

In vitro model systems to study androgen receptor signaling in prostate cancer

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Abstract

Prostate cancer (PCa) is one of the most common causes of male cancer-related death in Western nations. The cellular response to androgens is mediated via the androgen receptor (AR), a ligand-inducible transcription factor whose dysregulation plays a key role during PCa development and progression following androgen deprivation therapy, the current mainstay systemic treatment for advanced PCa. Thus, a better understanding of AR signaling and new strategies to abrogate AR activity are essential for improved therapeutic intervention. Consequently, a large number of experimental cell culture models have been established to facilitate *in vitro* investigations into the role of AR signaling in PCa development and progression. These different model systems mimic distinct stages of this heterogeneous disease and exhibit differences with respect to AR expression/status and androgen responsiveness. Technological advances have facilitated the development of *in vitro* systems that more closely reflect the physiological setting, for example via the use of three-dimensional coculture to study the interaction of prostate epithelial cells with the stroma, endothelium, immune system and tissue matrix environment. This review provides an overview of the most commonly used *in vitro* cell models currently available to study AR signaling with particular focus on their use in addressing key questions relating to the development and progression of PCa. It is hoped that the continued development of *in vitro* models will provide more biologically relevant platforms for mechanistic studies, drug discovery and design ensuring a more rapid transfer of knowledge from the laboratory to the clinic.

Key Words

- ▶ three-dimensional coculture
- ▶ castration-resistant
- ▶ cell culture
- ▶ stroma
- ▶ tumor

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Introduction

Prostate cancer (PCa) is the most commonly diagnosed cancer in men and second leading cause of male cancer death in Western societies (Siegel *et al.* 2012). Since the androgen dependence of PCa was discovered, intensive efforts have focused on better understanding of androgen receptor (AR) signaling with our current knowledge being largely derived from experimental cell culture and animal

models. *In vitro* cell cultures have the advantage of being relatively cheap and typically have a high replicative capacity ensuring sufficient material for long-term use. By contrast animal models, although expensive, more closely recapitulate the *in vivo* paracrine and endocrine environment of human PCa and also permit investigation of stromal–epithelial interactions, angiogenesis

and metastasis. However, recently developed three-dimensional (3D) coculture systems now permit investigation of such processes *in vitro*.

A number of different *in vitro* and *in vivo* models have been established to aid studies into prostatic disorders such as benign prostatic hyperplasia (BPH) and various stages of PCa, including castration-resistant (CR) and metastatic disease. Each model system displays its own characteristics regarding androgen responsiveness and AR expression making it often difficult to select the most appropriate model system for a particular question. This review summarizes *in vitro* cell models currently available to study AR signaling with particular focus on their use in addressing key questions relating to the development and progression of PCa. It is beyond the scope of the current review to discuss *in vivo* models, which have been reviewed recently elsewhere (Hensley & Kyprianou 2012, McNamara et al. 2012, Toivanen et al. 2012).

The AR

Androgens play a critical role in the development of the male phenotype during sexual differentiation but also in

the development and progression of PCa (Sampson et al. 2007, Green et al. 2012, Yadav & Heemers 2012). Androgenic action in the prostate is primarily mediated by dihydrotestosterone (DHT), which derives predominantly from the reduction of testicular testosterone but also adrenal dihydroepiandrosterone (DHEA) catalyzed by locally produced 5α -reductase enzymes (Wilson 1996, Mohler et al. 2004). The cellular response to androgens is mediated via the AR, a ligand-inducible transcription factor that comprises a C-terminal ligand-binding domain (LBD), a highly conserved DNA-binding domain, a hinge region and N-terminal transactivation domain (Brinkmann 2011, Bennett et al. 2012, Green et al. 2012). Upon ligand binding, cytosolic AR undergoes conformational changes, including interaction of the N- and C-terminal domains and dissociation from heat shock proteins, enabling the AR to interact with coregulatory molecules such as ARA70 and importin- α , which facilitate nuclear translocation and dimerization (Fig. 1; Rahman et al. 2004, Schaufele et al. 2005, Cutress et al. 2008). In the nucleus, AR binds to the promoters of androgen-regulated genes (ARGs), such as prostate-specific antigen (PSA) and recruits various coactivators and RNA polymerase II to induce transcription

