

Review Article

# Prostate cancer as a dedifferentiated organ: androgen receptor, cancer stem cells, and cancer stemness

Xiaozhuo Liu<sup>1</sup>, Wen (Jess) Li<sup>1,2</sup>, Igor Puzanov<sup>3</sup>, David W. Goodrich<sup>1,2</sup>, Gurkamal Chatta<sup>3</sup> and  Dean G. Tang<sup>1,2</sup>

<sup>1</sup>Department of Pharmacology & Therapeutics, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, U.S.A.; <sup>2</sup>Experimental Therapeutics (ET) Graduate Program, Roswell Park Comprehensive Cancer Center and the University at Buffalo, Buffalo, NY 14263, U.S.A.; <sup>3</sup>Department of Medicine, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, U.S.A.

**Correspondence:** Xiaozhuo Liu (Xiaozhuo.Liu@Roswellpark.org) or Dean G. Tang (Dean.Tang@Roswellpark.org)



Cancer progression is characterized and driven by gradual loss of a differentiated phenotype and gain of stem cell-like features. In prostate cancer (PCa), androgen receptor (AR) signaling is important for cancer growth, progression, and emergence of therapy resistance. Targeting the AR signaling axis has been, over the decades, the mainstay of PCa therapy. However, AR signaling at the transcription level is reduced in high-grade cancer relative to low-grade PCa and loss of AR expression promotes a stem cell-like phenotype, suggesting that emergence of resistance to AR-targeted therapy may be associated with loss of AR signaling and gain of stemness. In the present mini-review, we first discuss PCa from the perspective of an abnormal organ with increasingly deregulated differentiation, and discuss the role of AR signaling during PCa progression. We then focus on the relationship between prostate cancer stem cells (PCSCs) and AR signaling. We further elaborate on the current methods of using transcriptome-based stemness-enriched signature to evaluate the degree of oncogenic dedifferentiation (cancer stemness) in pan-cancer datasets, and present the clinical significance of scoring transcriptome-based stemness across the spectrum of PCa development. Our discussions highlight the importance to evaluate the dynamic changes in both stem cell-like features (stemness score) and AR signaling activity across the PCa spectrum.

## Introduction

### Histopathological grading of human tumors is evaluated based on the level of dedifferentiation

Differentiation in biology describes the processes by which immature cells become mature cells with specific biological functions, while in cancer, this defines how much or how little tumor tissue looks like the normal counterpart it originated from under a microscope. If the cells in the tumor and the organization of the tumor tissue closely resemble those of normal cells and tissue where they start, the tumor is called ‘well-differentiated’. These tumors tend to grow and spread more slowly than ‘poorly differentiated’ or ‘undifferentiated’ tumors, which have abnormal-looking cells and lack normal tissue organization and histological structures. As an indicator of how quickly a tumor is likely to grow and spread (‘tumor malignancy’ or ‘aggressiveness’), the differentiation level, or histopathological grade of human tumors is evaluated routinely in the clinic, with poorly differentiated tumors generally having a worse prognosis [1].

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## Tumor development and growth is an evolving process of dedifferentiation

As tumor progresses, its cellular organization and histological presentations gain an architecture that resembles earlier or the less differentiated (immature) state in normal tissues, with gradual loss of a differentiated phenotype [1,2]. In addition to these histological and cellular phenotypes, how about the gene expression patterns in poorly differentiated tumors? Pioneer work using molecular profiling of cancer cells at different tumor stages, such as breast cancer, glioma, and bladder cancer, revealed that histologically poorly differentiated tumors possess gene sets and transcription factors enriched in human embryonic stem cells [1,3] and adult stem cells [3–5]. In addition, higher pathological grading in breast cancer correlates well with higher frequencies of cancer cells expressing a breast stem cell phenotype [4]. These studies demonstrate the link between tumor pathogenesis and the stem cell state, i.e., cellular expression signatures and molecular regulatory networks that define human stem cell (HSC) identity are also active in more aggressive human tumors. Moreover, these studies suggest phenotypic and functional plasticity of cancer cells in that differentiated cancer cells may undergo dedifferentiation (reprogramming) to revert to stem-like cancer cells under the therapeutic pressure and/or due to genetic/epigenetic alterations [6,7].

