, JAK/STAT, NF-κB, NOTCH, and PI3K/AKT/mTOR signaling pathways were found to be the regulators of CSC biology in prostate tissue and therefore are candidate targets. The idea of inhibiting signaling that induces proliferation and survival could mean an effective therapy for PC [77].

Proteins acting in the WNT signaling pathway are usually over-expressed in PCSCs. Hence, tumorigenesis is promoted and prostaspheres which have self-renewal capacity exhibit proliferation, differentiation, and heterogeneous expression of stem cell-associated markers such as CD44, ABCG2 and CD133. When WNT inhibitors are applied the size of prostaspheres and their self-renewal ability can be reduced; plus, the CD133 and CD44 expressions are down-regulated. WNT activity also regulates the self-renewal capacity of PC cells that have stem cell-like features and inhibition of WNT signaling potentially reduces the self-renewal ability of PCSCs with an enviable therapeutic outcome [76].

The JAK/STAT signaling pathway seems to be important in PCSC biology. Than, when PCSCs expressing aldehyde dehydrogenase (ALDH⁺), which is involved in the formation of bone metastasis, were treated *via* a galiellactone- a specific STAT3 signaling inhibitor-; apoptosis of cancerous cells could be induced [78]. Besides, *in vivo* targeting of STAT3 in a drug treated DU145 xenograft gave also desired results. Therefore, targeting of JAK/STAT signaling pathway components might be a promising therapeutic resulting in ALDH1A1 expressional down-regulation in PSCSs [78]. The importance of the NF-κB signaling pathway came up after the finding of enhanced functional signaling in purified naïve stem-like human prostatic TICs. When cells were treated with small molecular inhibitors that targeted the NF-κB

signaling pathway secondary sphere formation *in vitro* and tumor-initiation *in vivo* could be inhibited [59].

Cell fate specification, initiation of differentiation, and SC maintenance is regulated by the NOTCH signaling pathway in many tissues [79]. The over-expression of various proteins that function in the NOTCH signaling cascade has been found in a number of different tumors including PC. For example JAGGED-1, a NOTCH receptor ligand, has been found to be significantly more expressed in metastatic PC when compared with localized PC or benign prostatic tissue samples. This up-regulation also correlated with clinical features like recurrence, progression and metastasis of PC [80]. When Jagged-1 expression was downregulated with small interfering RNAs (siRNAs) cell growth was inhibited and cell cycle arrest achieved in the S phase of cell division [81].

The PI3K/AKT/mTOR signaling pathway member PTEN was first identified as a candidate tumor suppressor gene that was frequently mutated in brain, breast, and prostate tumors [82]. Introduction of PTEN into cancer cells that lack PTEN function down-regulated cell migration and survival, and induced cell cycle arrest and apoptosis [82]. PTEN is the most mutated gene in metastatic PC that is advanced and has an aggressive tumor phenotype; and has been associated with cancer progression in 30–60% of PC cases [83]. An association between androgen-independent tumor growth and PTEN mutations has also been discovered [84]. A number of mouse models for PC suggested that PTEN might play a role in the initiation or early progression of this disease. PTEN heterozygous mice are likely to develop epithelial dysplasia and hyperplasia resembling high-grade PIN and adenocarcinoma [53, 85]. While PTEN mutations lead to a predisposition for PC in mouse models, such an association could not be shown for human yet [83, 84].

4.5. Endocrine effects on PCSCs

In PC, the stromal niche or microenvironment plays a critical role in regulating differentiation of CSCs, probably by altered endocrine and/or paracrine signaling. Direct androgen binding to epithelial ARs is not required for epithelial differentiation, but is essential for the induction and maintenance of a secretory activity [11].