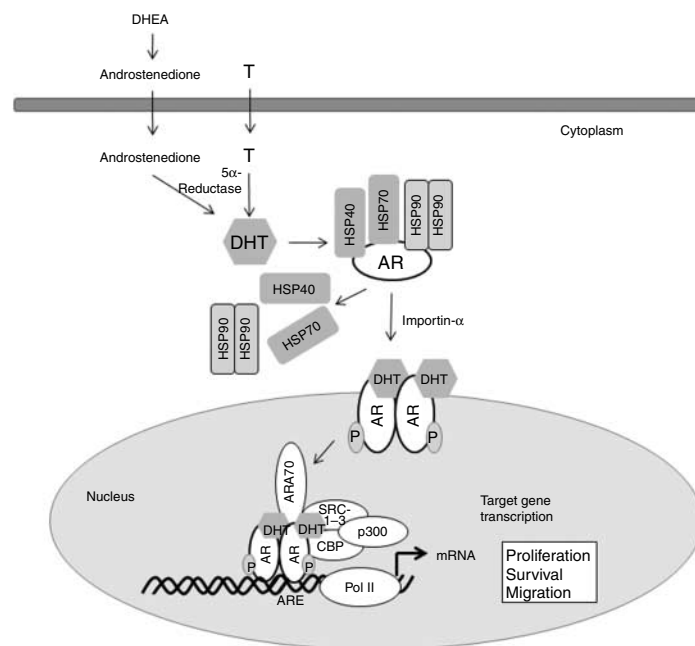


Figure 1

Classic androgen receptor (AR) genomic activity via androgen. Androgens derive predominantly from the testis (90–95%) but also to a lesser extent from the adrenal glands (5–10%) and mediate their effects via binding to the AR. Testicular testosterone (T) and adrenal DHEA or androstenedione are converted locally in the prostate into bioactive DHT by the enzymes 5α -reductase 1 and 2. In the classic mode of AR genomic activity, androgen binding to the AR induces a conformational change that leads to the

dissociation of chaperone and heat shock proteins (HSP40, HSP90) and its subsequent interaction with coregulatory molecules and importin- α , which facilitate nuclear translocation of AR–ligand complexes. In the nucleus, the AR undergoes phosphorylation and dimerization, which permits chromatin binding to androgen-responsive elements (ARE) within androgen-regulated target genes. The AR recruits a variety of coactivators (ARA70, SRC-1, -3, and CBP/p300) and RNA polymerase II (Pol II) to induce gene transcription.

(Veldscholte *et al.* 1992, Smith & Toft 1993, Truss & Beato 1993, Tsai & O'Malley 1994). This classic genomic mode of AR action promotes the transcription of a variety of genes encoding proteins necessary for the development, growth and maintenance of the normal prostate. A comprehensive list of androgen-regulated target genes has been recently published (Lamont & Tindall 2010). The AR can also act via less well-understood nongenomic mechanisms through reciprocal cross talk with numerous signaling molecules at the plasma membrane. These nongenomic AR actions have been recently reviewed in detail elsewhere (Thomas 2012, Nyquist & Dehm 2013).

The role of the AR in PCa

Inhibiting AR signaling remains one of the most common and effective systemic methods to treat PCa (Miyamoto *et al.* 2004). However, many patients relapse and succumb to CR-PCa within 3 years (Molina & Beldegrun 2011). Despite low circulating androgen levels, AR signaling is

frequently reactivated in CR-PCa and plays a key role in disease progression (Chen *et al.* 2004). Several mechanisms have been identified by which AR reactivation can occur, including AR hypersensitivity, promiscuous/constitutive AR activation via cross talk with other signaling pathways or alternative splicing, elevated tumoral androgen production/uptake and altered recruitment/expression of AR coregulators (Fig. 2 and Table 1; Dehm *et al.* 2008, Steinkamp *et al.* 2009, Wegiel *et al.* 2010, Hu *et al.* 2011, Lamont & Tindall 2011, Reis 2011, Green *et al.* 2012, Sampson *et al.* 2012).

PCa and CR-PCa remain largely dependent on the AR for growth (Chen *et al.* 2004). Thus, targeting AR signaling is considered one of the most promising therapeutic approaches and supported by the findings of phase III clinical trials that AR targeting can improve survival of patients with metastatic CR-PCa (Kim & Ryan 2012). A number of agents have been developed that inhibit androgen signaling either by directly targeting the AR or by intervening with androgen synthesis (Schweizer &

