The complex interactions between the tumor microenvironment and prostate and oropharyngeal cancer

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CHAPTER 2

Androgen Receptor Moonlighting in the Prostate Cancer Microenvironment

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ABSTRACT

Androgen receptor (AR) signaling is vital for normal development of the prostate, and is critically involved in prostate cancer (PCa) progression. AR is not only found in epithelial prostate cells, but is also expressed in various cells in the PCa-associated stroma, which constitute the Tumor MicroEnvironment (TME). In the TME, AR is expressed in fibroblasts, macrophages, lymphocytes, and neutrophils. AR expression in the TME was shown to be decreased in higher grade and metastatic PCa, suggesting that stromal AR plays a protective role against PCa progression. With that, the functionality of AR in stromal cells appears to deviate from the receptor's classical function as described in PCa cells. However, the biological action of AR in these cells and its effect on cancer progression remains to be fully understood. Here, we systematically review the pathological, genomic and biological literature on AR actions in various subsets of prostate stromal cells, with the aim to better understand the consequences of AR signaling in the TME in relation to PCa development and progression.

PROSTATE CANCER

PCa is the second-most frequently diagnosed tumor type in men worldwide and the most-common male malignancy in developed countries [1]. Annually, there are an estimated 1.1 million new prostate cancer cases worldwide and 300.000 cancer-related deaths [2]. The main risk factors for PCa are ethnicity, family history and genetic predisposition. Moreover, prevalence increases with age, with the highest incidence between 70 and 75 years of age [3].

The majority of patients present with localized disease, in which the tumor is confined to the prostate. PCa is often detected after the development of lower urine tract complaints, while an increasing percentage of patients are being diagnosed before developing any symptoms as a result of Prostate-Specific Antigen (PSA) testing [4] . PSA is a serine protease, specifically secreted by epithelial prostate cells, which remains expressed in PCa cells. Likelihood of recurrence is commonly estimated by the TNM classification of malignant tumors (tumor, lymph nodes involvement and metastasis) and the Gleason Score (GS); both prognostic scores are based on histopathological features of the tumor.

Treatment options largely depend on stage and grade of the disease, as well as age, health condition and expected life-span of the patient. For primary local treatment, a choice is made between radiotherapy and radical prostatectomy. Radical prostatectomy can be combined with extended pelvic nodal dissection [5], while radiotherapy can be combined with adjuvant Androgen Deprivation Therapy (ADT) [6]. Both treatment modalities are considered equally effective in curing the disease at first [7-9], however, approximately 35% of patients will develop a rise in PSA and a smaller proportion will develop metastatic disease [10]. These patients cannot be cured, but the disease can be treated with ADT to which virtually all patients will respond.

THE PROSTATE CANCER MICROENVIRONMENT

Stromal cells present in an organ are all non-epithelial cells that jointly constitute the connective tissue. During normal tissue development, epithelial-stromal interactions are fundamental in order to maintain organ homeostasis. In PCa, the tumor is surrounded by a large variety of stromal cells including resident fibroblasts, myofibroblasts, endothelial cells as well as innate and adoptive immune cells [11]. Apart from cells, tumors are influenced by soluble factors such as cytokines and other extracellular molecules that constitute the ExtraCellular Matrix (ECM). Components of the ECM are secreted by both tumor and stromal cells, and they can regulate tumor cell proliferation and migration [12]. Moreover, cytokines released in the TME can control stromal cell polarization towards tumor-suppressive or tumor-promoting phenotypes [13].

In multiple tumor types, including PCa, tumor-associated stromal cells are considered to be highly plastic compared to the normal-associated stromal cellsn [14-16]. During PCa development and progression, stromal cells show an altered phenotype which leads to an increased ECM remodeling, angiogenesis, protease activity and immune cells infiltration [17]. Tumor-associated stromal cells have been shown to undergo genetic alterations in the presence of a tumor, which might sustain the malignant phenotype [18].

Many studies have addressed the microenvironment as a prognostic factor in PCa [19-22]. Moreover, the TME gained a lot of interest as a therapeutic target over the last decades. Targeting various components of the TME might represent an alternative approach as compared to the classical therapies targeting cancer cells. This is an attractive concept, since (in contrast to tumor cells) stromal cells are normally regulated and do not show genetic instability. Based on this concept, new potential drugs targeting the crosstalk between cancer cells and stromal cells, such as Src kinase inhibitors, TGF-ß inhibitors, and angiogenesis such as the vascular endothelial growth factor (VEGFR) inhibitors are now being tested in clinical trials [23-25].

AR EXPRESSION IN PCA CELLS

Nuclear receptors such as AR, but also estrogen receptor (ER), glucocorticoid receptor (GR) and progesterone receptor (PR) are all expressed in prostate tissue and play a role in PCa development and progression [26-28]. AR is expressed in the epithelial cells of primary and metastatic PCa and regulates a variety of cellular functions [29]. AR is a steroid hormone receptor located on chromosome Xq12 and a member of the nuclear receptor family [30]. Its transcript comprises of 8 exons and 3 functional domains: the N-terminal transactivation domain (NTD) in exon 1, the DNA-binding domain (DBD) in exon 2 and 3, and the C-terminal ligand-binding domain (LBD) in exon 5 to 8. The AR transcript is translated into an 110kDa ligand-dependent transcription factor [31] that plays a critical role in PCa development and progression by regulating the transcription of genes involved in cell proliferation, migration, differentiation, cell cycling, and apoptosis [29].

After entering the target cell, testosterone is converted into dihydrotestosterone (DHT) by 5-α-reductase, which has a high affinity for AR (Figure 1). Upon DHT binding, AR dissociates from the heat-shock protein 90 complex (Hsp90) and undergoes intra-molecular conformational changes at the N- and C-terminaI of the receptor (N/C interactions) [32]. Consequently, the AR-DHT complex translocates into the nucleus where the receptor dimerizes [33, 34], and binds the DNA at Androgen Responsive Elements (ARE) of promoter and enhancer regions of various target genes [35].

