Androgen receptor and prostate cancer stem cells: biological mechanisms and clinical implications

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Endocrine-Related Cancer

Abstract

Prostate cancer (PCa) contains phenotypically and functionally distinct cells, and this cellular heterogeneity poses clinical challenges as the distinct cell types likely respond differently to various therapies. Clonal evolution, driven by genetic instability, and intraclonal cancer cell diversification, driven by cancer stem cells (CSCs), together create tumor cell heterogeneity. In this review, we first discuss PCa stem cells (PCSCs) and heterogeneity of androgen receptor (AR) expression in primary, metastatic, and treatment-failed PCa. Based on literature reports and our own studies, we hypothesize that, whereas PCSCs in primary and untreated tumors and models are mainly AR^-, PCSCs in CRPCs could be either AR^+ or $AR^{-/lo}$. We illustrate the potential mechanisms AR^+ and AR^- PCSCs may employ to propagate PCa at the population level, mediate therapy resistance, and metastasize. As a result, targeting AR alone may not achieve long-lasting therapeutic efficacy. Elucidating the roles of AR and PCSCs should provide important clues to designing novel personalized combinatorial therapeutic protocols targeting both AR^+ and AR^- PCa cells.

Key Words
- androgen receptor
- prostate cancer
- cancer stem cells
- prostate cancer stem cells
- castration-resistant prostate cancer

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Cancer stem cells and tumor heterogeneity

Tumors contain genetically heterogeneous cellular clones, which constantly evolve during disease progression and clinical treatment. Clonal evolution, driven by genetic instability of cancer cells, generates cellular heterogeneity and promotes tumor progression. For instance, genome-wide DNA sequencing of three individual prostate tumors revealed the existence of three or more clones within each cancer (Cooper *et al*. 2015). Even morphologically normal regions could possess as many as ten genetic mutations (Cooper *et al*. 2015). In untreated primary prostate cancer (PCa), genetic alterations such as TMPRSS2-ERG fusion and PTEN deletion within tumor clones could activate critical signaling pathways such as ERG and PI3K, thus driving clonal evolution (Berger *et al*. 2011, Haffner *et al*. 2015). In a longitudinal tracking of a castration-resistant PCa (CRPC) patient with nine prostate tumor foci at the initial presentation, it was found that during the 17 years of tumor progression, only the tumor clones with *PTEN*, *P53*, and *SPOP* mutations gained additional genetic alterations and gave rise to lethal metastatic tumors. Surprisingly, the lethal clone (defined by the presence of the same *PTEN*, *P53*, and *SPOP* mutations) in this patient was found to arise from a morphologically low-grade (Gleason 3) tumor focus rather than the predominant Gleason 4 tumor foci (Haffner *et al*. 2013). Whole-genome

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exome sequencing in 50 lethal and heavily pretreated metastatic CRPCs also confirmed the monoclonal origin of lethal CRPC (Grasso *et al.* 2012). These examples highlight the importance of genetically driven clonal evolution in driving PCa progression.

On the other hand, there is also strong evidence that tumor cells within a genetically identical clone possess different tumorigenic ability and, in most cases, are organized in a hierarchical manner (reviewed in Tang (2012), Kreso & Dick (2014) and Yang *et al.* (2014)). Sitting at the apex of this tumorigenic hierarchy is the small subset of stem-like cancer cells, or cancer stem cells (CSCs) that possess high self-renewal and differentiation ability. In other words, CSCs sustain an established tumor clone through unlimited self-renewal and maintain intraclonal heterogeneity through generating both tumorigenic and less or non-tumorigenic cancer cells. Similar to normal hematopoietic stem cells (HSCs), which are among the best understood adult stem cells, the best characterized CSCs are CSCs in leukemia or leukemic stem cells (LSCs; Kreso & Dick 2014). Like HSCs, LSCs are undifferentiated, lacking the expression of lineage differentiation markers. Subsequent studies have led to the identification of CSCs in multiple human solid tumors, and a common phenotypic feature of these CSCs seems to be the lack of differentiation markers and regulators (Tang 2012, Kreso & Dick 2014, Yang *et al.* 2014).

In a strict sense, CSCs in human tumors are defined as a population of cancer cells that, when prospectively purified out from patient tumors, xenografts, and even long-term cultures, can regenerate and also indefinitely propagate human tumors in immunodeficient mice. In reality, the CSC properties of a candidate population of human tumor cells are best assessed by performing limiting dilution tumor-regeneration assays combined with serial tumor transplantations and cell biological (e.g., clonal in 2D, clonogenic in 3D, sphere formation, single-cell division, differentiation, etc.) as well as molecular (e.g., RNA-Seq and ChIP-Seq) characterizations (reviewed in Rycaj & Tang (2015)). The tumor cell population that can initiate or regenerate tumors at low cell doses is considered to be tumor-initiating or tumor-regenerating cells, and the tumor cell population that can propagate human xenograft tumors long-term is called tumor-propagating cells (Rycaj & Tang 2015). Unfortunately, many of the reported CSC populations do not fully satisfy this strict definition. For example, some studies only utilized cell lines to perform *in vitro* assays without tumor experiments, whereas some others only performed tumor experiments without further carrying out serial transplantations. Such shortcomings have created a lot of confusions in the field and led many to even disbelieve the presence of CSCs. Recent lineage tracing studies in genetically driven mouse model tumors (i.e., glioblastoma and intestinal and skin tumors) have provided definitive evidence for CSCs (Rycaj & Tang 2015).