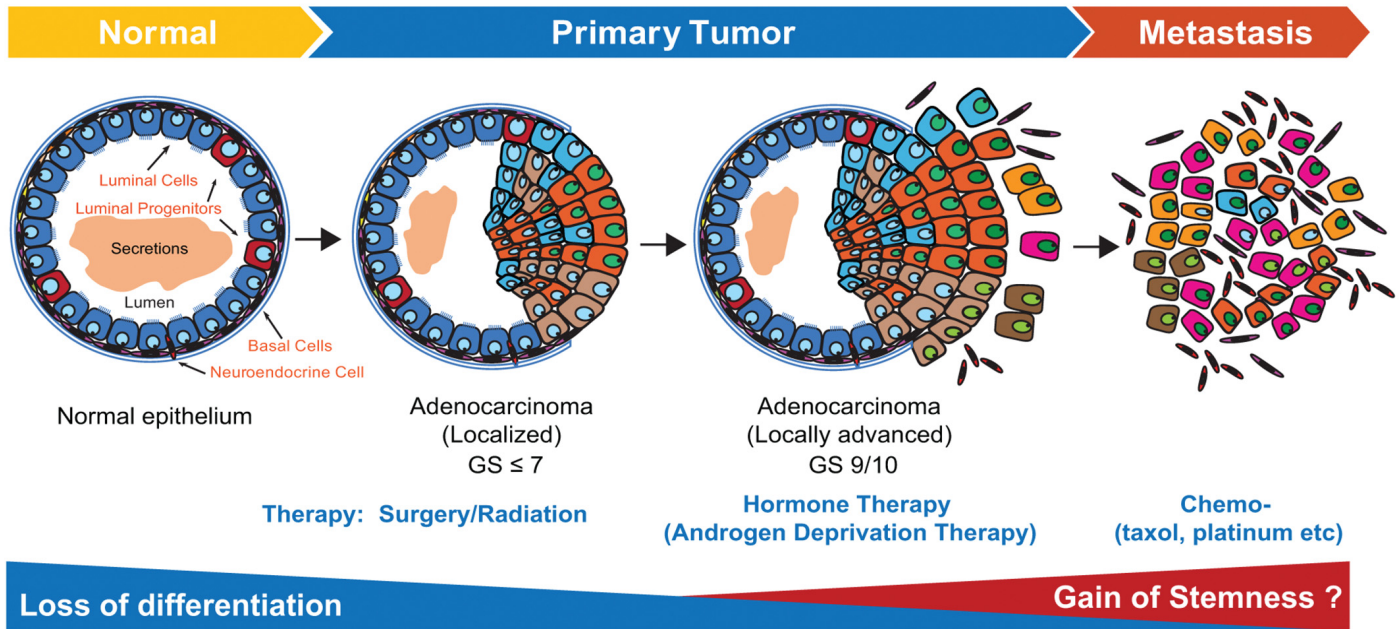
The unique ability of embryonic and adult stem cells to self-renew and differentiate into multiple cell lineages constituted the basic definition of ‘stemness’ [3]. Strong evidence indicates that tumors may harbor stem-like cancer cells called cancer stem cells (CSCs), which are generally defined as a small subpopulation, in treatment-naïve tumors, of the bulk tumor cells capable of self-renewal, regeneration, long-term maintenance of tumors, and recapitulating (at least partially) cellular heterogeneity seen in parental tumors [6,8–10]. CSCs are commonly identified and enriched using similar strategies for identifying normal stem cells, which include flow cytometry-based sorting using cell surface markers and functional approaches, including the side population analysis, Aldefluor assay, and sphere formation coupled with serial sphere passaging [6]. CSCs have been identified in a wide variety of cancers, as demonstrated by their capability to initiate and perpetuate tumor growth and reconstitute the cellular heterogeneity in serial transplantation experiments in immunocompromised mice [11–13]. The CSC hypothesis provides plausible explanations for clinical observations including therapeutic resistance, tumor dormancy, minimal residual disease (MRD), and metastasis [14]. Although it remains unclear whether the enriched stemness signature in poorly differentiated tumors reflects the abundance of bona fide CSCs in the bulk tumors, cancer stemness has become an important phenomenon because of its strong association with poor prognoses in different cancers, suggesting it is a fundamentally important property of malignancy during cancer evolution [5,14,15].

## Prostate tumor as an organ of increasingly deregulated differentiation

Prostate cancer (PCa) is the most common malignant tumor in the male reproductive system, usually diagnosed in the elderly (age > 65 years) [16,17] and ranking the second leading cause of cancer-related death in men [18]. The normal human prostate is a walnut-sized, pseudostratified two-layer epithelial glandular organ that contains an inner layer of secretory luminal epithelial cells surrounded by a layer of basal cells interspersed with rare neuroendocrine cells [19,20] (Figure 1). The luminal or glandular cells, characterized by the expression of cytokeratin 8 (CK8), androgen receptor (AR), prostate-specific antigen (PSA), constitute the ‘functional’ exocrine compartment of the prostate, secreting into the glandular lumen prostatic fluid as a major component of the ejaculate [19]. The basal cells are CK5<sup>+</sup>, CK14<sup>+</sup>, and p63<sup>+</sup> and AR<sup>-</sup> cells (Figure 2). PCa predominantly displays a luminal cell phenotype and histologically presents as adenocarcinomas with significantly reduced numbers of basal cells [21].

Tumorigenesis resembles abnormal organogenesis, and prostate tumor is an organ of increasingly deregulated differentiation. The aggressiveness or the level of malignancy of primary PCa in a biopsy can be pathologically assessed by a combined GS grading system with respect to their degree of differentiation, i.e., the higher the GS, the more malignant (less differentiated) the tumor is [19,22]. This most commonly used grading system in the clinic relies on the architectural pattern of cancerous glands, with Gleason pattern 1—representing most well-differentiated glandular structures and Gleason pattern 5—representing the most poorly differentiated cells (lack of glandular structures). Given that PCa is often multifocal, the combined GS is the sum of the two most prevalent patterns, with a sum score less than 7—representing low-grade tumors and a sum score more than 8—representing high-grade PCa (Figure 1). The concept of prostate tumors as a dedifferentiated organ is also supported by the gradual decrease and loss in prostatic basal cells expressing CK5, CK14, and p63 [19].

PCa treatment is decided largely based on the pathologic evaluation of a prostate biopsy (Figure 1). About 75% primary PCa are diagnosed as low to intermediate grade (GS ≤ 7) and treated by the surgical procedure called radical prostatectomy and/or radiation with a good prognosis. Many patients with high-grade tumors (GS ≥ 8) have



**Figure 1. A schematic illustrating spectrum of PCa initiation, development, and progression**

Left: The normal human prostate is a pseudostratified two-layer epithelial glandular organ that contains an inner layer of secretory luminal epithelial cells surrounded by a layer of basal cells with rare neuroendocrine (NE) cells scattered in between. Also depicted are rare luminal progenitor cells in the luminal cell layer.