AR/DNA binding is mediated by pioneering transcription factors (TFs) including Forkhead Box Protein A1 (FOXA1), GATA Binding Protein 2 (GATA2) and Homeobox Protein 13 (HOXB13), that render ARE regions accessible for AR to bind [36]. Subsequently, a variety of co-regulators are recruited to the complex that can either activate (co-activators, e.g. steroid receptor co-activator 1 (SRC-1), Androgen Receptor Co-activator 70-alpha (ARA70-alpha) [37, 38] or repress (co-repressors, e.g. Flightless I (FLI1), Nuclear Receptor Co-Repressor 1 (NCoR1) [34] the expression of downstream-responsive genes.

AR AS A THERAPEUTIC TARGET

Since AR signaling modulates the expression of critical genes involved in PCa proliferation and migration, inhibiting AR action is the mainstay of anti-hormonal treatment for both metastasized and adjuvant treatment [39]. Understanding the molecular mechanisms of AR

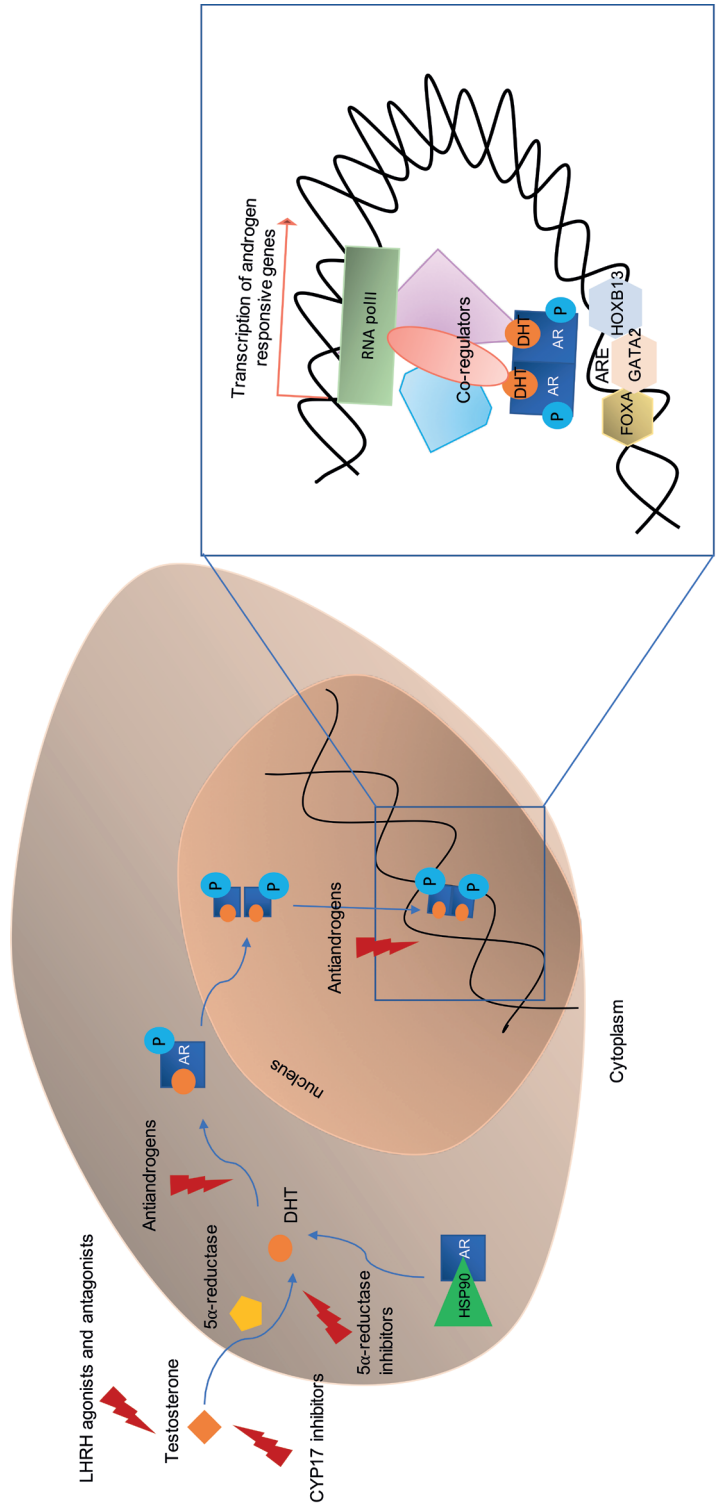

Figure 1. Schematic view of AR genomic function in PCa) cells. Testosterone diffuses into the target cell, where it is converted in the cytosol into dihydrotestosterone (DHT) by 5α-reductase. Subsequentially, DHT binds AR and induces the dissociation of Heat Shock protein 90 (HSP90). DHT binding to AR promotes translocation into the nucleus, where AR dimerizes and binds the chromatin at Androgen Responsive Elements (AREs). AR binding at AREs is facilitated by other Transcription Factors (TFs) that regulate chromatin accessibility, such as FOXA1, GATA2 and HOXB13. Finally, chromatin-bound AR recruit's co-regulators that promote the interaction and activation of RNA polymerase II (RNA pol-II) to regulate transcription of AR-responsive genes. Therapeutic interventions with anti-hormonal drugs are aimed to block the various steps of the AR genomic cascade: LHRH agonists and antagonists lower the levels of testosterone synthesized in the testicles; CYP17 inhibitors interfere with the biosynthesis of androgens in the gonads and adrenal glands; 5'-reductase inhibitors blocks the testosterone-DHT transformation by inhibiting the 5'-reductase enzyme; and anti-androgens bind AR and prevent the ligand-mediated AR translocation into the nucleus and binding to the DNA.