PCa stem cells

The CSC model helps explain the generation of tumor cell heterogeneity from the viewpoint of stem cell maturation and differentiation. PCa is well known to be a highly heterogeneous malignancy with each tumor harboring many tumor clones (Haffner *et al.* 2013, Cooper *et al.* 2015). Therefore, it is not surprising that many PCa stem cell (PCSC) populations have been reported (reviewed in Chen *et al.* (2013) and Rybak *et al.* (2015)). PCSCs are defined, more or less, using a spectrum of *in vitro* and *in vivo* assays used to define other CSCs (see above). *In vitro*, PCSCs preferentially express stem cell and CSC-associated molecules and self-renewal genes (e.g., Bmi1, Stat3, Nanog, Sox2, Oct4) and possess high clonal and clonogenic capacities, and *in vivo*, PCSCs possess higher tumor-initiating and serial tumor-propagating activities than non-PCSCs in immunodeficient mice (Chen *et al.* 2013, Kroon *et al.* 2013, Rybak *et al.* 2015). Three papers, published in 2005, simultaneously provided the earliest proof-of-principle evidence for PCSCs: the side population (SP) in the LAPC9 human xenografts was enriched in tumor-initiating cells (Patrawala *et al.* 2005); ABCG2, a surface pump protein normally involved in cellular detoxification, mediated efflux of androgen in putative PCSCs (Huss *et al.* 2005); and the CD44$^+$ $\alpha 2\beta 1^+$CD133$^+$ PCa cells from patient prostate tumors possessed high clonogenic survivability in methylcellulose (Collins *et al.* 2005).

Since 2012 our lab has been employing and developing a variety of experimental strategies to elucidate the cellulose basis and molecular regulation of PCa cell heterogeneity and to link PCa cell heterogeneity to therapy resistance and tumor relapse. In virtually all of our PCSC studies, we have performed tumor regeneration and, in many cases, serial tumor transplantation assays. Using the SP analysis, we provided the very first piece of evidence that the SP in certain PCa xenograft models is enriched in tumor-regenerating and tumor-propagating cells and thus satisfies the strict definition of CSCs (Patrawala *et al.* 2005). Using cell surface markers, our systematic studies have provided convincing evidence that the CD44 high-expressing (i.e., CD44$^+$) PCa cell population in most, though not all, PCa models we have studied is significantly enriched in PCSCs with enhanced tumor-regenerating, tumor-propagating, and metastatic capacities (Patrawala *et al.* 2006, 2007, Liu *et al.* 2011, 2015). Using holoclone assays, we have shown that the PCa cell holoclones, like stem cell-enriched primary keratinocyte holoclones, possess long-term tumor-propagating CSC properties (Li *et al.* 2008*a*). Using lentiviral-mediated lineage tracing, we have recently demonstrated that the phenotypically undifferentiated PCa cell population that lacks the expression of prostate-specific antigen (PSA; i.e., PSA$^{-/\mathrm{lo}}$) harbors self-renewing long-term tumor-propagating PCSCs, which express stem cell gene expression and epigenetic profiles, can undergo authentic asymmetric cell division, and are intrinsically refractory to castration treatments (Qin *et al.* 2012, Liu *et al.* 2015).

Similar to the heterogeneity of CSC populations in other tumor systems (Tang 2012), the PCSC pool is heterogeneous containing CSC subsets with distinct tumor-regenerating and tumor-propagating capabilities (Liu *et al.* 2015), potentially explaining many different PCSC populations reported by others (e.g., Collins *et al.* 2005, Miki *et al.* 2007, Dubrovska *et al.* 2009, Rajasekhar *et al.* 2011, Domingo-Domenech *et al.* 2012). Also similar to the undifferentiated nature of LSCs and other CSCs (Tang 2012), a common phenotypic trait of the reported PCSC populations is the lack of expression of differentiation regulators and markers such as androgen receptor (AR) (see below), PSA (Qin *et al.* 2012), and MHC molecules (Domingo-Domenech *et al.* 2012).

One of the major unresolved questions related to PCSCs is whether any subpopulation of PCa cells acutely purified from primary patient tumors or CRPCs truly possesses hardcore CSC properties such as regenerating tumors at the single-cell level and enabling serial tumor transplantations. Although patient tumor or early patient-derived xenograft (PDX) cells have been demonstrated in many experimental settings to possess at least certain CSC properties (especially *in vitro*), this question has dodged a direct answer mainly due to our current technical difficulty in reconstituting human PCa development in immunodeficient mice (Chen *et al.* 2013).

AR heterogeneity in PCa

AR is a master regulator of normal prostate differentiation and development. The human *AR* gene, located on chromosome Xq11-12, encodes a protein with four functional domains: the NH2-terminal domain (NTD), the DNA-binding domain (DBD), the hinge domain, and the ligand-binding domain (LBD) (Fig. 1). The prostate is one of the main organs that express AR, and the AR protein is expressed in the luminal cell layer of the prostatic glands. AR signaling critically regulates development, differentiation, and maintenance of the prostate as documented in both human and animal studies.