AR is a member of the steroid hormone receptor family and its over-expression is involved prostate tumorigenesis. Consequently, androgen deprivation therapy (ADT) has been used to treat locally advanced and metastatic PC [86]. Despite initial regression of the tumor the majority of patients inevitably develop castrate-resistant prostate cancer (CRPC), which establishes metastases relatively rapidly and is subsequently incurable by current treatment strategies. Mouse model studies revealed that androgen ablation can select for more aggressive and metastatic disease, which means that current hormonal therapies do not affect the AR⁻-CSCs [87]. ADT may promote disease progression by causing an increase in the castrate-resistant SC pool and/or activating quiescent SCs to repopulate the tumor with androgen-independent SCs. Vander *et al.* reported that unlike normal adult human prostate SCs, CD133⁺ PCSCs are AR⁺ and suggested that AR⁺ prostate TICs are derived from a malignantly transformed intermediate cell that acquired "stem-like activity". The AR signaling pathway might therefore comprise another therapeutic target, especially for prostate TICs [88].

In addition to androgens, estrogens play key roles in prostate carcinogenesis and progression. However, the mechanisms are not fully understood. Although there is still no direct evidence that estrogens initiate PC in humans, there is accumulating evidence pointing towards a central role for estrogens in PC [89]. To give just some examples are the rising E2:T ratio in aging men, association of estrogen metabolizing gene polymorphisms and elevated urine hydroxy-estrone ratios with higher PC risk, progressive increase in aromatase expression in PCs upon advancement to metastatic disease, and marked alterations in estrogen receptor expression with cancer progression. Normal human prostate progenitor cells are responsive to estrogens with increased rates of self-renewal, implicating them as direct estrogen targets.

The importance of estrogen receptor (ER) expression, *e.g.* ERα and ERβ, is unknown; but, is of interest based on the integral role of estrogens in prostate carcinogenesis. The expression of ERα is low and hard to detect in prostatic epithelial cells, where ERβ is predominantly expressed. An ERβ agonist compound could selectively induce apoptosis in castrate-resistant CD133+ basal cells, providing a rationale for further exploring the role of ERβ in PC and PCSCs [90].

Prolactin (PRL) is a peptide hormone that is secreted by the pituitary gland. It regulates several physiological functions, many of which relate to male and female reproduction. In humans PRL is also produced by prostate epithelial cells under normal physiological conditions. Local PRL profoundly affects the prostate epithelial compartment, with dramatic expansion of basal and stem-like epithelial cells, markedly enhanced epithelial cell proliferation, and strong activation of the STAT5 pathway as three hallmarks of tumorigenesis [91].

4.6. Potential role of PCSCs in metastasis

PC is the second leading cause of cancer death in male; but, because of the progress made in the diagnosis and treatment of primary PC, mortality in 70 - 80% of the patients is increasingly linked to its metastatic disease. The bone marrow is the most frequent site for metastasis in PC; and stem cells, besides their role in tumorigenicity, are highly migratory cells that are involved in bone metastasis formation [92].

CSCs contain a subpopulation of cells that are exclusively capable of disseminating and subsequently providing the substrate for tumor metastasis; *e.g.* CD44+ PC cells are more tumorigenic and metastatic than the corresponding CD44- cells [93]. Stromal cell derived factor and its C-X-C chemokine receptor type 4 (CXCR4) form a critical regulatory axis for SC migration, engraftment and homing, and also function in the metastasis of breast and prostate cancer [94]. Using a mouse/human comparative translational genomics approach an 11-gene signature that consistently displays a stem cell–like expression pattern in metastatic lesions of prostate carcinomas could be recovered from multiple distant target organs [95].

On the other hand, some incidents do not support the CSC involvement in metastasis. For example, CD44+CD24- and CD44+CD24+ breast CSCs have same metastatic potential [96]. Then, in an orthotopic pancreatic cancer model CD133+ cells were not metastatic, whereas CD133+CXCR4+ cells showed strong metastasis [97]. Also, CD133- colon cancer cells were

more aggressive and metastatic than their CD133$^+$ counterparts [98]. In conclusion, metastasis and tumor initiation might be processed by distinct cancer cell populations, probably by metastatic CSCs.

Tumor microenvironment facilitates cancer metastasis by several mechanisms. When human PC cells were injected into the dorsal prostate of a nude mouse more metastasis was generated, than when cells were injected subcutaneous [99]. Later, it was shown that dorsal prostate-implanted human PC cells over-express many CSC genes including osteoponin, CXCR4, CD133, ABCG2, CD44 and CD24. Some of these genes clearly have functional roles in PC metastasis [100]. But, the exact molecular mechanisms that account for the microenvironment regulated PC cell metastasis are still not known.