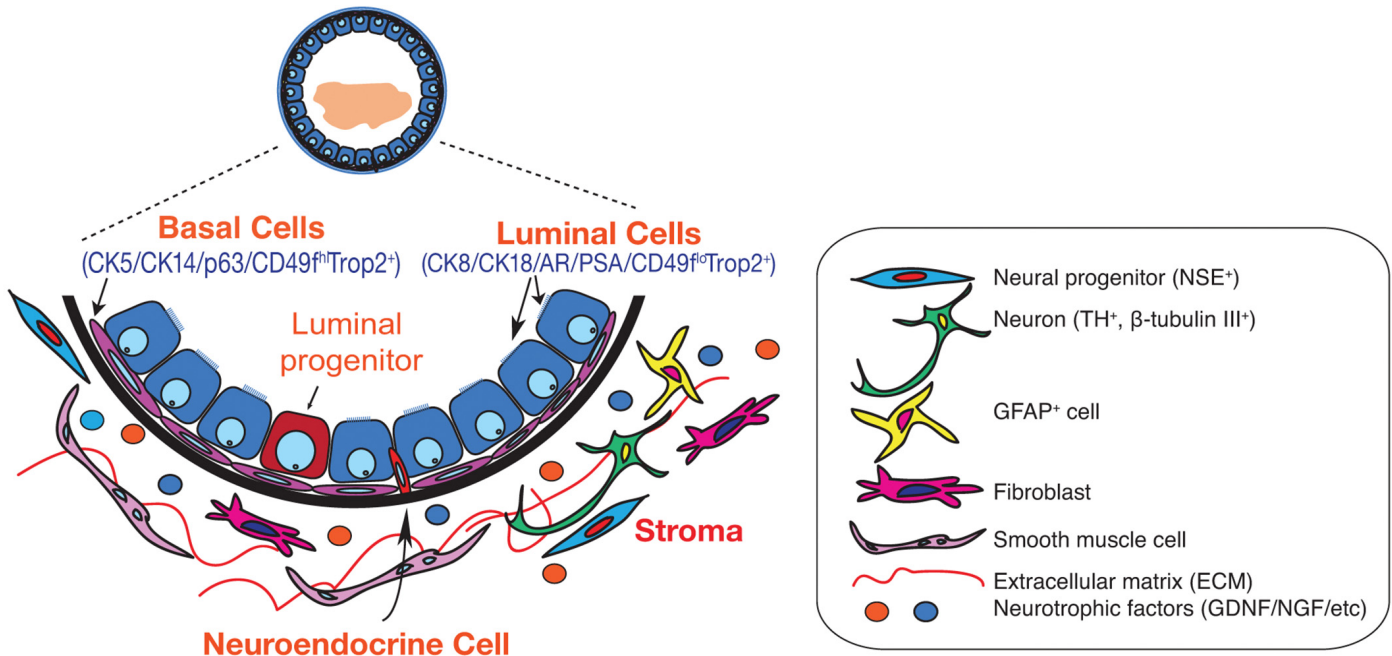
Middle: Localized PCa (i.e., tumors restricted to the primary organ, prostate). PCa gains more aggressiveness across the spectrum of PCa initiation, progression, relapse, metastasis, and therapeutic resistance. Primary PCa is commonly graded by the combined Gleason score (GS) system, which relies on the architectural pattern of cancerous glands, with Gleason pattern 1—representing most well-differentiated glandular structures and Gleason pattern 5—representing the most poorly differentiated cells (lack of glandular structures). Given that PCa is often multifocal, the combined GS is the sum of the two most prevalent patterns, with  $GS \leq 7$ —representing low-grade PCa and  $GS \geq 8$ —representing high-grade PCa. Illustrated here are two representative tumor glands with a decrease/loss in basal cells and expansion of luminal progenitor-like cells.

Right: PCa metastasis. Depicted is a metastatic castration-resistant PCa (mCRPC) with increased cellular heterogeneity (i.e., cells at a variety of epigenetic and phenotypic states) and increased stem-like and NE-like cells.

Below: Depending on the severity of the disease, current treatment options for PCa include active surveillance, surgery by prostatectomy, radiation therapy, hormonal therapy, or chemotherapy. Up to one-third of patients with a localized disease eventually fail on local therapies and progress to advanced-stage or metastatic PCa within 10 years. For locally advanced ( $GS \geq 9$ ) and metastatic PCa, androgen-deprivation therapy (ADT) is the standard of care, which uses LHRH agonists/antagonists to block testicular androgen synthesis. Although the majority of patients initially respond, most tumors become resistant to primary hormonal therapy within 14–30 months. Tumors that have failed this first-line therapy are termed CRPC and are further treated with AR signaling inhibitors such as enzalutamide (Enza) that interferes with AR functions. Enza only extends CRPC patients' lives by 4–5 months before tumor recurrence. For men with mCRPC, the median survival in phase III studies range from 15 to 19 months. For several years, the chemotherapeutic drug docetaxel was the only treatment option for mCRPC. Most mCRPC, including both CRPC-adenocarcinoma (CRPC-adeno) and CRPC-NE subtypes, remains lethal. Figure is modified from [85] and [86].

Bottom: The trajectory of PCa development and progression is accompanied by loss of differentiation with increasing malignancy and aggressiveness at both cellular and molecular levels as well as at the tumor level. Could PCa progression be associated with increasing stemness? This is an outstanding question elaborated in the present mini-review.

extraprostatic extension on histopathological examination (i.e., tumor cells beyond the borders of the prostate) and lymph node metastasis and are treated with drugs called AR signaling inhibitors (or ARSIs). There are two general subclasses of ARSIs: those that block the production of AR ligands in the testis or adrenal gland (e.g., Lupron) and those that block AR nuclear translocation and AR functions (e.g., enzalutamide or Enza). Up to one-third of the patients with localized tumors eventually fail on local therapy and progress to advanced-stage or metastatic PCa within 10 years. Advanced and metastatic PCa, which claims the lives of >30000 American men per year, is generally treated



**Figure 2.** A schematic illustrating the cellular architecture of the prostate epithelium and ECM and stromal cells in the normal prostate gland

The luminal epithelial cells are defined by expression of CK8 and CK18 and AR. The basal epithelial cells express high levels of CK5 and p63 and very low levels of AR. Neuroendocrine cells are a small population of endocrine–paracrine cells. Neuroendocrine cells express neuroendocrine markers such as synaptophysin and chromogranin A and do not express AR [86]. The stroma is populated by fibroblasts, immune cells, smooth muscle cells and various subtypes of neural cells, such as TH<sup>+</sup>, NES<sup>+</sup>, and β-Tubulin III<sup>+</sup> nerve fibers and abundant GFAP<sup>+</sup> cells [83]. Figure is modified from [85] and [83].

with ADT and antiandrogens such as Enza [23,24]. Although the majority of patients initially respond, most tumors become resistant to ADT/Enza and evolve into lethal CRPC within 14–30 months [25]. For men with mCRPC, the median survival in phase III studies ranges from 15 to 19 months. Most mCRPC, including both CRPC-Adeno and CRPC-NE subtypes, remain lethal. mCRPC patients have rather limited treatment options, which generally include chemotherapeutic drugs, docetaxel and cisplatin [19,23]. PCa progression is accompanied by increasing aggressiveness, from treatment-naïve primary tumors to ADT/Enza-treated tumors to mCRPC [19].