function is essential to develop new drugs targeting any of the steps of the AR signaling cascade. Currently, multiple drugs targeting these steps in the AR pathway are introduced in clinical practice or are in clinical development (Table 1). ADT is achieved by physical castration or Luteinising Hormone-Releasing Hormone (LHRH) agonists and antagonists. The latter two interventions lower the level of the testosterone produced by the testicles by inhibiting the production of Luteinizing Hormone (LH) from the pituitary gland [40] Furthermore, antiandrogen treatment is commonly prescribed to PCa patients. Antiandrogens such as bicalutamide, flutamide and enzalutamide act by directly blocking AR function, while others such as the CYP17A1 inhibitors ketoconazole and abiraterone acetate inhibit extragonadal and intratumoral synthesis of androgens [41]. Despite the very high response rate to AR targeting interventions in metastatic PCa patients, progression into metastasized Castration Resistant Prostate Cancer (mCRPC) is inevitable, which is hallmarked by high morbidity and mortality [42]. In contrast to the previous believe that PCa developed a hormone-refractory stage [43], we now know that prostate cancer cells develop a hypersensitivity to testosterone [44], resulting in activation of the AR cascade at castrate levels of circulating hormones. In this mCRPC stage of the disease, AR remains expressed as a driver of disease progression [45]. Enzalutamide and abiraterone have shown clinical activity in mCRPC in combination with ADT [46, 47], which confirms continued androgen dependence of PCa cells in this stage of the disease. Apart from further anti-hormonal interventions, also taxanes and radio nucleotides have shown activity in mCRPC in combination with ADT [48].

The underlying mechanism of this high sensitivity to testosterone in mCRPC has been unraveled in recent years. Altered AR functions commonly occur, which are thought to develop during continued selection pressure induced by treatment [49]. The molecular mechanisms by which impaired AR activity is associated with PCa development and progression is complex and includes AR amplification, constitutive active AR splice variants and extra testicular testosterone synthesis [34, 49-52], overexpression of AR cofactors [53, 54], gain-of-function AR mutations in the LBD [55] and intracrine androgen production [56]. Alternative spliced AR variants represent a key factor in resistance to hormonal intervention and are often found in mCRPC [57]. One of the best characterized AR variant is AR-V7 (AR3), which is composed only of exon 1 to 3 which encode the NTD and DBD and is therefore capable of DNA binding [58]. However, it is ligand-independent and constitutively active. AR-V7 was significantly up-regulated during PCa progression and expression was correlated with disease recurrence after radical prostatectomy [59]. Importantly, overexpression of AR-V7 in circulating tumor cells was associated with resistance to androgen ablation treatments in PCa patients [60]. Currently, occurance of AR-V7 in circulating tumor cells is being validated as a predictive biomarker for antihormonal treatment insensitivity [61].

Table 1. Hormone therapy for prostate cancer patients: established drugs and clinical trials. On the left side of the column, established treatments to lower the androgen levels (Androgen biosynthesis blockade, LHRH agonist and LHRH antagonists), anti-androgen therapies (Androgen receptor blockade and AR-targeted mustard conjugates) currently applied to treat PCa patients are listed. On the right side of the column, drugs currently in clinical trials are listed. These include the new interventions: AR NTD blockade and androgen therapy.

Established therapies	In clinical trial
Androgen biosynthesis blockade	
Ketoconazole [165]	TAK-700 [166]
Abiraterone [167]	
Androgen receptor blockade	
Bicalutamide [168]	Galeterone [169]
Nilutamide [170]	ARN509 [171]
Flutamide [172]	ODM-201 [173]
Enzalutamide [174]	
LHRH agonists	
Goserelin [175]	
Histrelin [176]	
Leuprolide [177]	
Triptorelin [178]	
LHRH antagonists	
Degarelix [179]	
Abarelix [180]	
AR NTD blockade	
-	EPI-506 (NCT02606123)
AR-targeted mustard conjugates	
Estramustine phosphate [181]	
Androgen therapy	
-	Testosterone cypionate [182]

AR EXPRESSION IN THE PROSTATE CANCER MICROENVIRONMENT

There is a growing interest in the impact of stromal AR signaling on the development and progression of PCa. While a large number of studies addressed the role of AR in epithelial cells, only a limited number of reports were focused on the role of AR in the stroma [62]. However, it is well established that AR is expressed in stromal cells (Table 2) and it is also known that stromal AR is lost during PCa progression [63-65]. In various studies, decreased stromal AR expression was shown to be associated with biochemical relapse and poor prognosis [66-68]. These results suggest a protective role of stromal AR against PCa progression which would be in contrast to the well-established role of AR in PCa cells. However, the role of AR in stromal cells of the TME remains largely unclear. Given the growing evidence for a key role of the TME in PCa development and progression, exploring the expression and function of

Table 2. Functional and genomic features of AR signaling in the various cells of the prostate cancer microenvironment. The table shows the actions of AR signaling in prostate cells and various cells of the PCa microenvironment and the current knowledge on the genomic regulation of AR in these cells.

Cell type	AR role	Possible effect in PCa	DNA binding location	Function	Pioneering factors	References
Prostate cells	Maintenance of prostate development and morphogenesis. Regulation of cell growth and migration.	Increased proliferation and migration	Distal intergenic and introns	Enhancer	FOXA1, GATA2, HOXB13	26-29
Fibroblasts	Regulation of cytokines secretion and cross-talk between stromal and epithelial cells (cytokine-cytokine receptor interaction, cell adhesion, ECM-receptor interaction)	Up-or down-regulation of tumor cell growth and migration	Distal intergenic and introns	Enhancer	c-Jun, c-Fos, ATF ZFX	5, 60, 86-90, 93-96
T lymphocytes	Suppression T cells proliferation. Promotion of differentiation of FoxP3+ T cells (Tregs) and Th2/Th1 T cells ratio (via downregulation of IL-2, IFN-γ, and IL-12)	Increased number of 'pro-tumor' T cells	-	-	-	103-121
B lymphocytes	Negative regulation of B cells development and differentiation and antibodies production	Reduced humoral immune response	-	-	-	106, 122-127
Neutrophils	Positive regulation of neutrophils maturation, proliferation and inflammatory cytokines production (IL1-ß, IL-6, TNF)	Increased innate immune reaction	-	-	-	129-130
Macrophages	Negative regulation of TLR4, CCL2 and FcγR expression. Increased CCL4 production. Up- and down-regulation of TNF	Decreased immune response and phagocytosis. Promotion of tumorigenesis. Up-or down-regulation of EMT genes.	-	-	-	58, 82, 132-141
Dendritic cells	Negative regulation of inflammatory cytokines production (TNF, IL1-ß, IL-6 etc)	Decreased antigen presentation	-	-	FOXO3	142-144
Endothelial cells	Induction of VEGF-mediated cell growth. Increased VCAM-1 expression. Increase TNF-α -induced apoptosis	Increased angiogenesis	-	-	-	145-150