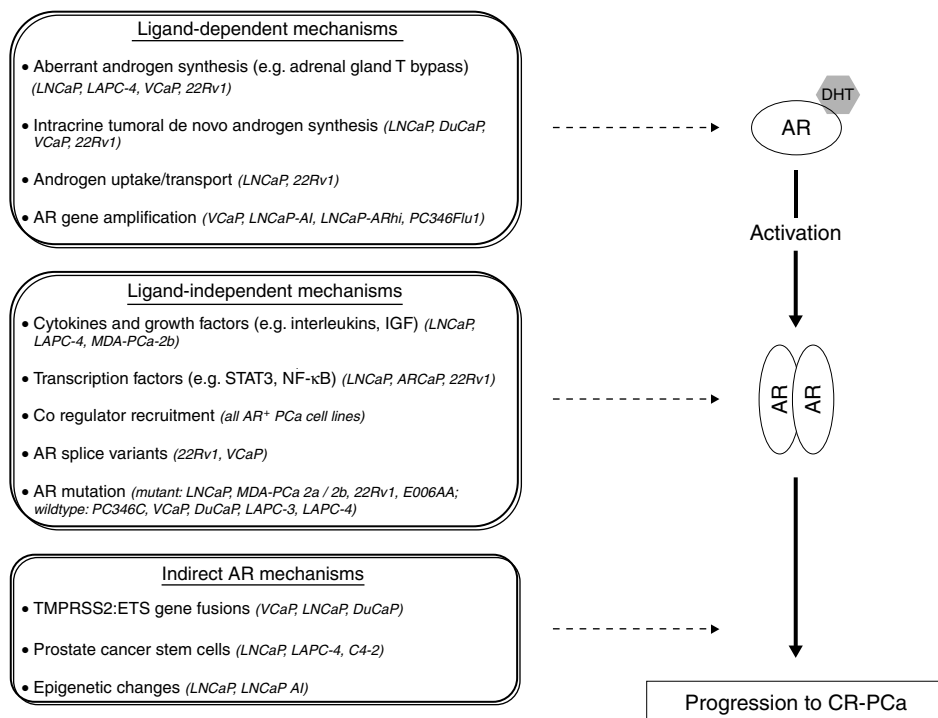


Figure 2

In vitro cell models exhibiting characteristics of androgen receptor (AR)-dependent and AR-independent mechanisms that promote prostate cancer (PCa) progression to castration-resistance. Several pathways have been identified by which PCa cells can overcome androgen depletion and thereby facilitate tumor progression to CR-PCa and can be divided into: i) ligand-dependent mechanisms, which promote AR activation despite castrate levels of androgens; ii) ligand-independent mechanisms, which

facilitate AR activation by nonandrogenic factors and/or altering the intrinsic behavior/sensitivity of AR; and iii) indirect mechanisms that act downstream of AR activation (e.g. chromatin remodeling via histone deacetylases, re-emergence of tumors via CSCs and AR-dependent expression of oncogenic ETS transcription factors). Cell lines that have been used to study these different mechanisms are indicated.

Table 1 Overview of AR point mutations in commonly used PCa cell lines.

Mutation	Location within AR	Functional consequence	Cell line	References
Wild-type AR	-	-	Benign: RC-165N/hTERT, PWR-1E, RWPE1, BPH-1-AR PCa: ARCaP, PC346C, VCaP, DuCaP, LAPC-3, LAPC-4, PC346Flu1	Webber et al. (1996a,b), Bello et al. (1997), Klein et al. (1997), Korenchuk et al. (2001), Lee et al. (2001), Gu et al. (2005), Marques et al. (2005, 2006), Kim et al. (2007), Waltering et al. (2009) and Yu et al. (2009) Marques et al. (2005) Koochekpour et al. (2004)
K311R	N-terminus	ND	PC346DCC	Navone et al. (1997) and Zhao et al. (1999)
S599G	DNA-binding domain	Dominant-negative loss of AR function	E006AA	Attardi et al. (2004)
L701H		Enhances transcriptional activity of AR to glucocorticoids	MDA PCa 2a/2b	Veldscholte et al. (1990), Navone et al. (1997) and Marques et al. (2005)
H874Y	Ligand-binding domain	Induces conformational change in LBD, modulates coregulator protein binding (enhances p160-mediated AR transactivation). AR activation by DHEA, E ₂ , P ₄ , flutamide	22Rv1	
T877A		Altered stereochemistry of LBD, AR activation by flutamide, E ₂ , progesterin, corticosteroids	LNCaP, MDA PCa 2a/2b, PC346Flu2	

E₂, estradiol; LBD, ligand-binding domain; P₄, progesterone; ND, not determined; AR, androgen receptor; PCa, prostate cancer.

Antonarakis 2012). For example, enzalutamide (MDV3100) directly binds to the AR, thereby preventing its nuclear translocation and coactivator recruitment to the ligand-receptor complex. By contrast, abiraterone acetate suppresses extragonadal androgen synthesis via blockade of the enzyme CYP17. These agents demonstrate high clinical potential. Nonetheless this remains an intense area of active research with several new AR antagonists and novel approaches under development, including antisense technology to inhibit AR expression (Cheng et al. 2006, Snoek et al. 2009, Desiniotis et al. 2010, Mohler et al. 2012). The most commonly used and pipeline AR-targeting agents have been comprehensively reviewed recently (Schweizer & Antonarakis 2012).

Cell lines established directly from PCa patient tissues

The first human prostatic tumor epithelial cell lines to be spontaneously established were LNCaP, PC3 and DU145, which were derived from PCa lymph node, bone and brain metastases respectively and remain the most commonly used PCa cell lines (Table 2; Bosland et al. 1996). Of these three cell lines, only LNCaP expresses significant levels of AR and consequently is the most widely used AR⁺ cell line. DU145 and PC3 cells are generally considered to be AR⁻ and thus commonly used as AR⁻ controls or to study androgen signaling by ectopic AR overexpression. Although LNCaPs are androgen responsive and produce PSA, it should be noted that they express a mutated AR (T877A), which results in altered AR signaling (Veldscholte et al. 1990). Recently, exome sequencing of LNCaP cells revealed significant genetic variation and a degree of genetic instability that should be considered when working with this cell line (Spans et al. 2012). In addition, AR signaling and androgen responsiveness of LNCaPs appear to be sensitive to serial passaging and culture conditions (Karan et al. 2001, Sieh et al. 2012). For example, when grown in 3D hydrogels with Arg-Gly-Asp (RGD) motifs (common recognition sites in extracellular matrix (ECM) proteins), LNCaP cells formed tumor-like structures and exhibited different kinetics of androgen-induced AR turnover and AR nuclear translocation with higher basal expression levels of ARGs, a finding also observed upon culture of LNCaPs on bone ECM (Robbins et al. 1996, Sieh et al. 2012). Thus, LNCaP cells may be particularly amenable for studies investigating the impact of tumor cell-ECM interactions on AR signaling.