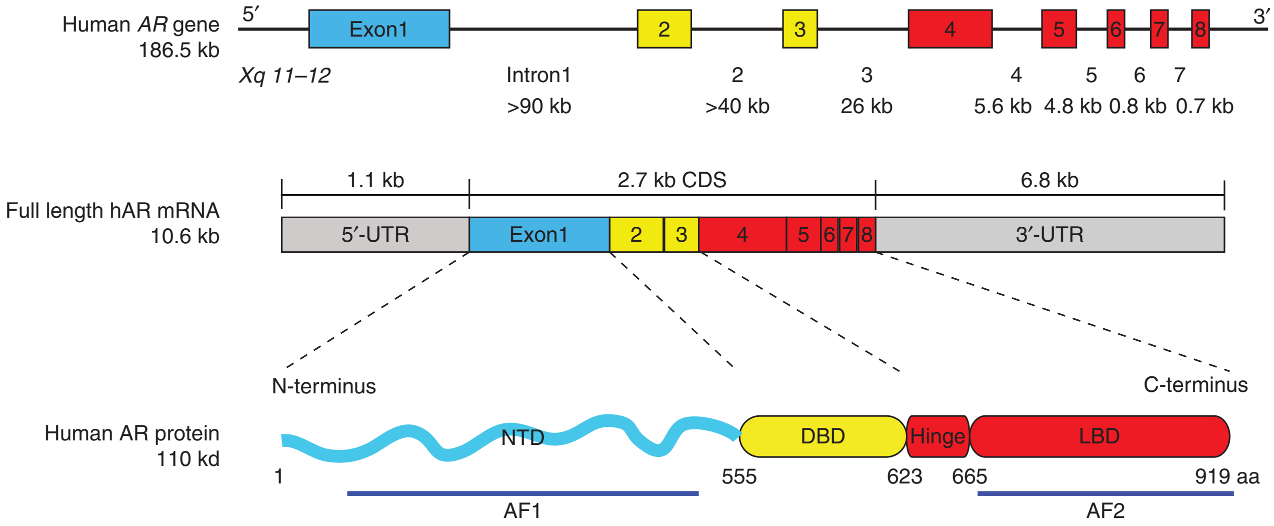

Figure 1

Genomic organization of the *AR* gene and overall domain structure of the androgen receptor (AR) protein. The *AR* gene is mapped to the long arm of the X chromosome and spans about 186.5 kb. It contains eight exons interrupted by introns of various lengths (indicated below). The mRNA of the *AR* gene is 10.6 kb with exon 1 coding for the NH2-terminal domain (NTD), exons 2 and 3 for the DNA-binding domain (DBD), and exons 4–8 for the hinge and ligand-binding domain (LBD). The full length AR protein contains 919 amino acids consisting of a very flexible NTD and a constant DBD, hinge domain, and LBD. The constitutively active AF1 domain is located in the NTD, and the LBD consists of the AF2 domain.

Somatic mutations of the *AR* gene lead to the malfunction of AR and androgen insensitivity syndrome in humans in which 46 XY individuals present female phenotype and the prostate is absent (Quigley *et al*. 1995). The *AR* NTD knockout male mice all have small immature testes and lack secondary reproductive organs (Kerkhofs *et al*. 2009).

Simanainen *et al*. (2007) established an *AR* exon 3 knockout mouse model and observed underdeveloped prostates in the male mice with delayed structural and functional differentiation of the prostate epithelium. There was also increased proliferation in the *AR* deficient epithelium (Simanainen *et al*. 2007). In another prostate-specific AR knockout mouse model, Wu *et al*. (2007) also reported increased proliferation and less differentiation of the epithelium. These genetic studies suggest that AR promotes prostate differentiation and suppresses epithelium proliferation in the mature prostate; in this way, AR signaling maintains the homeostasis and relative dormancy of mature prostate epithelium. Consistent with this pro-differentiation role of AR, the prostate epithelial-specific *AR* knockout promoted transgenic adenocarcinoma mouse prostate (TRAMP) tumor development, providing genetic evidence for a tumor-suppressive function of AR (Niu *et al*. 2008).

Somewhat paradoxically, however, AR expression is frequently overexpressed in PCa and, in fact, AR is thought to be required for prostate tumorigenesis and, hence, targeting AR and AR signaling has long been a therapeutic strategy. Androgen-deprivation therapy (ADT) aims to block androgen synthesis (e.g., abiraterone) or AR functions (e.g., bicalutamide, enzalutamide). Nevertheless, AR expression has been observed to be heterogeneous in primary and, in particular, treatment-failed patient tumors. Ruizeveld de Winter *et al*. (1990) examined AR by immunohistochemistry (IHC) staining in 26 primary PCas and found that 7 cases presented a considerable heterogeneity in AR expression and the proportion of AR-expressing cells was decreased in the more aggressive tumors. Similar AR IHC staining by Masai *et al*. (1990) showed that AR expression correlated inversely with grade. Also, Chodak *et al*. (1992) analyzed AR expression in 57 untreated PCas and observed that AR content was significantly higher in differentiated tumors compared to that of poorly differentiated tumors. Our own studies revealed AR^- PCa cells to be present in all nine primary PCa samples we examined representing approximately 5–30% of the total (Liu *et al*. 2015). Overall, these and many other studies suggest that, although AR^- cells may not be dominant in treatment naïve tumors, all primary prostate tumors nevertheless harbor both AR^+ and AR^- cells or clones (Fig. 2, bottom; Liu *et al*. 2015).

AR heterogeneity in hormone-refractory PCa has been observed since the early 1990s. van der Kwast *et al*. (1991) examined AR expression in CRPC and found that in 13 of 17 tumors, over 80% of the tumor cells were AR^+. However, three tumors showed a considerable heterogeneity in AR expression, and in one sample nearly all tumor cells appeared $AR^{-/lo}$. Sadi *et al*. (1991) observed similar AR heterogeneity in needle biopsy specimens of 17 patients with stage D PCa. Ruizeveld de Winter *et al*. (1994) examined AR expression in locally progressive CRPC and found that less differentiated PCa cells tended toward diminished AR expression. Computer quantification of nuclear AR levels in PCa patient samples showed that the AR concentration per cell was significantly more heterogeneous in poor responders (Sadi & Barrack 1993). Our own IHC staining of AR on a tissue microarray of CRPC samples revealed highly heterogeneous AR expression patterns across individuals: there were AR^+ as well as AR^- CRPC cores, and within one single CRPC, there were regions that were AR^+, AR^-, or a mixture of both populations (Liu *et al*. 2015).