AR in the TME is highly relevant. Also, the underlying mechanisms by which stromal AR expression is lost during PCa progression remains largely unknown. However, some hypotheses have been proposed: increased epithelial AR expression during PCa development might lead to increased uptake of androgens by epithelial AR which outcompetes stromal AR, possibly leading to reduced expression [62]. Another option is that distinct inactivating AR mutations might occur in stroma. However, this remains a relatively unexplored hypothesis as the only data available on AR inactivating point mutations were originated from PCa cells [69]. Finally, epigenetic modifications have also been proposed, such as changes in methylation status of the AR promoter [70]. Importantly, epithelial and stromal AR can also interact together to promote epithelial-to-mesenchymal transition (EMT) and influence PCa development. During EMT, epithelial cells gain the migratory properties characteristic of the mesenchymal stem cells, and this may strongly promote PCa progression [71, 72]. As shown in human and ARKO-mice (mice lacking AR expression) studies, stromal AR is believed to promote EMT differentiation in male urogenital tract via secretion of various growth factors and cytokines, such as keratinocyte growth factor, insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) [73, 74] [75]. Furthermore, tissue recombinant studies evaluated the mesenchymal induction of prostatic epithelium and show that stromal AR plays a key role in prostate development by modulating the epithelial differentiation, apoptosis and proliferation. More specifically, mice with recombinant tissue with wild-type urogenital sinus mesenchyme (UGM) and wild-type epithelium successfully develop the prostatic glands, however, mice with recombinant tissue composed of AR-deficient testicular feminization (Tfm)-UGM and wild-type-epithelium fail to form the prostate. This highlighted the critical role of mesenchymal AR in prostate formation [76, 77].

Interactions between stromal cells and PCa cells are frequently mediated by soluble factors, such as cytokines [78]. Testosterone and other sex hormones can modulate the adaptive and innate immune system; however, their effect might vary depending on the type of sex steroids. Indeed, estrogens are generally thought to promote pro-inflammatory cytokines production, whereas androgens are thought to suppress them [79]. The fact that males are in general more prone to infectious diseases and females are more prone to develop autoimmune diseases, supports this hypothesis [80-83]. However, relatively little is known about the mechanisms by which androgens affect the immune system.

Suppression of the pro-inflammatory signals by androgens might be mediated through reciprocal repression between AR and the NF-kB signaling pathway, which is a well-known regulator of immune functions [84, 85]. Studies in rats showed that NF-kB was implicated in repression of the AR gene [86]. Moreover, NF-kB activation was shown to block the proliferation of androgen-dependent PCa cells, but not androgen-insensitive PCa cells [87]. Also, in human benign prostatic hyperplasia cells, DHT-induced suppression of NF-kB–mediated inflammatory cytokine production was demonstrated [88, 89]. However, in LNCaP prostate cancer cells, no tethering of AR and NF-κB was observed in the chromatin binding upon stimulation with androgens and TNFα, suggesting that they would not compete for the same genomic locations [90]. Instead, redistribution of the AR pioneer factor, FOXA1 was observed together with

increased NF-κB binding sites in the chromatin. This phenomenon was suggested to possibly 'mask' the AR-binding sites due to redistribution of FOXA1 binding in the presence of inflammatory cytokines stimulation. Therefore, a potential negative regulation of AR function would be possible by activation of the NF-κB via inflammatory cytokines such as TNFα .

In conclusion, AR expression in the PCa TME might profoundly affect development of the disease. Therefore, the exact actions of AR in stromal cells warrants further characterization. Importantly, AR is expressed in various cells in the PCa stroma, which might have different kinetics and might affect PCa development in different ways (Figure 2). We will discuss the available data on AR actions in the various cells of the TME below.

AR EXPRESSION IN PROSTATE CANCER ASSOCIATED FIBROBLASTS

Fibroblasts represent one of the most abundant cell populations in the TME and one of their primary functions is to produce the structural and regulatory components of the ECM and a large variety of cytokines [91]. During PCa development and progression, the stroma becomes reactive and undergoes structural and functional changes which might affect progression of the disease [91]. Relatively little is known about the exact mechanisms by which fibroblasts become activated into Cancer Associated Fibroblasts (CAFs), however, their role in modulation of tumorigenesis and progression is well documented [92, 93]. A recent study showed that CAFs in the TME of cutaneous squamous cell carcinoma are characterized by unique Nuclear Receptor (NR) expression profiles as compared to Normal-Associated Fibroblasts (NAFs), which might affect cancer cell invasiveness, proliferation and response to chemotherapy [94, 95].

Recent studies described the genomic action of AR in immortalized stromal cells from benign prostatic hyperplasia (PshTert-AR) and CAFs. Using Chromatin Immunoprecipitation, followed by massive parallel sequencing (ChIP-seq) in PshTert-AR cells, it was shown that AR binds the DNA upon testosterone stimulation via the activating protein-1 (AP-1) complex [95]. However, this could not be confirmed in CAFs or primary Embryonic Prostate Fibroblasts (EPFs) [96]. In CAFs and EPFs, AR binding was reported proximal to the known AR-responsive genes ATAD2 and ARL8B, which was shared with PCa cells [96]. However, the vast majority of AR chromatin binding sites in CAFs were specific for this cell type and not shared with prostate cancer cells. In the same study, the zinc-finger protein X-linked (ZFX) was identified as a potential AR co-factor in EPFs but not in CAFs. This suggests that ZFX may function as an AR-co-factor during embryonic development of the prostate which disappears during differentiation. However, also in prostate tumors ZFX was shown to be elevated and to drive cell proliferation and survival [97, 98].