For many years, LNCaP was the only cell line available for *in vitro* studies of AR signaling. Several additional

Table 2 Origins, characteristics and culture conditions of prostate epithelial cell lines.

Name	Origin	Characteristics	References
Benign cell lines			
BPH-1	Immortalized with SV40	AR ⁻	Hayward <i>et al.</i> (1995)
PWR-1E	Immortalized with human papilloma virus 18	AR ⁺ , androgen responsive, express PSA, nontumorigenic in nude mice	Bello <i>et al.</i> (1997)
RC-165N/hTERT	Immortalized with human telomerase reverse transcriptase	AR ⁺ , androgen responsive, express PSA, nontumorigenic in nude mice	Kim <i>et al.</i> (2007)
RWPE1	Immortalized with SV40	AR ⁺ , androgen responsive, express PSA, nontumorigenic in nude mice	Webber <i>et al.</i> (2001)
AR⁺ PCa cell lines			
ARCaP	Ascites fluid of the same patient as MDA PCa cells	Low levels of AR and PSA, growth inhibited by androgens	Zhau <i>et al.</i> (1996)
DUCaP	Brain metastasis	Wild-type AR, androgen sensitive	Lee <i>et al.</i> (2001)
E006AA	Primary PCa from an African-American patient with hormone naïve localized PCa	Mutated AR, do not express PSA, insensitive to androgens	Koochekpour <i>et al.</i> (2004) and D'Antonio <i>et al.</i> (2010)
LAPC	Locally advanced or metastatic PCa	Wild-type AR, express PSA, different sublines available	Klein <i>et al.</i> (1997) and Craft <i>et al.</i> (1999)
LNCaP	Lymph node metastasis	Mutated AR, produce PSA, androgen responsive	Horoszewicz <i>et al.</i> (1980)
MDA PCa cells	Bone metastasis	Mutated AR, produce PSA, less responsive to androgens	Navone <i>et al.</i> (1997)
PC346	Transurethral resection of localized advanced PCa	Wild-type AR, different sublines available	Marques <i>et al.</i> (2006)
22Rv1	Primary PCa	Mutated AR, low levels of AR and PSA	Sramkoski <i>et al.</i> (1999) and Attardi <i>et al.</i> (2004)
VCaP	Bone metastasis	Wild-type AR, androgen sensitive	Korenchuk <i>et al.</i> (2001)
AR⁻ PCa cell lines			
DU145	Brain metastasis	AR ⁻ , do not respond to androgens	Stone <i>et al.</i> (1978)
PC3	Bone metastasis	AR ⁻ , do not respond to androgens	Kaighn <i>et al.</i> (1979)

AR⁺ PCa cell lines have now been established, including MDA PCa 2a and 2b, which were derived from a bone metastasis of a patient with PCa (Navone *et al.* 1997). Like LNCaP cells, MDA PCa cells also express AR and PSA but are less responsive to androgens and the agonist effects of nonandrogens (e.g. estrogens and progesterone) possibly due to the additional L701H mutation in the AR LBD (Tables 1 and 2; Navone *et al.* 1997, Zhao *et al.* 1999). By virtue of their androgen dependence, LNCaP and MDA PCa AR⁺ cell lines are useful models to investigate mechanisms underlying CR. In addition, these cell lines have been employed to investigate the efficacy of novel therapeutic compounds, such as the histone deacetylase inhibitor valproic acid and the GH-releasing hormone antagonist MZ-J-7-138 (Chou *et al.* 2011, Stangelberger *et al.* 2012). Interestingly, ARCaP cells, which were established from ascites fluid of the same patient as MDA PCa cells, form tumors with high incidence when injected s.c. or orthotopically into intact or castrated male nude mice (Zhau *et al.* 1996). Moreover, unlike MDA PCa cells, ARCaPs express low levels of AR and PSA, are highly metastatic and

growth was inhibited by androgens due to G1 cell cycle arrest and AR-dependent regulation of c-Myc, Skp2, and p27Kip (Zhau *et al.* 1996, Chung *et al.* 1997, Chuu *et al.* 2011).

E006AA is one of the few cell lines established from primary PCa and originates from an African-American patient with hormone naïve localized PCa (Table 2; Koochekpour *et al.* 2004). A stromal cell line (S006AA) established in parallel from the same patient material further extends the experimental value of E006AA by enabling autologous epithelial-stromal interactions to be studied *in vitro*. E006AA cells express a mutated AR (harboring an S599G mutation in the AR DBD) but do not express PSA and display loss of AR-dependent growth suppression with cell growth insensitive to AR knockdown, androgens and antiandrogens (D'Antonio *et al.* 2010). This has important clinical implications since patients with PCa tumors harboring such AR loss-of-function mutations will not benefit from hormone or anti-AR therapies despite AR protein expression. Thus, E006AA cells represent an interesting *in vitro* model for dominant negative AR loss-of-function in hormone-naïve PCa.