AR expression varies in metastases as well. Shah *et al*. (2004) investigated AR expression by IHC in the metastatic lesions of 30 CRPC patients who underwent warm autopsy and observed wide variations in AR expression between tumor samples. Specifically, 31% (83 of 265) of the metastatic samples had $<50\%$ AR^+ cells and 41.5% (100 of 265) metastases had $<10\%$ AR^+ cells. Five patient metastases had $<1\%$ AR^+ cells (Shah *et al*. 2004). Similarly, Davis *et al*. (2006) reported that both AR^+ cells and AR staining intensity decreased in metastatic CRPC cells compared with benign tissues or untreated PCa. Of note, two commonly used PCa cell lines, Du145 and PC3, which were derived from brain and bone metastasis, respectively, and possess high tumorigenic and metastatic capacities, lack AR expression. ARCaP cells, derived from the ascites fluid of a disseminated CRPC, express little AR (Zhau *et al*. 1996). Bone metastases MDA PCa 118a/118b also completely lack AR (and PSA) expression (Li *et al*. 2008*b*).

Similar AR heterogeneity has also been observed in prostatic-specific transgenic mouse models. In a ARR_2Pb driven c-Myc (i.e., Hi-Myc) model (Ellwood-Yen *et al*. 2003), the residual tumors 5 months post-castration expressed low and heterogeneous levels of cytoplasmic AR compared to the intact mice. These castration-resistant Hi-Myc tumor cells were also quiescent as shown by negative Ki67 staining (Ellwood-Yen *et al*. 2003). In a prostate-specific *Pten*-deleted mouse prostate, although

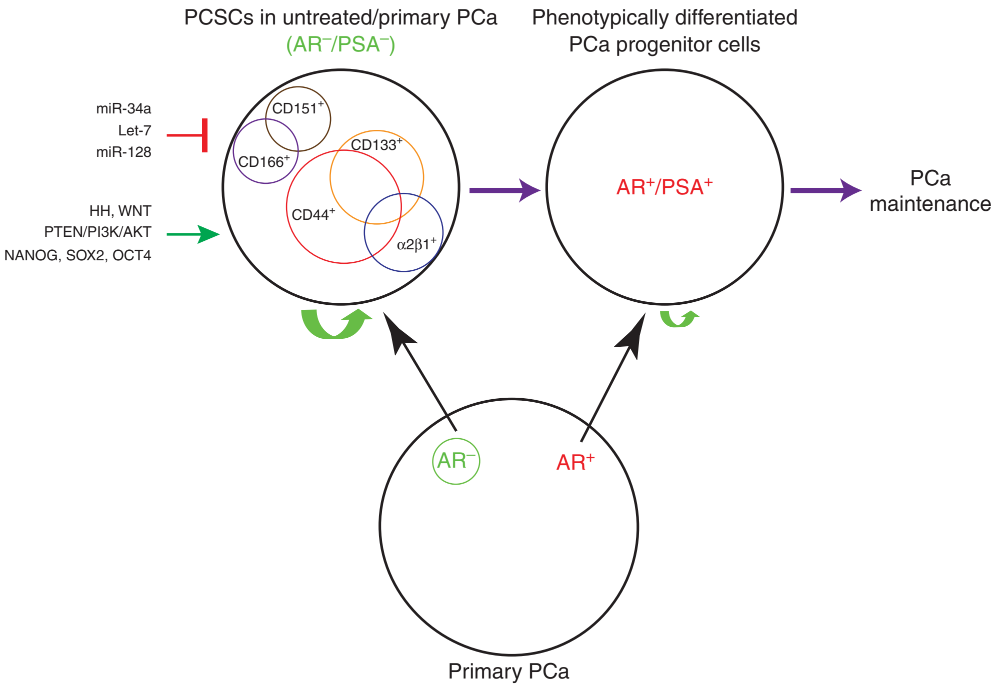

Figure 2

Prostate cancer stem cells (PCSCs) in untreated/primary prostate cancer (PCa). Primary PCa contains androgen receptor (AR)$^+$ PCa cells as the majority and AR$^-$ PCa cells being the minority (below). Depicted on top (left) are several representative PCSC populations reported in primary PCa and untreated prostate tumor models, which are mostly AR$^-$ and PSA$^-$ but have the capacity to differentiate into more mature AR$^+$/PSA$^+$ PCa cells (right). The PSA$^{-/lo}$ PCSC population has unlimited self-renewal potential (indicated by a large green arrow), whereas differentiated AR$^+$/PSA$^+$ PCa progenitors cells have more limited self-renewal activity (indicated by a small green arrow) (Qin *et al*. 2012, Liu *et al*. 2015). The PCSCs can be positively regulated through HH (Hedgehog), WNT, and PTEN signaling pathways, as well as by transcription factors such as NANOG, SOX2, and OCT4. On the other hand, several miRNAs including miR-34a, let-7, and miR-128 have been reported to negatively regulate PCSCs.

most tumor cells expressed AR after 10 weeks' castration, the expression level was weaker and more diffuse compared to the hormonally intact prostate (Wang *et al*. 2003).

AR heterogeneity in CRPCs has a genetic basis. A recent sequencing study of 150 metastatic PCa and CRPCs suggests that genetic alterations of AR (mutations, amplifications) (approximately 63% patients) become enriched in CRPCs compared to those in untreated tumors (Robinson *et al*. 2015). In addition to mutations in *AR* itself, alterations of members in the AR signaling pathway were also observed in metastatic CRPCs, including FOXA1 and NCOR1/2, among others. Similarly, by comparing 50 lethal CRPCs and 11 primary cancers, Grasso *et al*. (2012) identified mutations in FOXA1 and MLL2 in CRPCs that likely change the AR signaling in treatment-failed tumors.