## AR is a master regulator of normal prostate differentiation

Androgens, acting through the receptor AR, are the major growth factors for the pseudostratified prostate epithelium ([26,27]; reviewed in [28]). Androgen signaling is activated when circulating or locally made androgens bind to AR in normal prostate epithelial and stromal cells or in PCa cells. AR is a steroid hormone receptor with ligand-binding domain and DNA-binding domain. Upon ligand binding, AR dimerizes and translocates from the cytoplasm to the nucleus. The dimerized-liganded AR then binds to androgen-response elements (ARE) on ARE-driven genes to increase their transcription. AR regulates the expression of the genes with diverse functions, including secreted proteins (KLK2, KLK3), transcription factors (NKX3.1, FOXP1), cell cycle regulators (UBE2C, TACC2), growth stimulators (IGF1R, APP), and PI3K modulation (FKBP5) [29]. Thus, AR signaling intensity or AR activity (AR-A) can be measured, as a proxy, by weighted linear sum of the expression levels of a cassette of AR target genes. In fact, AR-A has been semiquantitatively presented using this strategy (gene expression signature) in large PCa patient cohort with transcriptomic profiling data to compare AR activity [30–33]. It should be noted that AR does not act alone but functions in a complex, i.e., AR collaborates with chromatin-associated proteins and nuclear cofactors, including transcription factors, which bind to specific DNA elements in regulatory regions of AR-responsive genes [34]. AR signaling activates various pathways for normal prostate development, including those that regulate cellular proliferation, differentiation, and survival. The prostatic luminal cells, representing the major cell type in normal and



hyperplastic epithelium, express high levels of AR, and depend on androgens for their survival and functions [35]. Dysregulated AR signaling, on the other hand, has been implicated in PCa growth and progression [36].

Androgen/AR signaling is a critical regulator of normal prostate differentiation and development as documented in both human and animal studies [37]. For example, somatic mutations of the *AR* gene, such as deletion of exon 3, lead to AR signaling dysfunctions and androgen insensitivity syndrome in humans in which individuals with male karyotype (46, XY) lack the prostate and present female phenotypes [38]. Consistently, male mice with the *AR* exon 3 knockout manifest underdeveloped prostates with delayed structural and functional differentiation of the prostate epithelium [39]. These studies suggest a prodifferentiation role of AR.

Abnormal AR signaling will break the balance between the rate of cell proliferation and cell death of prostate epithelial cells, and drive neoplastic cell proliferation [40]. AR expression and activity is frequently overexpressed in PCa and AR is thought to be required for prostate tumorigenesis and, hence, targeting the AR signaling axis has been the mainstay of PCa therapy for nearly 80 years [19,37]. ARSIs are among the most effective targeted anticancer therapeutics, given that most of the PCa patients respond with clear clinical evidence of tumor regression [19]. The dramatic initial PCa response to ARSIs can be potentially explained by several considerations: (1) the majority (>90%) of prostate tumors at diagnosis are adenocarcinomas; (2) prostate adenocarcinoma predominantly displays a luminal phenotype with most tumor cells expressing high levels of AR; (3) AR<sup>+</sup> tumor cells rely on AR signaling for their survival. Nevertheless, the clinical efficacy of ARSIs is generally short-lived and after the initial response, most treated tumors become resistant to ARSIs and develop lethal CRPC within 14–30 months [25]. Lack of enduring therapeutic efficacy in ARSIs may be closely related to inherent PCa cell heterogeneity [6].

Significant heterogeneity in the AR expression (AR<sup>+</sup>, AR<sup>-/lo</sup>) has been documented for decades, from treatment-naïve primary tumors to treatment-failed patient tumors to disseminated metastases [41–43]. In fact, AR<sup>-/lo</sup> PCa cells pre-exist in treatment-naïve primary tumors [42,44,45]. In support, recent molecular profiling of treatment-naïve primary PCa also defined a *de novo* low AR signaling-intensity subclass [32]. In addition, the percentage of AR-expressing cells tends to decrease in more advanced and aggressive prostate tumors [41,46,47]. Similarly, reduction in AR signaling intensity as measured by AR target gene expression has been observed during PCa progression from low-grade to high-grade diseases [48,49]. Alumkal et al. measured the AR signaling intensity in a cohort of 34 mCRPC patients before Enza treatment and evaluated the Enza treatment response based on a criterion of 50% reduction in serum PSA levels in a 12-week window [50]. In the present clinical study, nine of the 34 patients were classified as nonresponders (i.e., PSA decline <50%), indicating *de novo* Enza resistance. Strikingly, the nonresponder group showed significantly lower AR signaling intensity and enrichment in a stemness gene signature [50]. Indeed, PCa cells with low AR expression (i.e., AR<sup>-/lo</sup>) in treatment-naïve tumors frequently display stem cell phenotypes and properties (i.e., PCa stem cells or PCSCs; see below) and preferentially employ stem cell signaling program to support their survival and resistance to ARSIs [42,43,51,52].