LNCaP cell line variants representing CR-PCa

Progression to CR-PCa is a major clinical problem and subsequent treatment is mainly palliative. Several *in vitro* model systems have been developed to study mechanisms underlying the development of CR, including a panel of LNCaP variants. These sublines differ widely in the method of their establishment and culture conditions (summarized in Table 3) but can be largely divided into those established by long-term culture in androgen-deprived media vs those established after *in vivo* passage through athymic nude mice. In addition, some sublines have been established by coculture with other cell types. We observed that many of these variants (including the LNCaPabl subline generated in our laboratory) are less sensitive to apoptosis-inducing agents compared with parental LNCaP cells (Culig et al. 1999, Pfeil et al. 2004). We and others have used these sublines to gain an insight into molecular mechanisms underlying CR-PCa and demonstrate in proof-of-principle studies of the potential clinical efficacy of AR targeting in CR-PCa (Desiniotis et al. 2010, McCourt et al. 2012).

AR overexpression is a common phenomenon in CR-PCa that is mimicked in two LNCaP sublines, LNCaP-ARmo and LNCaP-ARhi, which stably overexpress AR at levels 2–4 and 4–6 times higher than parental LNCaPs respectively (Waltering et al. 2009). These sublines were recently used to demonstrate that AR overexpression sensitizes receptor binding to chromatin, thus, providing an explanation as to how AR signaling can be reactivated in CR-PCa (Urbanucci et al. 2012).

Cell lines established from xenotransplanted tumors

A number of cell lines have been established from human PCa tissue first heterotransplanted into immune-deficient host animals. For example, VCaP and DuCaP cell lines were established respectively from metastatic bone and brain lesions of the same patient with CR-PCa via xenografting into *Scid* mice and later harvested for *in vitro* culture (Table 2). Both VCaP and DuCaP cells are androgen-sensitive and express higher levels of wild-type AR than LNCaP cells (Korenchuk et al. 2001, Lee et al. 2001, Marques et al. 2006, Waltering et al. 2009). In addition, these cell lines also harbor a TMPRSS2:ERG gene fusion and thus are frequently used to investigate the functional significance of the genetic rearrangement involving ERG, a member of the ETS family of transcription factors, which is the most common genetic aberration

in PCa identified to date (Fig. 2; Tomlins et al. 2008, Paulo et al. 2012). In addition to wild-type AR, VCaPs also express several alternatively spliced AR isoforms in response to castration or androgen deprivation with one variant lacking the LBD (Dehm et al. 2008, Watson et al. 2010), which like other recently identified AR variants appears to act as a constitutively active, ligand-independent transcription factor to support AR reactivation in CR-PCa (Fig. 2; Dehm & Tindall 2011). Moreover, given their upregulation of AR and numerous enzymes involved in the metabolism of adrenal steroids following androgen deprivation, DuCaPs may represent an ideal *in vitro* model system to study intratumoral *de novo* androgen synthesis, a key mechanism underlying progression to CR-PCa (Fig. 2; Locke et al. 2008, Pfeiffer et al. 2011).

The LAPC cell lines were established from eight different patients with locally advanced or metastatic PCa following subcutaneous implantation into *Scid* mice in the presence of Matrigel (Table 2). Established tumors were then grown and serially passaged *in vitro* as eight distinct cell lines, which have been described in detail (Klein et al. 1997). Whilst LAPC-3 and LAPC-4 cells both express wild-type AR and PSA, the latter also expresses high levels of HER-2/neu receptor tyrosine kinase and consequently has been used to study ligand-independent AR activation (Craft et al. 1999). LAPC cells are important tools to investigate wild-type AR and have proved particularly useful in comparing drug efficacy (such as the antiandrogen abiraterone acetate and small molecule 1(3-(2-chlorophenoxy)propyl)-1H-indole-3-carbonitrile (CPIC)) with cell lines expressing mutated ARs (Cherian et al. 2012, Li et al. 2012a).

The PC346 panel of cell lines, which originate from a transurethral resection of localized advanced PCa, also represents an interesting model system to study AR signaling in different stages of PCa (Marques et al. 2006). Xenografts (PC346P) were established from primary tumor tissue subcutaneously implanted into male athymic mice from which the wild-type AR expressing and androgen-dependent cell line PC346C was established. Three CR PC346C sublines were generated following long-term culture in steroid-stripped medium alone (PC346DCC) or supplemented with the antiandrogen flutamide (PC346Flu1 and PC346Flu2). Unlike their parental counterparts, PC346DCC cells express low levels of AR and do not produce PSA. By contrast, both PC346Flu cell lines express high levels of AR and produce PSA. However, whilst PC346Flu1 expresses wild-type AR, PC346Flu2 expresses a T877A-mutated AR and PC346DCC expresses AR with a novel K311R mutation, although no difference

Table 3 Origins, characteristics and culture conditions of LNCaP subline variants.