PCSCs in primary and untreated PCa: AR negativity and signaling mechanisms

The preceding discussions highlight the presence of AR$^-$ PCa cells in untreated PCa (Liu *et al*. 2015). This is an important point as the AR$^-$ PCa cells are expected to not respond well to AR-targeting therapies. This point would be consistent with reports that androgen-independent PCa cells preexist in primary tumors, which may become selected during ADT (Issacs & Coffey 1981, Fiñoes *et al*. 2013, Liu *et al*. 2015). Interestingly, in many reported PCSC populations in untreated PCa models or primary tumors, AR expression is often low or undetectable (Fig. 2). For example, the CD44$^+$α2β1$^+$CD133$^+$ cells purified from seven human tumor samples (Collins *et al*. 2005), the ABCG2$^+$ putative PCSCs (Huss *et al*. 2005), and the CD44$^+$ cells in several PCa xenografts (Patrawala *et al*. 2006) were all AR$^-$. In fact, the AR$^-$CD44$^+$ PCSCs were shown to be able to differentiate, at the clonal level, into AR$^+$CD44$^-$ cells (Patrawala *et al*. 2006). Gu *et al*. (2007) also showed that the human prostate epithelial cells immortalized by overexpressing hTERT (HPET cells)-expressed stem cell molecules, such as CD44 and Nanog, could regenerate three prostate epithelial cell types and were AR negative. Miki *et al*. (2007) showed mutually exclusive expression patterns of CD133 and AR by IHC

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staining in 16 clinical specimens. Rajasekhar *et al*. (2011) reported both AR and PSA negativity in the TRA-1-60^+CD$151^+$and CD166^+ PCSC population, which possessed high tumorigenic ability and could generate differentiated AR^+ and PSA^+ tumors *in vivo*. The docetaxel-resistant PCSCs that lacked the expression of MHC molecules were also negative for AR and PSA (Domingo-Domenech *et al*. 2012). Likewise, the $PSA^{-/lo}$ PCSC population was enriched in AR^- PCa cells (Qin *et al*. 2012, Liu *et al*. 2015). These and many other studies (reviewed in Liu *et al*. 2015) suggest that PCSCs in primary and untreated tumors seem to be generally AR-; in other words, AR^- (and PSA^-) cells are highly enriched in primary and/or untreated PCSC populations (Fig. 2). Vice versa, loss of AR expression has been shown to promote PCSC generation through SATA3 signaling (Schroeder *et al*. 2014). It remains to be seen whether the AR^+ and AR^- PCa cells in untreated/primary PCa possess distinct self-renewal, tumor-propagating properties, and drug sensitivities as these two populations of PCa cells have not been prospectively separated, purified out, and compared for their biological properties.

PCSCs in untreated PCa remain AR^- presumably because these cells are simply less differentiated. Alternatively, molecules such as ABCG2 are preferentially expressed in PCSCs (Huss *et al*. 2005), which mediates the efflux of androgens leading to the degradation of ligand-less AR in PCSCs. We have shown that at least some of the PCSCs (e.g., SP, $CD44^+$, $ABCG2^+$, and $PSA^{-/lo}$) have been able to self-renew based on serial tumor-transplantation assays and asymmetric cell divisions using clonal and time-lapse analyses (Patrawala *et al*. 2005, 2006, Qin *et al*. 2012, Liu *et al*. 2015). A fraction of $PSA^{-/lo}$ PCa cells can undergo authentic asymmetric cell division regenerating a $PSA^{-/lo}$ daughter cell as well as a differentiated PSA^+ cell, which subsequently undergoes rapid proliferation (Qin *et al*. 2012, Liu *et al*. 2015). Self-renewal is a shared property for both normal stem cells and CSCs, and, not surprisingly, many molecules and pathways that regulate self-renewal in normal stem cells have been reported to operate in PCSCs (Fig. 2). For example, we have shown that NANOG is preferentially expressed in several PCSC populations and its expression is important for CSC properties as its knockdown severely impairs tumor regeneration (Jeter *et al*. 2009). In contrast, inducible expression of NANOG alone is sufficient to reprogram bulk cancer cells into stem-like cancer cells with enhanced tumor-regenerating and tumor-propagating activities (Jeter *et al*. 2011). Our results suggest that certain pluripotency molecules may also be functionally important for PCSC self-renewal and other properties. In support, several other studies have similarly implicated OCT4 and SOX2 in conferring on PCSC activities (Linn *et al*. 2010, Kregel *et al*. 2013). Interestingly, reciprocal relationships between AR and NANOG, OCT4, and SOX2 have been noted in these studies.

Hedgehog (HH) and WNT signaling often act together and play important roles in regulating self-renewal. The importance of WNT/β-catenin signaling is illustrated by the observations that treatment of LNCaP and C4-2 cells with WNT-3a increased their sphere formation rate and size, with increased nuclear β-catenin accumulation (Bisson & Prowse 2009). Although AR antagonist bicalutamide reduced the sphere size, the sphere formation rate did not change, thus suggesting a role of WNT signaling in PCSC self-renewal independently from AR (Bisson & Prowse 2009). Bmi1 acts downstream of HH and has been shown to be necessary for self-renewal of several populations of normal stem cells as well as CSCs (Lessard & Sauvageau 2003, Park *et al*. 2003). Lukacs *et al*. (2010) investigated the effects of Bmi1 loss in the presence of overactivated Wnt signaling on murine prostate stem cells (PSCs) and demonstrated that Bmi1 expression was required for the Wnt pathway to modulate self-renewal in the PSCs. In addition, several other signaling molecules and pathways may also be involved in regulating PCSCs. For example, the PTEN/PI3K/AKT pathway has been reported to be essential for PCSC proliferation independent of AR status (Dubrovska *et al*. 2009).