The consequences of AR signaling in CAFs for PCa development remains unestablished, since reports are not unequivocal. It was suggested that acceleration of human PCa growth and migration was mediated by soluble factors secreted by CAFs [99, 100]. Co-culture of CAFs in which AR was knocked down, with PC3 prostate cancer cells resulted in decreased epithelial growth, and diminished colony formation and invasion. This was mediated by

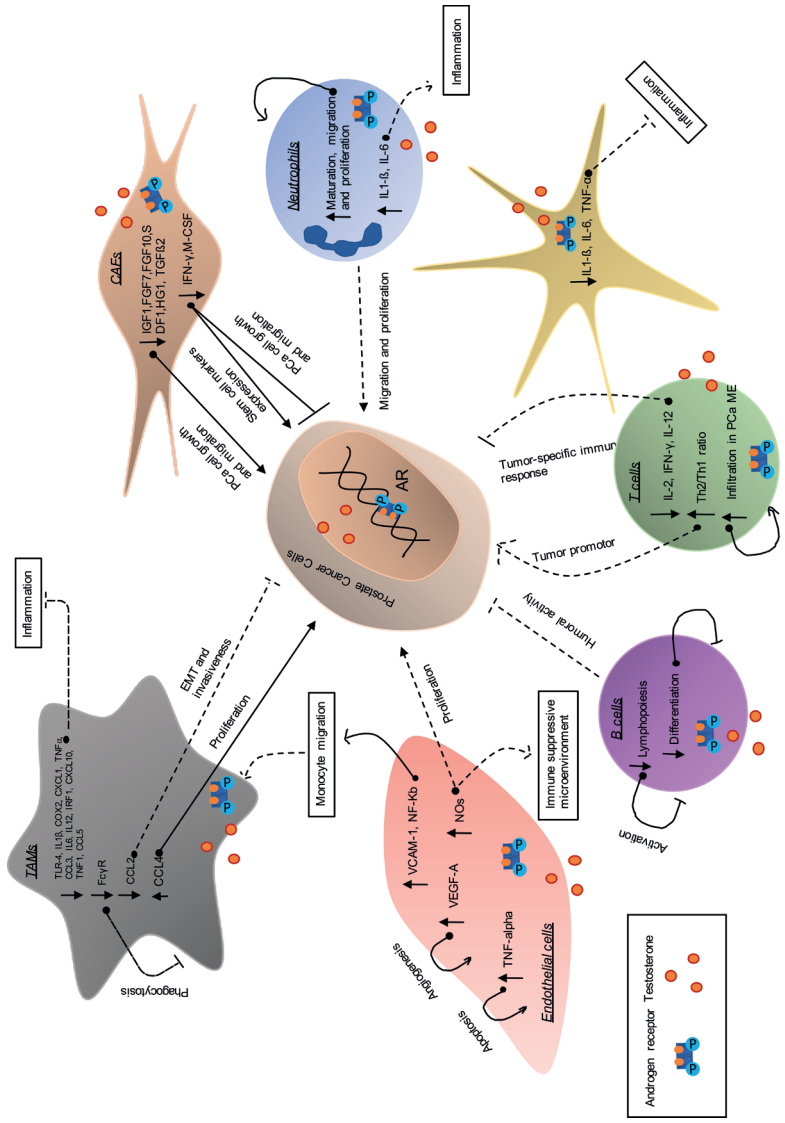

Figure 2. Potential role of Androgen Receptor (AR) signaling in the prostate cancer microenvironment. Schematic representation of AR signaling in PCa cells and stromal cells in the TME. In the TME non-immune cells, such as endothelial cells and Cancer-Associated Fibroblasts (CAFs) and immune cells, such as neutrophils, Dendritic Cells (DCs), T cells, B cells and Tumor-Associated Macrophages (TAMs) are found. As a result of AR actions, signals to the prostate cancer cells and to the microenvironment (boxed) can be activated. These signals might stimulate or inhibit processes (truncated line). Solid lines indicate functions that have been established in PCa, while segmented lines indicate putative actions based on known functions of cytokines. Signals that affect stromal cells, are indicated by a line looping back to the cell. In CAFs, opposite actions of AR signaling have been described (91-95 and 4,96).

reduced secretion of IGF1, FGF7, FGF10, SDF1, HGF and TGFß2 [101]. In agreement with these results, conditioned medium of DHT-stimulated AR-positive WPMY-1 immortalized normal human prostate fibroblasts, significantly increased LnCap prostate cancer cell proliferation compared to non-stimulated fibroblasts [102]. Moreover, invasion of PCa cells co-cultured with WPMY-1 fibroblasts in which AR was knocked down, was significantly lower compared to co-culture with AR wild-type WPMY fibroblasts [103]. In contrast, it was reported that antisense oligonucleotide AR-silenced CAFs promoted PCa cells growth, colony formation and expression of stem cell markers by increased IFN-γ and M-CSF expression [104]. Furthermore, castrated mice co-grafted with patient-derived PCa tissue and PshTert AR-positive myofibroblasts showed a significant increase of apoptotic PCa cells compared to PCa tissue co-grafted with AR-negative myofibroblasts, suggesting that loss of AR in myofibroblasts protects PCa cells from castration induced apoptosis [4]. There is no clear explanation why AR signaling in fibroblasts is both associated with increased and decreased PCa cell proliferation, migration and apoptosis. A potential explanation of these contrasting findings might be related to differences in the fibroblasts' origins (normal fibroblasts or CAFs) or variation in the duration of AR stimulation of the fibroblasts in the various studies.

If AR actions in fibroblasts protect against PCa development and metastases, this would impact the way we look at anti-hormonal treatments. Although AR inhibition is the mainstay of metastasized disease treatment, this would also imply that this treatment has unwanted effects by disrupting the protective function of fibroblasts against disease progression.

AR EXPRESSION IN ADAPTIVE IMMUNE CELLS

The cell-mediated adaptive immune system largely consists of B and T lymphocytes. Various subsets of T cells have been described, most prominently CD8+ cells (commonly referred to as cytotoxic T cells) and CD4+ cells, also called T helper cells [105]. Moreover, various sub-populations of CD4+ T helper cells have been described in tumor inflammation, including the anti-tumor Th1 CD4+ cells, the pro-tumor Th2 CD4+ cells and regulatory T cells (Tregs) [106].