4.7. MicroRNA-mediated regulation of PCSCs

For the identification of novel PC therapeutic targets it is important to evaluate functional genes that are related with CSCs self-renewal and survival abilities. The experiences with PC therapy showed that PC recurs frequently; meaning that chemotherapy, radiotherapy, androgen-ablation therapy, and radical prostatectomy are not sufficient enough to eliminate TICs or metastatic cells. PCSCs are androgen independent and therapy resistant cells. Thus, generating novel therapies that specifically target PCSCs may be more effective than those that target differentiated PC cells. New approaches depend on CSC exterminating rather than total tumor decay. The limitation for these studies is to be able to specifically target CSCs in normal tissue that also contains its specific SCs; since, they have similar expressional and antigenic profiles [101]. Consequently, new markers are needed to distinguish CSCs from tissue specific SCs. microRNAs (miRNA) can be considered as such novel therapeutic target molecules for distinguishing PCSCs from normal SCs. MicroRNAs are 21- to 25-nucleotide (nt)–long, noncoding RNAs that induce the target mRNA degradation or repress mRNA translation by imperfect binding to their 3'-untranslated region (UTR) [102].

Depending on their expressional profiles and their target-mRNA types miRNAs can be divided into two classes: one that act like oncogenes (oncomiRs) and the other that act like tumor suppressor genes. OncomiRs are commonly up-regulated in tumors and target tumor suppressor mRNA transcripts, causing a decrease of tumor suppressor protein syntheses and thus function. Tumor suppressor miRNAs on the other hand are mostly down-regulated in tumors and therefore cannot target and inhibit the syntheses of the specific oncogene mRNA transcripts into oncoproteins. When tumor suppressor miRNAs are experimentally over-expressed in cancer cells they inhibit their proliferation, invasion and proliferation capacity [103].

Expression profiling of miRNAs in PC have showed that some miRNAs were significantly up- or down-regulated when compared to normal prostate tissue, pointing to the importance of miRNAs in tumor progression and pathogenesis; *e.g.* miR-34a and miR-34c were found to have an important role in AR-dependent and p53-mediated apoptosis [104, 105]. miR-125b was an up-regulated miRNA in clinical PC samples and androgen independent cell lines; thus, its up-regulation might be related with androgen-independence and survival [103]. Another up-regulated miRNA in PC was miR-21; but, it affected tumorigenesis, invasion and metastasis by inhibiting the synthesis of proteins that normally function in these

pathways. miR-21 also inhibits apoptosis [103]; and, contributes to drug resistance of PC to docetaxel treatment [106, 107]. miR-148a was defined as an androgen-responsive microRNA that promoted growth when up-regulated in the PC cell line LNCaP and one if its mRNA targets was found to be the cullin associated and neddylation-dissociated 1 (CAND1) transcript, coding for a tumor suppressor protein [108].

In contrast, miRNAs like miR-15a and miR-16-1 were found to be down-regulated in PC; their over-expression achieved by intra-cell delivery methods showed significant tumor regression capacity *in vivo* [103]. Other down-regulated miRNAs with tumor suppressor function in PC were miR-125b, miR-99a, miR-99b and miR-100. Again, when their expressions were restored, PSA expressions could be reduced and PC cell proliferation was inhibited [109].

miR-145 and miR-143 are tumor suppressor miRNAs that are commonly dysregulated in all cancer types. miR-145 and miR143 are also first transcribed together on a cluster and cleaved off during the miRNA maturation process. In PC miR-145 is down-regulated and over-expression of it has an anti-tumorigenic effect, resulting with the inhibition of migration and invasion of PC cells [103].

Some miRNAs take part in formation of androgen-independent PC; and, by comparing androgen-dependent and -independent PC samples, miR-146a has been revealed as such [110]. Finally, an example of a miRNA that is regulated by its target is miR-34a. The tumor suppressor and transcription factor p53 directly regulates the expression of miR-34a, which is decreased in CD44+ PC cells. When normally expressed it could inhibit PC regeneration and metastasis by directly repressing CD44 [111, 112]. The list of miRNAs which expressions are most significantly altered in PC are given in Table 2.