The E-twenty-six (Ets)-related gene (ERG), which is essential to maintain adult HSC self-renewal during stress-induced hematopoiesis (Loughran *et al*. 2008, Ng *et al*. 2011), is deregulated in most PCa through the most common genetic event TMPRSS2-ERG fusion (Tomlins *et al*. 2005, Mosquera *et al*. 2009). TMPRSS2-ERG expression is associated with a relative increase in clonogenic PCa cells (Casey *et al*. 2012). Interestingly, although the expression of the TMPRSS2-ERG fusion gene is expected to occur in AR^+ PCa cells due to the TMPRESS2 regulation by AR, recent evidence suggests that the TMPRSS2-ERG fusion protein may also be expressed in the AR^- PCSCs. Polson *et al*. (2013) demonstrated that in $CD133^+\alpha2\beta1^+$primary tumor cells with stem cell properties, TMPRSS2-ERG and AR expression was not necessarily concordant. While most of the marker-positive cells were AR negative, they expressed ERG at both RNA and protein levels, which may help maintain the PCSC properties such as self-renewal in the marker positive cells (Polson *et al*. 2013).

Taken together, the above discussions indicate that many well-known signaling molecules and pathways can regulate and confer the CSC properties in AR$^-$ PCSCs (Chen *et al.* 2013, Rybak *et al.* 2015). These molecules and pathways represent obvious therapeutic targets, and therapeutics targeting these PCSC-specific signaling nodes could, in principle, be utilized in conjunction with the ADT regimens.

PCSCs in CRPC might be AR$^+$ or AR$^-$

It is well appreciated that AR heterogeneity becomes more pronounced in CRPCs than in the primary tumors (Liu *et al.* 2015) and activation of alternative AR signaling in PCa cells may promote PCa cell proliferation under androgen-deprived environment (Wang *et al.* 2009*a*). What is the cell of origin of CRPCs? AR$^+$ or AR$^-$ PCa cells? As early as 1981, Isaacs and Coffey (1981), working on the Dunning R3327H rat prostatic adenocarcinoma model, proposed that castration selected for androgen-insensitive cells that preexisted in the untreated tumors. Craft *et al.* (1999), working on the LAPC9 xenograft model, also provided histological evidence for the outgrowth of the androgen-independent clones in the later stages of CRPC development. Fiñones *et al.* (2013) demonstrated androgen-independent PCa cells in untreated early-stage prostate adenocarcinomas. These androgen-independent and androgen-insensitive PCa cells may not necessarily be AR$^-$, because PCa cells that overexpress AR and splice variants that lack the LBD may also be insensitive or refractory to androgen ablation. Our recent work provided direct evidence of AR$^-$ PCa cells in primary patient tumors (Liu *et al.* 2015). As many PCSCs have been shown to be AR$^-$ and resistant to castration and other therapeutics (Qin *et al.* 2012, Chen *et al.* 2013, Liu *et al.* 2015, Rybak *et al.* 2015), it is reasonable to postulate that the AR$^-$ PCa cells that preexist in untreated tumors could be favored as 'initiators' or the cells of origin of CRPCs (Fig. 3). These AR$^-$ PCa cells could be expanded on the ADT-induced elimination of AR$^+$ cells as well as due to the de-differentiation from AR$^+$ PCa cells (Fig. 3), much like therapy- or microenvironment-induced de-differentiation of non-CSCs in other tumor systems (Tang 2012, Kreso & Dick 2014). As a result, the AR$^-$ PCa cells in CRPCs may function as the CSCs for the AR$^-$ CRPC clones (Fig. 3). The best example is the PSA$^{-/\text{lo}}$ PCSC population, which has been evinced to possess significant tumor-regenerating and tumor-propagating activities in fully castrated male mice (Qin *et al.* 2012). Germann *et al.* (2012) showed that PCa cells expressing stem cell markers such as ALDH1A1 and NANOG became enriched in the BM18 castration model, and the castration-resistant stem-like PCa cells had a luminal progenitor phenotype but were negative for AR. Jiao *et al.* (2012) identified a CD166$^+$cell population in both human and mouse CRPCs, which was enriched in basal stem/progenitor cells that were CK5$^+$/p63$^+$/CK8$^+$/AR$^-$/TROP2$^{\text{hi}}$ and displayed enhanced sphere formation and tissue regeneration abilities. Also, studies on NANOG (Jeter *et al.* 2009, 2011) and SOX2 (Kregel *et al.* 2013) show that PCa cells expressing these molecules are castration resistant and express relatively low levels of AR. These observations raise the possibility that the AR$^-$ PCSCs may gain growth advantages in an androgen-deficient environment, leading to distinct AR$^-$ clones in CRPC (Fig. 3).

On the other hand, most CRPCs clearly have AR$^+$ cells and clones (Liu *et al.* 2015). Although these AR$^+$ cells in CRPCs can potentially be derived from the differentiation of AR$^-$ PCa cells (Fig. 2), it is very likely that at least some AR$^+$ PCa cells can survive androgen deprivation and function as the cells of origin as well as CSCs for CRPCs (Fig. 3). This is not very difficult to understand because the AR$^+$ PCa cells in most untreated primary tumors constitute the bulk cell population (Fig. 2). It is conceivable that due to their abundance, some of these AR$^+$ PCa cells, under the selective pressure from androgen deprivation, may selectively gain genetic alterations such as the *AR* gene amplification and TMPRSS2-ERG fusion, resulting in the expansion of AR$^+$ clones (Fig. 3, right). In the resultant AR$^+$ PCa cell clones, AR may likely be still functioning to regulate both conventional as well as new AR target genes (Wang *et al.* 2009*a*). The regulation of conventional AR targets can be achieved through intratumoral androgen synthesis. Alternatively, AR signaling in the AR$^+$ CRPC clones may be executed through ligand-independent AR splice variants and/or AR crosstalks with activated receptors such as the epidermal growth factor receptor (EGFR). In fact, there is evidence that certain AR$^+$ cell populations are refractory to castration and can function as the cell of origin for PCa in mouse models. Wang *et al.* (2009*b*) showed that castration-resistant Nkx3.1-expressing cells (CARNs) that expressed luminal markers including AR represented a rare population of androgen-resistant cells in the murine prostate that could function as the cells of origin for PCa caused by *Pten* deletion.