The CD8+ and CD4+ subsets of T lymphocytes are present in the PCa-affected prostate gland, however, an unequivocal correlation between CD8+ and CD4+ T lymphocyte infiltration and prognosis has not been established yet. The number of infiltrating CD8+ and CD4+ cells was shown to be increased in cancer compared to benign tissue, however, no correlation with malignancy grade (GS) was observed [107]. Moreover, the numbers of immunosuppressive Tregs were found to be increased in the prostate and peripheral blood of PCa patients compared to healthy men [108, 109]. However, a clear relation between T cell infiltration in the PCa TME and clinical outcome is yet to be established, as both increased and decreased numbers of infiltrating T cells into the PCa TME were found to correlate with shorter PSA recurrence-free survival after radical prostatectomy [110]. Expression of classical intracellular AR (iAR) has been documented in T and B cells, while in T cells also a surface AR (sAR) was described [111-115]. The functionality and significance of this sAR remains to be elucidated. A few studies have

suggested that AR signaling in immune cells alters cytokine production in T cells [116, 117], which may potentially affect prostate cancer development and progression [118].

Thymic cells of AR knocked-out (ARKO) mice showed a lower expression of CD80/CD86 (also known as B7-2) activation marks compared to AR proficient thymic cells [119], which are believed to be required for proper antigen-mediated activation of T cells [120]. AR-mediated activation of thymic cells was confirmed in another study where AR was shown to upregulate CD80 and CD86 by direct promoter binding [121]. However, in thymic cells of ARKO mice, an increased expression of IL-7 and CCL21 was shown, while TGFß1 and IL-6 expression was decreased. IL-7 and CCL21 were reported to promote thymopoiesis [122], while TGFß1 and IL-6 were suggested to inhibit it [123]. This would be in contrast to the suggested AR-mediated activation of thymic cells based on CD80/CD86 expression. However, reports on the role of CD80/CD86 activation marks in T cell activation are not unequivocal, since another study reported that double CD80/CD86 KO mice showed increased numbers of mature CD4 and CD8 splenic T cells [124], suggesting that increased CD80/CD86-mediated activation of T cells might negatively regulate the maturation and differentiation of T cells.

In mature T cells, AR signaling was shown to have a dual role. AR activation suppresses T cell proliferation in mice and in vitro [110,125], and it modulated the balance between CD4+ Th1 and Th2 (T helper cells) response, skewing the differentiation towards the Th2 phenotype in peripheral blood of ADT-treated PCa patients [126]. A possible explanation for AR-mediated Th2 polarization, is the suppression of IL-2, IFN-γ and IL-12 expression in T cells [126, 127], which are known to be key signals for the Th1 polarization [128, 129]. In CD4+ T cells, AR was found to bind an intronic region of the protein tyrosine phosphatase non-receptor type 1 (Ptpn1) locus [130]. Moreover, ptpn1 expression was decreased in CD4+ cells isolated from patients undergoing ADT [126]. In the same study, up-regulation of Ptpn1 in human and mouse androgen-treated CD4+ T cells was associated with IL-12 inhibition, which prevented STAT4 phosphorylation and ultimately blocked Th1 polarization, potentially contributing to a sustained tumor-specific immune response. Importantly, the AR – mediated Th1 to Th2 T cell switch would possibly support PCa development and progression as Th2 T cells have been widely described to favor pro-tumor and immunosuppressive microenvironment, producing cytokines that support the presence of MDSCs and TAMs [131, 132].

AR is also expressed in immature murine B cells [133]. In vitro and in vivo studies showed that blockade of AR signaling enhanced B cell lymphopoiesis, demonstrating that B cell development is negatively regulated by androgens and AR signaling [134-136]. These data suggest that hormonal therapy increases the generation of young B cells, however, it is not clear how this might affect PCa.

AR signaling seems to affect maturation of T and B cells. However, there is limited data on the relevance of AR signaling in T and B cells for PCa development, nevertheless in T cells, AR signaling could regulate the expression of various cytokines that might affect PCa development

AR EXPRESSION IN INNATE IMMUNE CELLS

Cells of the innate immune system (e.g. macrophages, dendritic cells (DCs), myeloid-derived suppressor cells (MDSCs) and neutrophils) promote phagocytosis and lysis of bacteria and virus-infected cells, and are critically involved in the immunological response to cancer development and progression. Within the same subset of innate immune cells, various phenotypes may be present that affect PCa development and progression. The effects of androgens on innate immune cells functions is largely unexplored, however, several androgen-driven mechanisms of action have been proposed, as further discussed below.

Neutrophils and polymorphonuclear cells

Neutrophils or polymorphonuclear neutrophils (PMNs) kill tumor cells by either phagocytosis or releasing toxic oxygen-free radicals [42]. It is suggested that neutrophils can be present in the tumor microenvironment as N1-like neutrophils (anti-tumorigenic) or N2-like neutrophils (pro-tumorigenic) and therefore, contribute to both suppression and promotion of the tumor [137]. Although a clear distinction between these two neutrophils phenotypes in PCa and their correlation with survival is lacking, an elevated neutrophils-to-lymphocytes ratio (NLR) in the peripheral blood was shown to be associated with lower response rates to abiraterone or docetaxel treatment in mCRPC patients [138].

A study in ARKO mice reported significant reduction of neutrophil proliferation and maturation [139], possibly via reduced phosphorylation of STAT3 and ERK, which are essential for myeloid cells differentiation. As a consequence, production of chemokines and cytokines such as IL1-ß, IL-6 and TNF-α was also reduced in granulocytes of ARKO mice. This would suggest that AR in neutrophils decreases the number of neutrophils and supports their immunosuppressive phenotype. However, in the same study, ADT did not significantly affect peripheral blood neutrophil count in patients. Despite no clear effect of AR function in neutrophils is known in relation to PCa development and progression, we speculate that, the AR-mediated neutrophils maturation might possibly be associated with a worse clinical outcome as high neutrophil-to-lymphocyte ratio in PCa patients was shown to be associated with shorter overall survival (OS) an increased chances of biochemical recurrence in several studies [140, 141].