4.8. New therapeutic approaches in targeting PCSCs

Despite progress in the therapeutic approaches that significantly increased the survival rate of PC patients, most prostate aggressive tumors become resistant to currently used treatment protocols. PC that initially responded well to a standard chemotherapy often recur with selective outgrowth of tumor cell subpopulations and get resistant not only to the original chemotherapeutic agent but also to other therapeutics. Thus, for most patients with relapse of castration-resistant metastatic PC currently no curative treatment exists. It has been suggested that AR expression in PC is modulated by CSCs and the CSC model may be responsible for the degree of sensitivity to anti-androgen therapy [114], [115].

The majority of studies to date have focused on the identification of characteristics that potentially could define CSCs. However, more questions have been raised on the issue which of these characteristics would be better suited as target and now research has seemed to shift towards identifying the way these CSCs behave that make them different from bulk tumor cells. Two important features of acute myeloid leukemia (AML) that allowed to discovery of new therapeutic agents were CD34+/CD38- and CD33+. Anti-CD33 antibodies have become an important aspect of CSCs targeted therapy. A drug called Gemtuzumab ozogamacin or Mylotarg, approved by the FDA in 2000, combines the cytotoxic antibiotic calicheamicin with the monoclonal anti-CD33 antibody [116].

Androgen-Independent miRNAs	
Up-regulated	*Down-regulated*
miR-184	miR-128b
miR-361	miR-221
miR-424	miR-222
miR-616	miR-146a/b
	miR-148a
	miR-663
Cancer Stem Cell, Invasion or Metastasis Related miRNAs	
Up-regulated	*Down-regulated*
miR-377	miR-34a
miR-141	miR-143
	miR-145
	miR-15
	miR-16
Common Cancer Related miRNAs	
Up-regulated	*Down-regulated*
miR-182	miR-125b
miR-96	miR-15a/16-1
miR-375	miR-34a
	miR-205
	miR-145
	miR-221
	miR-222
	miR-181b
	miR-31
	miR-200c

Table 2. Up- and down-regulated microRNAs in postate cancer [113]

Novel therapeutic strategies against locally advanced and/or metastatic hormone-refractory prostate cancers (HRPCs) by targeting different oncogenic signaling cascade elements are listed in Table 3. Recent studies have revealed that the blockade of these tumorigenic signaling cascades could be beneficial as adjuvant therapy in the early phases of PC for decreasing the risk of relapse as well as in the late stages for improving the efficacy of current androgen deprivation therapy, radiotherapy, and/or systemic chemotherapy and

patient survival rates [117]. Inhibition of the epidermal growth factor (EGFR) pathway by anti-EGFR antibody or EGFR tyrosine kinase inhibitor causes a cell cycle arrest, inhibits invasion and/ or induces apoptosis in metastatic PC cells when applied *in vitro* or *in vivo* [118-120]. Blockade of the SHH signaling pathway, which is important in stem cell self-renewal, by cyclopamine leads to long-term PC regression without recurrence, strongly suggesting a connection between this pathway and PCSCs [121]. Salinomycin, a structurally related compound to monensin, was recently identified as a potent PCSC inhibitor [122]. It inhibited the growth of PCs, but did not affect non-malignant prostate epithelial cells. That salinomycin impaired PCSC growth and function was evident by the findings of reduced CD44$^+$ cell fraction and ALDH activity. Moreover, salinomycin reduced the expression of MYC, AR and ERG; induced oxidative stress; and, inhibited NF-κB activity and cell migration.

Regulation of the cell cycle is frequently altered in PC, in part, by the interplay of activation of oncogenic cascades with diverse hormones, growth factors, and cytokines. Thus, inhibitors of cell cycle regulatory proteins have become an area of increased interest in targeting CSCs [123]. The cyclin-dependent kinase inhibitor VMY-1-103 inhibited at very low concentrations the Erb-2/Erb-3/heregulin-induced cell proliferation in LNCaP PC cells. [124]. It was also observed that VMY-1-103 induced apoptosis *via* decreased mitochondrial membrane polarity; and induced p53 phosphorylation, caspase-3 activation, and PARP cleavage in these PC cells, which do express endogen wild type p53. But, VMY-1-103 failed to induce apoptosis in the p53-null PC cell line PC3 [124]. These results, strongly suggest that VMY-1-103 may be an effective therapeutic agent, either alone or in combinations with other drugs, in treating PC.