Interestingly, expressing wild-type AR at physiological levels in AR$^-$ PC3 cells induced growth inhibition (Litvinov *et al.* 2006), whereas knocking down AR in AR-expressing metastatic PCa cells like LNCaP and its

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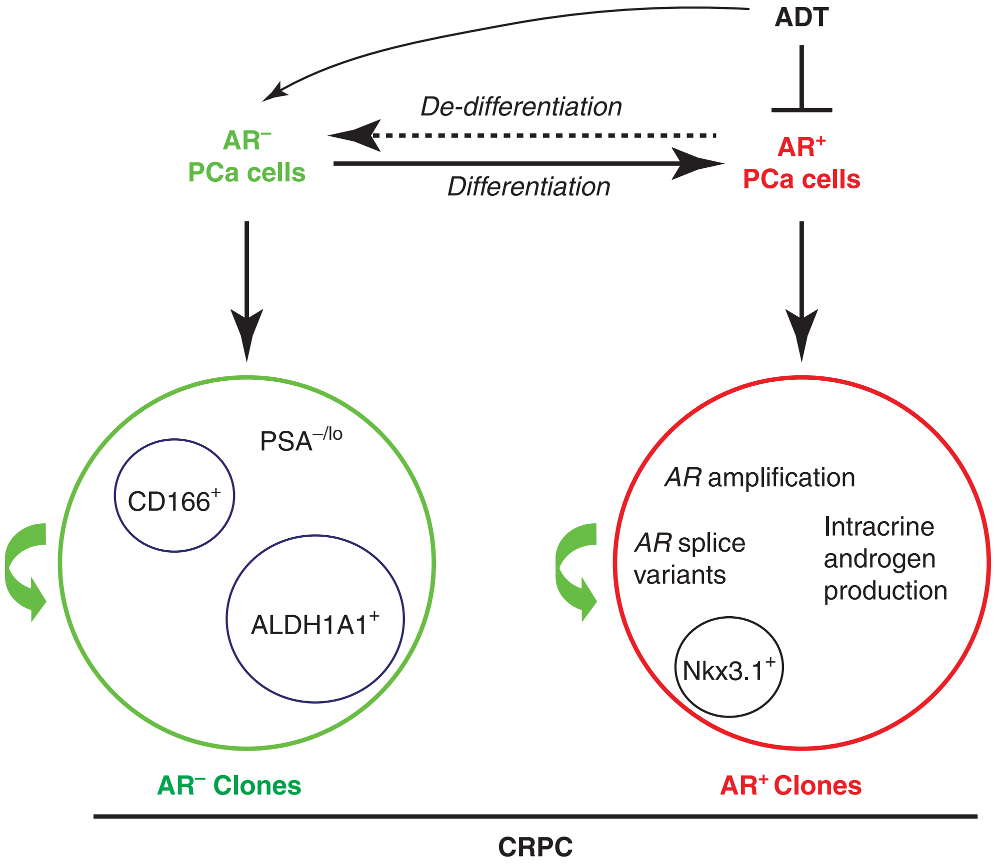

Figure 3

Hypothetical PCSCs in CRPC. Androgen-deprivation therapy (ADT) selectively targets androgen receptor (AR)$^+$ prostate cancer (PCa) cells and has been shown to enrich AR$^-$ PCa cells, which may result from preferential elimination by ADT of AR$^+$ cells as well as de-differentiation of AR$^+$PCa cells to AR$^-$cells (top). Clinical castration-resistant PCas (CRPCs) contain distinct AR$^+$ and AR$^-$ clones, both of which might contain their own CSCs. In AR$^+$clones, PCSCs could have *AR* amplification or ligand-independent AR signaling pathways to support the self-renewal in an androgen-deprived environment. Several potential cancer stem cell (CSC) subpopulations in AR$^-$ and AR$^+$ PCa cell clones are indicated.

derivative C4-2 resulted in growth inhibition and apoptotic cell death and compromised tumor development (Cheng *et al*. 2006, Snoek, *et al*. 2009). The contrasting roles of AR in AR$^-$ vs AR$^+$ PCa cell lines imply differential involvement of AR in AR$^+$ and AR$^-$ PCSCs in CRPCs. Regardless, the phenotype of PCSCs in CRPCs may well be context dependent, and both AR$^+$ and AR$^-$ clones, which possess their own intraclonal CSCs, likely coexist in hormone-refractory tumors (Fig. 3). The development of critical experimental tools that can allow the prospective separation of AR$^+$ and AR$^-$ CRPC cells is needed to clarify the precise functions of AR$^+$ vs AR$^-$ PCSCs in CRPC.