Our laboratory has recently observed three distinct patterns of AR expression in ~200 CRPC-derived biopsy TMA cores and whole-mount sections (from 89 patients): primarily nuclear AR<sup>+/hi</sup>, mixed nuclear/cytoplasmic AR and AR<sup>-/lo</sup> [43]. Studies using four pairs of androgen-dependent (AD) and androgen-independent (AI) xenograft models revealed that the AR<sup>-/lo</sup> CRPC model was refractory to castration/Enza and PCa cell heterogeneity in AR expression greatly impacted tumor response to ARSIs ([43]; see review in [19]). When the four xenograft AD tumors (i.e., LNCaP, LAPC4, VCaP, and LAPC9) were serially propagated in castrated mice, they manifested model-specific changes in AR expression and subcellular distribution during their progression to the AI or CRPC state. Specifically, the LNCaP AI tumors showed up-regulated nuclear AR (AR<sup>+/hi</sup> phenotype), and LAPC9 AI tumors showed loss of AR (AR<sup>-/lo</sup> phenotype), whereas the LAPC4 and VCaP AI tumors displayed mostly cytoplasmic AR (the mixed nuclear/cytoplasmic AR phenotype), which recapitulated the three AR patterns observed in patient CRPC [43]. Therapeutic studies in mice bearing these AI tumors showed that the three AR<sup>+</sup> AI models (LNCaP, LAPC4, and VCaP) initially responded to Enza treatment prior to emergence of Enza resistance after 6–13 weeks of Enza treatment [43]. On the other hand, the AR<sup>-/lo</sup> LAPC9 AI model showed *de novo* resistance to Enza [43]. Under persistent selective pressure from ARSIs, PCa cells may adaptively change the target protein AR in order to survive, including overexpressing (AR<sup>+/hi</sup>), losing (AR<sup>-/lo</sup>), or redistributing (cyto-AR) the AR [43]. With the use of more specific and potent ARSIs in the clinic, more AR<sup>+</sup> adenocarcinomas are being reprogrammed to AR<sup>-/lo</sup> neuroendocrine-like tumors called CRPC-NE [53,54] or AR pathway-independent AR<sup>-</sup>NE<sup>-</sup> mCRPC called double-negative PCa (DNPC) [30]. These studies illustrate cancer cell plasticity, yet another cellular mechanism accounting for short-lived therapeutic efficacy.

## PCSCs and tumor progression

At the cellular level, cancer cell heterogeneity may result from clonal evolution driven by genomic alterations and/or from intraclonal phenotypic differentiation and functional maturation of CSCs [51]. CSCs possess some or most of the normal stem cell properties such as relative quiescence but with great proliferative potential, the ability to self-renew and differentiate, and, importantly, the capability to regenerate and long-term propagate tumors with recapitulation of the cellular heterogeneity seen in parental tumors [6,55]. Many PCSC subpopulations have been reported partly due to multiple tumor foci and diverse subclones within each tumor [56,57]. PCSCs are defined using a spectrum of phenotypical assays (such as using stem cell or CSC-associated marker expression; e.g., CD44, CD133, ABCG2) and/or functional assays (such as high clonal and clonogenic capacities *in vitro*, high tumor-initiating and serial tumor-propagating activities in immunodeficient mice) (reviewed in [37]). Collins et al. reported putative PCSCs in human prostate tumors using the cell surface marker profile of CD44<sup>+</sup>α2β1<sup>+</sup>CD133<sup>+</sup>, as evidenced by the observations that: (1) this cell population represented a small fraction of the tumor bulk; (2) they exhibited a high potential for self-renewal and proliferation; and (3) the CD44<sup>+</sup>α2β1<sup>+</sup>CD133<sup>+</sup> cells were able to differentiate to heterogeneous cancer cells in *ex vivo* culture [58]. However, it was unclear whether the CD44<sup>+</sup>α2β1<sup>+</sup>CD133<sup>+</sup> cells in treatment-naïve prostate tumors possess high tumor-initiating and long-term tumor-propagating properties, the most important biological traits expected of CSCs. Our systematic studies using PCa xenograft and PDX (patient-derived xenograft) models showed that the CD44<sup>+</sup> PCa cell population is significantly enriched in PCSCs with enhanced metastatic capacities in addition to tumor-propagating ability.

PCSCs are heterogeneous as multiple subpopulations expressing different phenotypic markers have been reported (reviewed in [6,10]). For instance, the PCa cell population that lacks the expression of PSA (PSA<sup>-/lo</sup>) harbors PCSCs, which express stem cell genes, can undergo Notch-regulated asymmetric cell division, and are resistant to castration treatments [42,51]. Also, high levels of expression of drug-resistant genes such as ATP-binding cassette (ABC) transporter ABCG2 can further enrich PCSCs from CD44<sup>+</sup>CD133<sup>+</sup>CD24<sup>-</sup> subpopulation. These enriched PCSCs have increased clonal and sphere-formation abilities, highlighting the intrinsic therapy resistance as another important and shared feature of CSCs [51,59–61].

There is evidence that the frequency of PCSCs increases accompanying PCa progression. For example, aldehyde dehydrogenase 1 family member A1 (ALDH1A1) is a well-documented stem cell marker and increased ALDH activity is found in PCSCs [62,63]. PCa cells expressing ALDH1A1 were more often seen in samples with advanced GS [64]. In addition, a positive correlation was found between ALDH1A1 expression and primary tumor stage [64]. More recently, CD117<sup>+</sup> PCa cells are shown to represent a PCSC subpopulation by using several functional assays including xenograft limiting-dilution and serial tumor transplantation assays [65]. In addition, activation and phosphorylation of CD117 increases in PCa patients with higher Gleason grades, and the expression of CD117 is also associated with decreased overall- and progression-free survival [65]. Gene expression profiling by microarray revealed that common basal/stem or progenitor cell markers CK5, c-Kit, nestin, CD44, c-Met, ALDH1A1, α2-integrin, CD133, ABCG2, CXCR4, and POU5F1 (or Oct3/4) were up-regulated in clinical CRPC [61]. These studies suggest that such stem cell markers could potentially be exploited for targeted therapy of PCa, especially in advanced CRPC.