Adhesion receptors of the integrin family, particularly $α_v$-integrins, have functions including bone homing by cancer cells, tumor-induced angiogenesis, and osteoclastic bone resorption. Targeting of integrins by an $α_v$-integrin antagonist (GLPG0187) could inhibit the *de novo* formation and progression of bone metastases in PC by antitumor (including inhibition of epithelial-to-mesenchymal transition and the size of the PCSC population), antiresorptive, and antiangiogenic mechanisms [125].

Targeting the local microenvironment niche and stromal components of the CSCs would comprise two other promising therapeutic approaches. For instance, it is known that particularly the combined use of antiangiogenic agents with cytotoxic drugs inhibits tumor growth and invasion. Combining docetaxel with the EGFR-targeting agent cetuximab and the antiangiogenic agent sunitinib (SUTENT) inhibits tumor growth approximately 50% at the end of the 3rd week dosing schedule [126]. Targeting the fibroblast-to-myofibroblast transition with halofuginone (inhibitor of collagen type I) may also synergize with low doses of chemotherapy in achieving a significant antitumor effect, avoiding the need of high-dose chemotherapy and its toxicity without impairing treatment efficacy [57]. These results all support the idea that targeting PCSCs, their further differentiated progenies, and microenvironment could be more effective to counteract PC transition to invasive and metastatic stages.

Target	Effect	Molecules	Reference
EGFR signaling pathway	Anti-EGFR antibody	Cetuximab, Erbitux, mAb-C225, IMCC225	[118, 120]
	EGFR tyrosine kinase inhibitor	Gefinib, Erlotinib, EKB-569	
SHH signaling pathway	Signaling inhibition	GDC-0449	[127]
		Cyclopamine	[121]
		Anti-SHH antibody	[128]
Cell signaling pathway	Reducing ALDH activity and CD44$^+$ cell fraction	Salinomycin	[122]
STAT3 signaling pathway	STAT3 signaling inhibitor	Galiellalactone	[78]
WNT/β-Catenin signaling pathway	Suppression of the WNT co-receptor LRP6 expression	Silibinin	[129]
Cell cycle	Cyclin-dependent kinase inhibitor	VMY-1-103	[124]
Adhesion receptors	α$_v$-integrin antagonist	GLPG0187	[125]
Niche and stromal components	Collagen type I inhibitor	Halofuginone	[57]
	Anti-angiogenic agent	Sunitinib, SUTENT	[126]
	Telomerase reverse transcriptase (hTERT) promoter-induced CXCR4 knockdown	siRNA	[130]

Table 3. Novel targets for therapy against advanced prostate cancer

5. Conclusion

Despite all recent developments in cancer diagnosis and therapy, PC still remains one of the leading causes of cancer related deaths in men. Nevertheless, designed new tools for precise diagnosis will enable researchers to distinguish patients "who will be recurred earlier, but will require more extensive treatments" from those "who will have lifespan less effected from their disease". Unlike some other solid tumors, PC is one of these tumor types in which limited treatment options are available so far and gain of drug-resistance is seen more often. That is why there is an urgent need for alternative and novel therapies.

CSCs are believed to be a subpopulation of cancer cells that modulate malignancy and show resistance to current anticancer treatments, which make them indicators of poor prognosis. There are still many aspects of CSCs that remain to be discovered; like, which main mechanisms regulate normal SC function and how are they used by malignant cells to propagate the disease? A careful dissection of the main differences between normal adult SCs and CSCs as well as of their overlapping aspects are important to distinguish how cancers proceed. Transforming the gained knowledge in CSC biology into effective therapies would

then help patients to regain their health much earlier. Altogether, that is the reason why the relation between the expressed CSC markers and resulting malignant behavior needs to be sufficiently understood, as they are primarily relevant with the prognosis of cancer.

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