Name	Establishment	Culture conditions	Characteristics	References
Sublines established by culture under steroid-depleted conditions <i>in vitro</i>				
LNCaP 104 (-5, -I, -R)	Individual colonies selected after seeding serially diluted wtLNCaP; one colony designated 104-5 continuous passaging of which in androgen-depleted medium generated two variants: 104-I, passage 40–70; 104-R, passage 80–100	104-5, 10% FBS + 1 nM DHT; 104-I and 104-R, 10% CS-FBS	Slow growing in androgen-depleted medium (104-S); hypersensitive to androgen treatment; increased AR expression and transcriptional activity; PSA positive; AR expression/activity elevated with increasing passage numbers	Kokontis et al. (1994, 1998)
AI LNCaP	wtLNCaP cultured in androgen-depleted medium for 6 months	10% CS-FBS	Increased AR levels; very low PSA levels; highly resistant to drug-induced apoptosis; increased Bcl-2 expression	Gao et al. (1999)
LNCaPabi	wtLNCaP cultured in androgen-depleted medium for ≥ 40 passages	10% CS-FBS	Increased AR levels; hypersensitive to androgen up to passage 75; antiandrogens (Bic and flutamide) show agonistic effects; less sensitive to apoptosis-inducing agents	Culig et al. (1999) and Pfeill et al. (2004)
LNCaP AI	wtLNCaP cultured in androgen-depleted medium for 6 months	10% CS-FBS	More resistant to apoptosis-inducing agents; increased Bcl-2 expression compared to wt	Lu et al. (1999)
LNCaP-CS10	wtLNCaP treated with Bic under androgen-depleted conditions for 4 months	10% CS-FBS + 10 μ M Bic	Bic-resistant, androgen-independent cell line: androgen-sensitive	Ishikura et al. (2010)
LNCaP C-81	Serial <i>in vitro</i> passaging of wtLNCaP in normal growth medium, C-81, cells of passage > 81	5% FBS	Decreased androgen responsiveness above passage 33; no apparent difference in AR protein levels	Igawa et al. (2002)
LNCaP-19	wtLNCaP cultured in androgen-depleted medium, an androgen-independent subline appeared after passage 19	10% CS-FBS	Androgen-independent growth, lower PSA but higher VEGF levels than wt; <i>in vivo</i> tumors show increased microvessel density and altered vessel morphology	Gustavsson et al. (2005)
CL-1, CL-2	CL-1, wtLNCaP cultured in androgen-depleted medium; CL-2, CL-1 re-cultured in androgen-containing medium	10% CS-FBS	Potentiate growth of endothelial and bone marrow stromal cells in coculture; resistance to radiation and cytotoxic agents; tumorigenic and metastatic in nude mice without Matrigel; decreased AR levels; overexpression of Bcl-2, bFGF, IL6, 8, TGF β 1, β 2, EGFR, VEGF, loss of E-cadherin, decreased p53	Tso et al. (2000)
Sublines established after <i>in vivo</i> passage through athymic nude mice				
LNCaP-Pro1-5	Isolated from prostate tumors after orthotopic implantation of wtLNCaP cells into athymic mice; serial passage through mouse prostate	10% FBS	Growth inhibited in androgen-ablated medium; do not grow in castrated mice; low potential to metastasize in athymic mice; apoptosis resistant; produce less PSA than wtLNCaP	McConkey et al. (1996) and Pettaway et al. (1996)
LNCaP-LN1-4	Isolated from lymph node metastasis after orthotopic implantation of wtLNCaP cells into athymic mice; serial passage through mouse lymph node	10% FBS	Grow in castrated mice; high potential to metastasize in athymic mice; apoptosis resistant; produce more PSA than wtLNCaP	McConkey et al. (1996) and Pettaway et al. (1996)
LNCaP-M, LNCaP-C4, -C5, LNCaP C4-2	Cells isolated from tumors established by co-injection of wtLNCaP cells with human osteosarcoma cells into intact (-M) or castrated (-C4, -C5) male mice C4-2, obtained from tumors established by co-injection of C4 and osteosarcoma cells into castrated male mice	10% FBS	PSA; LNCaP-M are nontumorigenic, LNCaP-C4, LNCaP-C5 are tumorigenic in castrated mice when co-injected with fibroblasts; C4-2 are tumorigenic in castrated mice without fibroblasts and are able to form soft agar colonies in serum-free medium; C4-2 have low AR expression and are nonresponsive to androgen; they form metastases after s.c. and orthotopic injection	Thalmann et al. (1994, 2000)

Table 3 Continued

Name	Establishment	Culture conditions	Characteristics	References
LNCaP-C4-2-B, LNCaP-C4-2-Ln	Isolated from bone (B) or lymph node (Ln) metastases developed after s.c. or orthotopic injection of C4-2 cells into athymic male mice	10% FBS	Cytogenetics revealed translocation between 12p and 17q in C4-2-Ln; C4-2-B contain both human and murine metaphases	Thalmann <i>et al.</i> (1994, 2000)
LNCaP-Pre, LNC-RECA, LNC-SF	wLNCaP cells injected into intact (LNCaP-Pre) or castrated <i>Scid</i> mice (LNC-RECA) LNC-SF: wLNCaP cultured in androgen-depleted medium for 6 months	Pre and RECA, 10% FBS SF, 5% CS-FBS	Similar AR levels compared with wLNCaP Better growth of RECA and SF in castrated mice than wt cells	Iwasa <i>et al.</i> (2007)