Macrophages

Macrophages are found in most organs of the human body and are derived from circulating monocytes which differentiate into macrophages when entering the tissue [142]. Unlike neutrophils, macrophages are capable of repeated phagocytosis and can secrete inflammatory cytokines [42]. However, similar to neutrophils, phenotypic subsets occur with contrasting actions on tumor cells. M1-like and M2-like macrophages are so called pro-inflammatory and tumor-associated macrophages (TAMs), respectively [42]. The number of infiltrating TAMs in the PCa microenvironment was predictive for disease progression after hormonal therapy [143]. Moreover, increased numbers of cancer associated M2-macrophages was associated with extra capsular tumor extension [15].

In general, androgens are thought to inhibit macrophage function in vivo and in vitro [144]. For instance, AR knockdown suppresses migration of the macrophage cell line THP-1 [145], suggesting that AR in macrophages might support the migration ability of these cells. However, AR knockdown in THP-1 cells also induces expression of CCL2, which promotes EMT and enhances invasiveness of malignant cells [89, 146].

Moreover, androgen stimulation of murine macrophages reduced the expression of toll-like receptor 4 (TLR4) [147]. Downregulation of TLR4 expression alters the MyD88-dependent and-independent pathways which leads to decreased expression of various pro-inflammatory molecules such as IL1-ß, COX2, CXCL1, TNF-α, CCL3, IL-6, IL-12, IRF1, CXCL10, TNF1 and CCL5 [148]. Furthermore, expression of receptors for the Fc region of IgG (FcγR) on macrophages was reduced in guinea pig models after testosterone stimulation [149]. As FcγR expression in innate cells is crucial for phagocytosis and the release of inflammatory mediators [150], these data suggest that AR activation in macrophages decreases antibody-mediated phagocytosis via reduction of FcγR expression.

Interestingly, co-culture of normal prostate epithelial RWPE-1 cells with the THP-1 macrophage cell line induced prostate tumorigenesis in 3D-culture [151]. In the presence of THP-1 macrophages, RWPE-1 cells differentiated into a disorganized aggregate structure, suggesting that soluble factors secreted by THP-1 cells interfere with the normal development of well-organized spheroids of glandular prostate epithelial cells also called prostaspheres. These observations were confirmed in vivo as all mice injected with both RWPE and THP-1 cells developed tumors, while none of the mice injected with either RWPE or THP-1 alone developed tumors. In the same study the expression of several EMT-associated genes in RWPE-1 cells was increased after co-culture with THP-1 macrophages, including CCL4. CCL4 was previously identified as an AR-responsive gene and proposed as a main driver of tumorigenesis and EMT [152]. In this study, antibody-based blockade of CCL4 in the co-culture experiments showed significant reduction of THP-1-mediated cell migration and EMT-related gene expression in RWPE-1 cells. AR expression in THP-1 macrophages was proposed to be responsible for the cross-talk between THP-1 cells and RWPE-1 cells, as knocking down AR in THP-1 cells reduced of CCL4 expression. This key role of AR-mediated CCL4 expression was confirmed in vivo, since macrophage-AR knockout (M-ARKO)/PTEN$^{+/-}$ mice showed decreased CCL4 levels and reduced preneoplastic Prostatic Intraepithelial Neoplasia (PIN) formation when compared to tumors from PTEN$^{+/-}$ mice. Although these findings demonstrate a role of AR in macrophage-associated inflammatory response, the underlying mechanisms remains unclear.

In conclusion, these results suggest that AR signalling affects multiple functions of macrophages, including migration, cytokine production and phagocytosis. All of these might affect PCa development. Moreover, macrophages might increase prostate cancer cell EMT mediated through AR regulated CCL4 expression.

Dendritic cells

Dendritic cells (DC) share many features with macrophages and play an important role in T cell activation and assist their regulation into Th1 and Th2 differentiation. Very little is known about

the role of AR in DCs. DCs do express AR and androgens were shown to decrease production of the inflammatory cytokines IL1-ß, IL-6 and TNF-α [153]. In agreement with this, DHT stimulated bone marrow-derived dendritic cells (BMDCs) showed decreased IL-6 expression, which is fundamental for the maturation of DCs, while production of anti-inflammatory cytokines IL-4 and IL-10 was increased upon DHT treatment [154]. However, the genomic mechanisms of AR signalling in DCs, as well as the possible effect thereof on PCa development and progression is yet to be explored. The possible AR-mediated impairment of DCs maturation suggest that AR function in these cells suppress the DCs-mediated activation of CD8+ T cells, which is required for the proper tumor killing. Therefore, we speculate that AR function in DCs might suppress the progression of PCa via preventing CD8+ T cell tumor specific activation.

AR EXPRESSION IN ENDOTHELIAL CELLS

Endothelial cells are key components of blood vessels. Abnormalities in growth, function and organization of endothelial cells often occur in concert with the development and progression of atherosclerosis and cancer. Very little is known about the effects of androgens on endothelial cells. Human umbilical vein EA.hy926 endothelial cells have a functional AR, and AR stimulation increases TNF-α –induced apoptosis of these cells [155, 156]. In contrast, another study reported that AR promotes endothelial cell proliferation through AR/VEGF-A/cyclin-A –mediated mechanisms [157]. Given the opposing conclusions of studies exploring the effects of AR signalling in endothelial cells on proliferation and survival, a clear role of AR in angiogenesis and consequently PCa development remains elusive. AR stimulation induced vascular cell adhesion molecule 1 (VCAM-1) expression in endothelial cells which led to increased monocyte binding to the endothelium, promoting monocyte migration into the TME [158]. Testosterone was shown to rapidly induce nitric oxide (NO) production in human aortic endothelial cells (HAECs) [159], which is known to support an immune suppressive microenvironment, promote cancer cell growth and prevent apoptosis [160].

Altogether, these data would suggest a potential role of endothelial AR in PCa development and progression, mediated by increased angiogenesis and accelerated recruitment of immune cells into the PCa microenvironment.