## Inverse correlation of PCSCs and AR signaling

The above discussions highlight the heterogeneous expression of AR (i.e., AR<sup>+</sup>, AR<sup>-/lo</sup>) in different stages of PCa. Interestingly, in many reported PCSC populations, AR expression is often low or undetectable. Indeed, similar to the undifferentiated nature of normal stem cells, another common phenotypic feature of the reported PCSC subpopulations from treatment-naïve tumors is the absence or low expression of differentiation regulators and markers such as AR, PSA, and MHC molecules [6,37]. In fact, there appears to exist an inverse correlation between PCSCs and AR signaling. For example, in a recent study, Giridhar et al. reported that preferential expression of the oncogene MDM2, an E3 ubiquitin ligase, in AR<sup>-/lo</sup> PCa cells led to constant AR degradation and maintenance of the PCSC properties in AR<sup>-/lo</sup> cells [66]. Intriguingly, MDM2 also positively regulated the CSC properties such as self-renewal as well as stem cell gene expression in AR<sup>-/lo</sup> PCa cells [66]. Strikingly, MDM2 knockdown or inhibition in AR<sup>-/lo</sup> cells reinduced full-length AR expression and even caused their ‘terminal’ differentiation [66]. In another study, inhibition of AR by either antiandrogens or gene-silencing promoted both phenotypic and functional manifestations of PCSCs through the up-regulation of IL-6/STAT3 signal transduction [67]. In human PCa specimens, high levels of STAT3 expression coincided with high levels of CSC markers but low or absent AR expression [67]. As yet another example, Han et al. recently conducted transcriptomic profiling in 94 PCa PDX, cell line and organoid models and reported three transcriptional subtypes of mCRPC: ARPC (i.e., AR<sup>+</sup> PCa), MSPC (AR<sup>lo</sup> mesenchymal/stem-like PCa), and (AR<sup>-</sup>) NEPC [68]. The majority of clinical mCRPC specimens analyzed represent mixed ARPC and MSPC. Significantly,

AR blockade with Enza in LNCaP cells caused a rapid decline in the expression of *TP53*, *MDM2*, *p21*, *BAX*, and several additional canonical *TP53* targets, and this functional inactivation of *TP53* induces LNCaP cells to dedifferentiate to a hybrid epithelial and mesenchymal (E/M) and stem-like state [68]. Also, AR blockade with Enza inhibited BMP-SMAD signaling, which paradoxically promoted the emergence of drug-resistant PCa cells [68]. Finally, ADT has been shown to reprogram bulk PCa cells into PCSCs [69,70]. These studies are consistent with our studies [43,71] and with recent molecular profiling of patient tumors before treatment that identified a low-AR activity but a high stemness program to be associated with *de novo* Enza resistance [50].

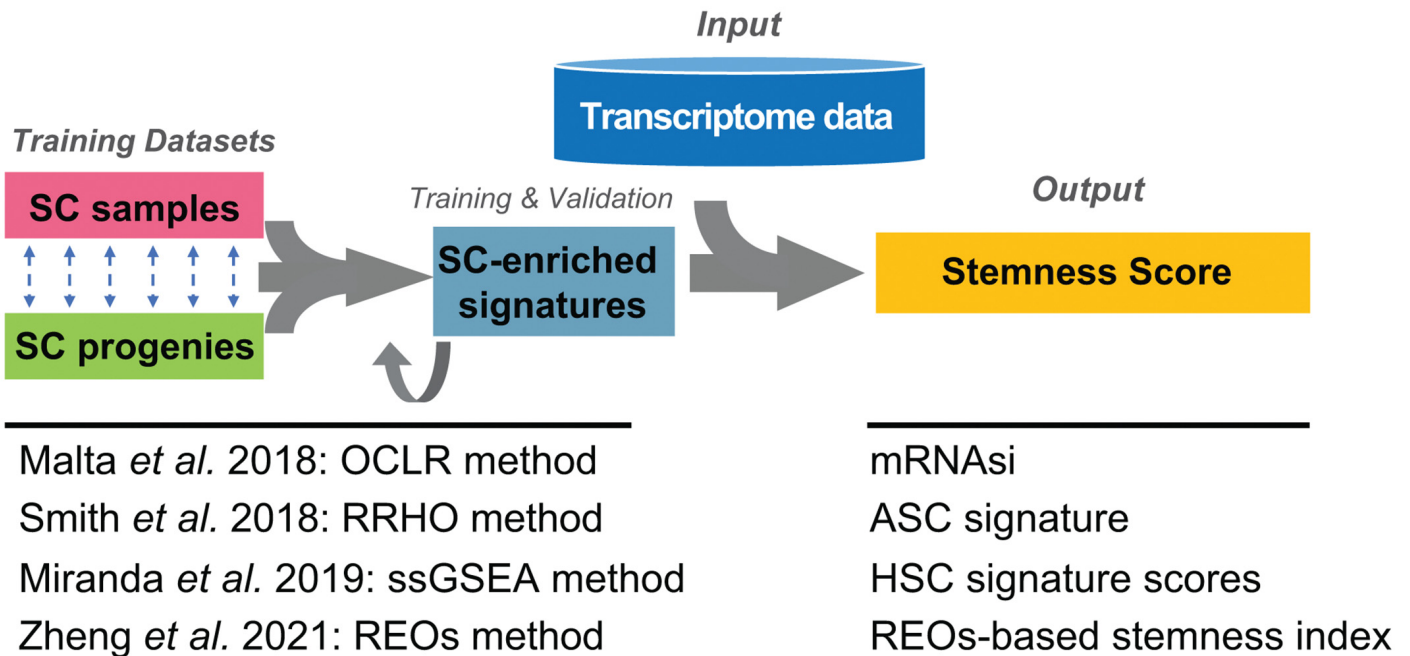
## Could PCa progression be associated with increasing stemness?