Bic, bicalutamide; FBS, fetal bovine serum; PSA, prostate specific antigen; wLNCaP, wild-type LNCaP; VEGF, vascular endothelial growth factor; TGF β , transforming growth factor β ; bFGF, basic fibroblast growth factor; IL6, interleukin 6; EGFR, epidermal growth factor receptor.

in AR transactivation was observed (Tables 1 and 2; Marques *et al.* 2005). The distinct growth and androgen-sensitivity properties of these CR-PCa sublines have been exploited to characterize the AR transcriptional response and identify AR bypass pathways during progression to CR-PCa revealing that the AR regulates different functional groups of genes at different stages of PCA progression (Marques *et al.* 2010, 2011).

22Rv1 is an androgen-responsive cell line derived from primary PCA that was xenografted and serially propagated in mice after castration-induced regression and relapse of the parental, androgen-dependent CWR22 xenograft (Sramkoski *et al.* 1999). Compared with LNCaPs, 22Rv1 cells secrete low levels of PSA and express lower levels of AR, which also harbors a rare H874Y mutation (Table 2; Attardi *et al.* 2004). In addition, 22Rv1 cells harbor two AR forms, a larger one expressing three zinc finger motifs due to duplication of exon 3 and a C-terminally truncated, constitutively active form, both of which have been functionally investigated by a number of groups (Dehm *et al.* 2008, Guo *et al.* 2009, Marcias *et al.* 2010, Watson *et al.* 2010, Dehm & Tindall 2011). Consequently, 22Rv1 has become a valuable model system to study AR function, the efficacy of existing drugs and to design novel anti-AR therapies that also target nontruncated regions of AR (Laschak *et al.* 2012, Li *et al.* 2012b). However, these cells produce high titers of the human retrovirus xenotropic murine leukemia virus-related virus, which has implications not only for handling and biosafety but also should be considered when interpreting experimental results (Knouf *et al.* 2009).

PCa stem/progenitor cells

In recent years the concept of a small population of tumor cells that gives rise to the entire tumor has been increasingly explored as a potential explanation for phenomena such as cancer therapy resistance, tumor recurrence and metastasis (Oldridge *et al.* 2012, Yu *et al.* 2012). These putative cancer stem cells (CSCs) are broadly functionally defined as cells within a tumor that: i) possess self-renewal capabilities and ii) can give rise to the heterogeneous lineages of cancer cells that comprise the tumor. Subpopulations of tumor cells with such CSC characteristics have been identified in PCA and other solid malignancies (Oldridge *et al.* 2012, Yu *et al.* 2012). However, the notion of CSCs remains controversial largely due to differences in experimental systems used to determine their self-renewal capacity and uncertainty regarding their cellular origin. For example, PCA stem cells (PCSCs)/progenitor cells could potentially arise from

AR⁺ luminal differentiated cells (which constitute the major cell type within PCa) or AR⁻ basal cells. Although experimental data suggest that PCSCs can derive from either of these cell types, prevailing evidence supports the basal cell-of-origin theory in PCa whereby a small proportion of transformed AR⁻ basal stem cells can still differentiate but form abnormal AR⁺ luminal tumor masses (Oldridge et al. 2012, Yu et al. 2012). In particular, cells that display functional characteristics of PCSCs/progenitor cells express markers typically associated with normal prostate stem cells, e.g. CD44, CD133 and integrins. Notably, CD44 is also enriched in CSCs from tumors other than the prostate, including colon, breast and ovary. Given the low to nondetectable expression of AR in CD44⁺ PCa cells, such basal cell-derived PCSCs/PCa progenitor cells would be expected to form a resistant core after androgen ablation therapy, a notion consistent with the frequent recurrence of PCa (Oldridge et al. 2012, Wang et al. 2012, Yu et al. 2012). Interestingly however, recent data indicate that CD44 may itself be subject to androgenic regulation, raising the possibility that AR regulation of putative stem cell markers may contribute to malignant transformation (Marcinkiewicz et al. 2012). Thus, there is an urgent need to better understand PCSC biology and develop therapeutic strategies to deplete the PCSC pool in PCa. Whilst it is generally preferable to isolate PCSCs from primary cancer cells rather than PCa cell lines, tissue availability is often a limiting factor. Interestingly, several established PCa cell lines such as LNCaP, LAPC-4 and C4-2 may also contain CD44⁺ PCSCs (Miki & Rhim 2008, Lee et al. 2013). Further studies are required to determine whether established PCa cell lines contain bona fide PCSCs and to assess the functional contribution of AR signaling on PCSC behavior.