OTHER NUCLEAR HORMONE RECEPTORS IN THE PCA MICROENVIRONMENT

Other nuclear hormone receptors (NHR) such as ER, GR and PR have also been described in the PCa stroma, however, their role in specific cell type is largely unknown. Both Erα and Erβ were shown to be expressed in the PCa-associated stroma [161], however, Erα is predominantly found in the stromal compartment, while Erβ is mainly expressed in the basal-epithelial cells [162, 163]. Indeed, a previous study showed that only increased expression of Erα and not Erβ in the stroma was shown to be correlated with advanced disease [164]. However, another study showed that PCa patients with Erα-positive stroma had a significantly lower risk of biochemical recurrence [165]. In the same study, in vitro experiments, revealed that stromal Erα reduced PCa

cell invasion possibly by downregulation of matrix metalloproteinase 3 (MMP3) and increased expression of thrombospondin 2 (Thbs2).

Glucocorticoid receptor expression was found in the stroma of both human benign prostatic hyperplasia (BPH) and PCa [166]. Importantly, glucocorticoids play a key role in immune cells as they are potent anti-inflammatory agents that act via transrepression of GR through tethering to various transcription factors, such as the AP-1 and NF-κB [167]. Therefore, targeting GR in PCa patients might also potentiate the efficacy of current therapies.

Expression of PR was also shown in stromal compartment of PCa biopsies, and levels were decreased when compared to normal prostate stroma. Furthermore, conditioned medium from PR-positive stromal cells was able to inhibit PCa cell migration and invasion, possibly via downregulation of CXCL12 and IL-6 cytokines production [168]. Also, PR was shown to inhibit prostate stromal cell proliferation [169].

All together, these data suggest that the composition of NHRs in the stromal compartment might strongly affect PCa development and progression, however, more studies should be performed to explore the exact role of NHRs in specific cell type.

NEW PROSPECTIVE FOR HORMONE THERAPY AND IMMUNOTHERAPY IN PCA

Immunotherapy was chosen by Science's editors as the "breakthrough of the year 2013" and represents a new potential weapon for fighting cancer by exploiting the immune system. Although effective in melanoma, non-small cell lung cancer and bladder cancer, thus far immunotherapy has shown limited efficacy in PCa patients [170-173].

One potential explanation for this lack of efficacy is the low mutational load of PCa cells, limiting the repertoire of neo-antigens that are required for recognition of cancer cells by activated T cells [174]. Another potential explanation for the low success rate of immunotherapy in PCa is the presence of an immunosuppressive tumor microenvironment. CD25+ and FoxP3+ Tregs as well as PD1+ exhausted T cells were found to surround prostate cancer islets in untreated PCa patients [175]. Moreover, levels of circulating immune suppressive CD14+HLA-DR$^{low/-}$ MDSCs were significantly increased in the blood of PCa patients, compared to healthy controls [176]. Importantly, MDSCs become more immune suppressive in the tumor and can differentiate into TAMs, supporting tumor growth [177].

Non-immune stromal cells might also contribute to immune suppression by releasing specific stromal factors. Myofibroblasts present in the PCa microenvironment were shown to release CCL2, IL-6 and TGF-ß, promoting differentiation of DCs into tumor-associated DCs (TADCs) via increased expression of IL-10 and PD-L1. This reduced the cross-presentation of tumor antigens to CD8+ T cells and TADCs-mediated T cell proliferation [178].

The effect of ADT therapy on the immune system remains elusive. ADT treatment is reported to increase the level of T cells in peripheral blood of mice [125] and in human PCa tissue [179, 180]. However, recent studies demonstrated that ADT suppresses T-cell differentiation and activation, which hampers the efficacy of immunotherapy [119, 181, 182]. Moreover, others

demonstrated that ADT also promote the expansion of immunosuppressive Tregs and TAMs [183, 184] which counteract the accumulation of tumor-infiltrating lymphocytes (TILs) observed upon ADT treatment.

Mouse prostate tumor (Myc-Cap) bearing mice treated with CpG, a TLR9 agonist which activates DCs, showed a suppressed tumor specific CD8+ T cells immune response upon treatment with the AR antagonist flutamide. More specifically, AR antagonist treatment was shown to suppress T cells priming [181]. These data suggest that AR inhibition impairs the efficacy of immune checkpoint inhibitors treatment of PCa patients. Furthermore, it was recently reported that ADT decreases the expression of the immune checkpoint marker, programmed death ligand 1 (PD-L1) possibly limiting the effect the anti-PD-L1 immune checkpoint therapy [185].

Recent advances of vaccination therapy in PCa treatment led to the development of sipuleucel-T; a therapeutic vaccine shown to prolong OS of mCRPC patients treated with ADT [186]. Clinical outcome of sipuleucel-T treatment was not affected by ADT, as no difference in efficacy was found between patients treated with ADT compared to patients that were treated with ADT after completion of sipuleucel-T treatment [187]. However, a small study in sipuleucel-T treated patients suggested that vaccination followed by treatment with the anti-androgen nilutamide improved survival, as compared with patients who first received anti-androgen therapy and then vaccine [188].

All together, these data suggest that AR blockade impairs the tumor-specific immune response, and additional studies are required to optimize combination strategies for the treatment of PCa patients.

CONCLUSIONS

In this review we explored the role of AR in various cells of the PCa microenvironment and its potential effect on the development of the disease. Understanding the functional mechanisms of AR expression in the stroma is relevant, since unwanted effects of hormone therapy can be expected, as AR in the epithelial and stromal compartments controls different signalling pathways. Opposing effects on PCa growth of stromal and epithelial AR signalling might be targeted through the development of endocrine agents with cell type-selective actions. Cell-specific AR targeting has been described in mice studies [189, 190] using Cre/Lox recombinant system, however no evidence of cell specific AR targeting has been provided in human. Nevertheless, it might be possible to target cell-specific downstream genes of AR signalling pathway to specifically inhibit the effect of AR activation in different cells. For instance, hormonal therapy could be combined with agents blocking the AR-mediated release of specific cytokines in different stromal cells which might support PCa development and progression.

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