As discussed in the first section, early work using molecular profiling of cancer cells (including breast cancer, glioma, and bladder cancer) from different tumor stages revealed that poorly differentiated human cancers are enriched in genes, transcription factors, and gene expression profiles that characterize human embryonic stem cells [1], highlighting the positive correlation of oncogenic dedifferentiation and tumor progression or aggressiveness. Given that stem cell markers are important for purification and identification of CSCs, can we develop and quantitatively measure the degree of oncogenic dedifferentiation or ‘cancer stemness’ using a universal single CSC marker or a set of CSC-related markers? The answer is probably no, as none of the CSC markers may be universal to all CSC subpopulations from the same tumor type in different cancer patients nor to CSCs in different tumor types [72]. However, with the advances of more sophisticated next-generation sequencing techniques, more and more transcriptomic data from different types of stem cells and their differentiated progeny become available [15], which allow researchers to define core gene-expression programs of normal ‘stemness’ and further develop cancer stemness quantification metrics across diverse cancer types [5,15,73–75].

Several transcriptome-based methods for stemness quantification have been developed [5,14,15,76,77]. In general, these transcriptome-based methods for stemness quantification include three steps: (1) development of stemness-enriched signatures from training datasets by comparing transcriptomic data from normal stem cells (including both embryonic and adult stem cells) with the data from their differentiated progeny; (2) validation of the stemness signature in validation datasets by its ability to distinguish stem cells from their differentiated counterparts; and (3) application of the stemness signature on cancer sample datasets to identify and stratify cancer progression and predict clinical outcomes (Figure 3).

Malta et al. utilized an innovative OCLR machine-learning algorithm [78] to develop the stemness-enriched signature by comparing molecular datasets in normal stem cells and defined populations of differentiated progeny in Progenitor Cell Biology Consortium (PCBC) [15]. The authors named this transcriptome-based stemness-enriched signature or stemness index mRNAsi, and validated the mRNAsi in an external dataset composed of both pluripotent stem cells and somatic differentiated cells (17 distinct tissue types and several primary cell lines of diverse origin) [78]. The mRNAsi values of given samples were measured using the Spearman correlation, followed by a linear transformation. The mRNAsi values can clearly distinguish stem cells from their differentiated counterparts—all stem cell samples displayed higher mRNAsi values than the differentiated counterparts [15]. The authors applied mRNAsi to analyze cancer stemness extensively in 12000 samples of 33 tumor types from The Cancer Genome Atlas (TCGA) database, which is an important and powerful resource composed of multiomics data along with histopathological and clinical annotations [15]. The results showed that tumor samples have higher mRNAsi values than normal/benign samples from the same tumor type and that tumor samples with aggressive pathological subtypes and poor clinical outcomes have higher mRNAsi values. For example, high mRNAsi values were observed in basal breast carcinomas, and HER2 and Lum B subtypes, which are more aggressive than the hormone-dependent Lum A subtype [15,79,80]. These results suggest that cancer stemness mRNAsi is positively correlated with tumor dedifferentiation as reflected in histopathological grade [15].

Miranda et al. adapted and developed a stemness-enriched signature including 109 genes [14,74] and they conducted ssGSEA on the 109 stemness-related genes to calculate the stemness index by using GSVA package in R [14]. The authors validated the ssGSEA-derived stemness in pan-cancer datasets by showing good concordance with OCLR-derived mRNAsi [15]. Consistent with previous studies showing stemness being a negative prognostic factor [5,81], the authors observed a strong negative relationship between median stemness and median overall survival across cancer types [14]. And ssGSEA-based stemness is substantially better predictive of survival as compared with OCLR-based mRNAsi using pan-cancer Cox regression modeling. ssGSEA-based stemness also showed a negative association with immune cell infiltration (CD8<sup>+</sup> T, NK, and B cells) across 21 solid cancers, but a positive correlation



**Figure 3. Schematic presentation of transcriptome-based stemness quantification methods**

Several transcriptome-based methods for stemness quantification have been developed [5,14,15,76,77], which generally include three steps: (1) development of stemness-enriched signatures from training datasets by comparing transcriptomic data from normal stem cells with differentiated progeny; (2) validation of the stemness signature in validation datasets by its ability to distinguish stem cells from their differentiated counterparts; and (3) application of the stemness signature on cancer sample datasets. Malta *et al.* utilized a one-class logistic regression (OCLR) machine-learning algorithm to develop the stemness-enriched signature score, namely, mRNAsi [15]. Smith *et al.* applied a rank-rank hypergeometric overlap (RRHO) algorithm to develop an adult stem cell (ASC) signature [5]. Miranda *et al.* conducted single-sample gene set enrichment analysis (ssGSEA) on the 109 stemness-related genes to calculate the stemness index (HSC signature score) by using GSVA package in R [14]. Finally, Zheng *et al.* calculated the stemness index based on relative expression ordering (REOs) of gene pairs within a sample [76].

with cell-intrinsic suppression of endogenous retroviruses and type I IFN signaling. These results suggest that gaining in stemness may represent a fundamental process in cancer progression with poor clinical outcome, and stemness may provide a mechanistic link between cancer progression and immune suppression across cancers [14].

More recently, Zheng *et al.* provided a robust stemness quantification method to overcome the batch effect and sample normalization, which are limitations for ssGSEA-derived stemness and OCLR-derived mRNAsi [76]. This improved method is developed based on the REOs of gene pairs within a sample. The REO-based method enabled the stable application on the evaluation of the absolute stemness index with a 0–1 range. Moreover, Smith *et al.* applied a RRHO algorithm to allow the identification of significantly concordant transcriptional profiles from independent transcriptome data regardless of sequencing platform or other variables [5]. Their results revealed that aggressive epithelial cancers with worse overall survival and alternations of oncogenic drivers are enriched for a transcriptional signature shared by epithelial adult stem cells (*i.e.*, ASC signature) [5].