AR and PCSCs in PCa metastasis

Metastasis is common in CRPC patients. The acquisition of invasive properties through epithelial-mesenchymal transition (EMT), a normal development process, is crucial for the evolution of metastatic populations (Tam & Weinberg 2013, Puisieux *et al*. 2014). There is accumulating evidence supporting the fact that ADT may induce an EMT in PCa cells (Jennbacken *et al*. 2010, Tanaka *et al*. 2010, Sun *et al*. 2012, 2014, Wu *et al*. 2012, Jacob *et al*. 2014), and EMT is well known to promote CSC traits. Studies by Tanaka *et al*. (2010) and Jennbacken *et al*. (2010) showed that N-cadherin was upregulated in castration-resistant LNCaP, LAPC4, and LAPC9 xenograft models. Sun *et al*. (2012) interrogated EMT marker expression in mouse and human CRPC samples and observed overall higher levels of mesenchymal markers in CRPC compared to non-castrated samples. They proposed a negative feedback loop model between ZEB1 and AR to explain the ADT-induced EMT. To some extent, AR signaling may be involved in the EMT switching in PCa cells. The study on AR and ZEB2 suggests that AR may function differently between AR$^+$ and AR$^-$ cell lines (Jacob *et al*. 2014).

Specifically, ZEB2 expression positively correlate with AR expression in LNCaP cells, but the opposite is true in PC3 and DU145 cells. In addition, the AR splice variants AR3 and ARv567es were shown to promote EMT in PCa cells (Wu *et al.* 2012, Sun *et al.* 2014).

CSCs not only play an important role in tumor initiation and treatment resistance but also seem to be involved in distant metastases. Tanaka *et al.* (2010) have shown that the castration-resistant, N-cadherin positive PCa cells are enriched in stem cell markers including CD44 and NANOG. *Vice versa*, $\text{Lin}^-\text{CD44}^+\text{CD133}^+\text{Sca-1}^+$ CD117^+ mouse PSCs express higher levels of mesenchymal markers N-cadherin and vimentin compared to the non-stem cells (Sun *et al.* 2012). On the other hand, EMT may also suppress the stemness in PCa cells (Celia-Terrassa *et al.* 2012). This is not entirely surprising because mesenchymal-epithelial transition (MET) is equally important and required for metastatic colonization. Research on the role of MET in PCa metastasis is very limited.

Clinical implications and perspectives

Studies about the potential prognostic role of AR in PCa are controversial, and most evidence suggests that AR is not prognostic in PCa (Ford *et al.* 2003, Fleischmann *et al.* 2011, Minner *et al.* 2011, Tamburrino *et al.* 2012, Lu-Yao *et al.* 2014). Minner *et al.* (2011) examined the AR expression in more than 2800 treatment-naïve PCa patient samples and observed no significant correlation between the AR expression level and the risk of biochemical recurrence. Studies by Fleischmann *et al.* (2011) of 382 lymph node metastases showed that AR is not prognostic in node positive PCa although higher AR does correlate with a larger size of metastases. Despite significant improvements in the efficiency of the ADT to block AR signaling, up to now, there is also no clear correlation between androgen signaling ablation and patient prognosis. A study by Ford *et al.* (2003) in 24 CRPC patients showed that 33% of patients have AR amplification and the patients with AR gene amplification had a recurrence 5 months earlier than those without amplification; however, no statistically significant survival disadvantage was observed in the AR amplified patients. More recently, Lu-Yao *et al.* (2014) performed a median 110 months follow-up study of a cohort consisting of 66 717 PCa patients who underwent primary ADT or conservative management and found that primary ADT was not associated with improved long-term overall or disease-specific patient survival. Furthermore, the AR heterogeneity in PCa indicates that targeting AR signaling alone may be of a limited role in preventing disease recurrence in the long term.

PCSCs may represent the driving force of tumor progression and metastases. A number of studies have shown that the expression of stem-cell markers has prognostic significance in PCa, as well as other cancer types (Kakarala & Wicha 2008, Li, *et al.* 2010). Studies on $\text{PSA}^{-/\text{lo}}$ PCSCs suggest that intratumoral PSA expression is inversely correlated with the tumor Gleason score and patient survival (Qin *et al.* 2012). Multiple studies have shown that the AR^- tumor cells are enriched in PCSC populations, implicating a pivotal role of PCSCs in ADT resistance. Hence, targeting PCSCs specifically in an adjuvant setting might be helpful in preventing CRPC. Preclinical studies in PCSCs targeting have provided promising results. For instance, we have demonstrated that microRNA-34a (miR-34a) potently inhibits the PCa progression and metastasis via directly targeting CD44 (Liu *et al.* 2011). We have also reported several other microRNAs including let7b and miR-128 in suppressing PCSC self-renewal and tumor progression (Liu & Tang 2011, Liu *et al.* 2012, Jin *et al.* 2014). At the same time, direct inhibition of WNT, PTEN/PI3K/AKT, and others cell-signaling pathways has shown tumor suppressive effects via lowering PCSCs population (e.g., Dubrovska *et al.* 2010, Rybak *et al.* 2015).

Understanding and elucidating the roles of and the interrelationship between AR heterogeneity and PCSCs could offer fresh insight on designing novel therapeutics to target lethal CRPC and metastasis. Recent evidence suggests that in untreated tumors, PCSCs seem to be largely AR^-,whereas in CRPCs, PCSCs may be either AR^+ or AR^-. In other words, both AR^+ and AR^- PCa cell clones coexist in most CRPCs (Fig. 3). In principle, PCSCs, whether AR^+or AR^-, are endowed with the fundamental trait of stemness, which is regulated by unique cohorts of genes, epigenetic landscape, and environmental factors (Kreso & Dick 2014). It is high time for us to develop novel therapeutics that target the stemness of PCSCs, which, when used in conjunction with ADT, should help prevent tumor recurrence.

Declaration of interest

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

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Author contribution statement

Q Deng and D G Tang conceptualized the paper; Q Deng wrote the draft; D G Tang finalized the manuscript.

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