Collectively, these transcriptome-based stemness quantification methods demonstrate that higher stemness was associated with tumor dedifferentiation reflected by histological grade and poor prognosis across diverse cancer types, indicating the potential therapeutic and diagnostic implications of transcriptome-based stemness quantification.

Could PCa progression be associated with increasing stemness? The answer may likely be yes based on the increasing stemness in aggressive cancer subtypes from pan-cancer transcriptome-based stemness analysis [5,14,15,76]. In fact, several studies have assessed the stemness scores of PCa in a pan-cancer setting [5,15,76]. For instance, the RRHO-derived ASC signature [5] was significantly enriched in high-grade (GS8–10) as compared with low-grade (GS6) PCa. Interestingly, the authors also found that the ASC signature significantly improved determination of PCa stage beyond GS alone [5], suggesting a higher sensitivity of molecular profiling in grading tumors, possibly because



the degree of tumor differentiation could have changed at the molecular level but had yet to be reflected at histological level [82,83]. Molecular profiling of primary human PCa cell populations revealed that advanced PCa subtypes are preferentially enriched in a prostate basal/stem cell signature with small-cell and neuroendocrine PCa being the most basal/stem-like [82,83]. These tumors have a clinically aggressive behavior, tend to lack AR expression, and are refractory to ARSIs [82,83]. NEPC/CRPC-NE and the normal prostate basal/stem cells shared a transcriptional program associated with the E2F-Rb pathway and specific transcription factors such as SOX2 [82,83].

PCa becomes more and more aggressive across the spectrum of tumor evolution, i.e., from tumor initiation to spontaneous cancer progression, metastasis, and development of therapeutic resistance [19]. It would be important for understanding PCa biology by evaluating whether PCa progression is also accompanied by gain of stemness across the PCa spectrum as most of the transcriptome-based stemness analysis was limited to comparisons between high-grade to low-grade tumors or between metastatic and primary tumors. Moreover, with the recent advances in various spatial sequencing, such as single-cell RNA sequencing (scRNA-seq), these powerful approaches should allow us to reconstruct cellular differentiation trajectories by characterizing single-cell transcriptomes in diverse tissues, organs, and whole organisms. For example, Gulati et al. developed a computational framework called CytoTRACE from nine 'gold-standard' scRNA-seq datasets with experimentally confirmed differentiation trajectories, which allow the prediction of differentiation states in transcriptome-based datasets [84]. Quantification of stemness level in PCa will have several benefits for potential clinical applications: (1) higher sensitivity and earlier prediction: the degree of tumor (de-)differentiation could have changed at the molecular level but yet to be manifested in histological grade; (2) applicable to metastatic tumors: Gleason grading is limited to localized PCa, but transcriptome-based stemness can evaluate the PCa spectrum including both localized tumors and distant metastases; and (3) providing an alternative method to gauge PCa progression since Gleason scoring is somewhat subjective and suffers from suboptimal inter-observer and intraobserver variability [22]. AR is a master regulator of normal prostate development, deregulated AR signaling represents a driving force in PCa growth, and targeting AR signaling pathway has been the standard treatment for PCa. However, AR signaling at the transcription level has been reported to decline in more aggressive, high-grade PCa as compared with low-grade PCa. Also, loss of AR expression promotes a stem-like cell phenotype implying an inverse relationship between AR signaling and PCa stemness. Therefore, it would be important to understand the dynamic changes in stem-like features and AR signaling activity at the global transcriptomic level, and the relationship between the AR signaling activity and stemness across the spectrum of PCa development and progression. This will provide new insights for drug development to prevent drug resistance and relapse.

## Summary

- Cancer progression is characterized by gradual loss of a differentiated phenotype and gain of progenitor and stem cell-like features.
- PCa progression resembles an organ with increasingly deregulated differentiation.
- AR is a master regulator of normal prostate differentiation.
- AR signaling is attenuated during PCa progression.

## Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Author Contribution

X.L. and D.T. conceptualized the framework of the manuscript. X.L. collected data from published studies and wrote the manuscript. X.L. and W.L. generated schematic figures. X.L., W.L., and D.T. discussed the review content and critically reviewed

the manuscript draft. I.P. and G.C. reviewed the manuscript draft and provided clinical insight. D.W.G. participated in brainstorm meetings, offered insight from studies using genetically engineered murine PCa models, and also reviewed the manuscript. D.T. aided in manuscript writing and finalized the manuscript. All the authors have read and agreed to the published version of the manuscript.

## Abbreviations

ABC, ATP-binding cassette; AD, Androgen-dependent; ADT, Androgen-deprivation therapy; AI, Androgen-independent; ALDHA1, Aldehyde dehydrogenase 1 family member A1; AR, Androgen receptor; AR-A, AR activity; ARE, Androgen response element; ARPC, AR+ PCa; ARSI, AR signaling inhibitor; ASC, Adult stem cell; CK8, Cytokeratin 8; CRPC, Castration-resistant PCa; CRPC-Adeno, CRPC-adenocarcinoma; CSC, Cancer stem cell; DNPC, AR- and NE- double-negative PCa; ECM, Extracellular matrix; Enza, Enzalutamide; GS, Gleason score; HSC, Human stem cell; LHRH, Luteinising-hormone releasing hormone; mCRPC, Metastatic castration-resistant PCa; MHC, Major histocompatibility complex; MRD, Minimal residual disease; MSPC, Mesenchymal/Stem-like PCa; NE, Neuroendocrine; NK cells, Natural killer cells; OCLR, One-class logistic regression; PCa, Prostate cancer; PCSC, Prostate cancer stem cell; PDX, Patient-derived xenograft; PSA, Prostate-specific antigen; REOs, Relative expression orderings; RRHO, Rank-rank hypergeometric overlap; scRNA-seq, Single-cell RNA sequencing; ssGSEA, Single-sample gene set enrichment analysis.

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