Benign prostate epithelial cell lines

In contrast to the abundance of PCa cell lines, there are relatively few cell lines derived from benign prostatic epithelium suitable for investigating AR signaling. This is primarily due to difficulties in *in vitro* immortalization and the terminally differentiated nature of the androgen-dependent luminal epithelium such that primary epithelial cultures predominantly exhibit an androgen-independent but proliferative basal/intermediate phenotype (Untergasser et al. 2005, Niranjana et al. 2012). Nonetheless, there are currently three main AR⁺ normal prostate epithelial cell lines PWR-1E, RWPE1 and RC-165N/hTERT, which were immortalized using SV40, human papilloma virus 18 or human telomerase reverse

transcriptase respectively (Webber et al. 1996a, Bello et al. 1997, Kim et al. 2007). These cell lines are androgen responsive, express AR and PSA but do not form tumors when injected into nude mice (Table 2). This latter characteristic has been exploited to investigate the role of putative oncogenes and carcinogens on tumorigenesis indicating the suitability of these cell lines as potential model systems to study processes of oncogenic transformation (Kim et al. 2010, Rhim et al. 2011). It should be noted however that the process of immortalization itself can result in genetic alterations and/or mutation (Stepanenko & Kavsan 2012). To date, these cell lines have predominantly been used to compare gene expression levels and drug efficacy with PCa cell lines (Kim et al. 2007, Deep et al. 2008, Mishra et al. 2010).

A current limitation of primary and some immortalized prostatic epithelial cell lines is their low expression of AR and gradual loss of androgen-responsive differentiation phenotype after serial passage of parental cells (Berthon et al. 1997). AR promoter methylation was excluded as a possible mechanism for the lack of AR expression in primary prostate cell cultures (Grant et al. 1996, Tekur et al. 2001). Rather, it appears that the androgen-responsive phenotype of human prostatic epithelial cells is dependent on their correct differentiation, which can be maintained via 3D coculture with stroma and/or ECM (see Section 3D *in vitro* cell culture models to study AR signaling in PCa; Lang et al. 2001). As an alternative strategy to overcome some of these limitations, a new subline (termed BPH-1-AR) stably expressing AR was recently generated from BPH-1 cells (Yu et al. 2009). The parental BPH-1 cell line is a nontransformed AR⁻ human prostatic epithelial cell line immortalized with SV40 large T antigen and displays a luminal epithelial cytokeratin profile (Table 2; Hayward et al. 1995). The new subline BPH-1-AR, which is androgen responsive, was used to functionally evaluate novel nonsteroidal AR modulators demonstrating its potential suitability as a screening tool for drug discovery (Yu et al. 2009).

Benign and carcinoma-associated prostate stromal cells

The critical role of stromal cells in PCa development and progression was first demonstrated in stromal-epithelial recombination experiments in which nontumorigenic prostatic epithelial cells formed tumors when combined with carcinoma-associated stromal cells but not with benign fibroblasts (Hayward et al. 2001, Cunha et al. 2002, 2003). This stromal reaction is an early feature

common to many malignant epithelial neoplasms initiated via the action of cancer cell-derived secreted factors, in particular transforming growth factor β 1, that modify the surrounding stroma generating a micro-environment which in turn further supports tumor growth and progression (Barclay *et al.* 2005, Ao *et al.* 2007, Verona *et al.* 2007, Sampson *et al.* 2011). The tumor-associated 'reactive' stroma is characterized in particular by activation of fibroblasts but also by recruitment of inflammatory cells, ECM remodeling and enhanced angiogenesis. The inductive properties of reactive stroma are primarily due to the mitogenic secretome of activated fibroblasts, also termed myofibroblasts (Barron & Rowley 2012, Sampson *et al.* 2012).

Recombination experiments also demonstrated that stromal AR is required for the inductive properties of reactive stroma in PCa. For example, nontumorigenic prostate epithelial cells only formed tumors in the presence of functional mesenchymal AR (Cunha *et al.* 2004, Ricke *et al.* 2006). However, the role of stromal AR on PCa development and progression appears to be complex since on the one hand, stromal AR signaling is down-regulated in clinical PCa, whereas stromal AR has also been shown to suppress prostate tumorigenesis (Karlou *et al.* 2010). The underlying reason for these apparent conflicting findings remains unclear, although it may be noted that the transcriptome of stromal AR remains poorly studied, at least in part because only a subpopulation of stromal cells expresses AR, which additionally appear to require paracrine-acting epithelial signals (Lang *et al.* 2001, Cano *et al.* 2007, Berry *et al.* 2011). In general, most immortalized stromal cell lines express only low or undetectable levels of AR (Peehl 2005, Kogan *et al.* 2006). Consequently, most studies analyzing the role of stromal AR in prostate development and carcinogenesis have utilized mouse urogenital sinus mesenchyme (Shaw *et al.* 2006, Cunha 2008). Clearly, further investigations into the role and functional contribution of stromal AR on prostate carcinogenesis are needed. In this respect, the immortalized human prostatic myofibroblast cell line WPMY-1, which is derived from the same prostatic tissue material as RWPE1 cell line (Webber *et al.* 1999), was recently used to determine the stromal androgenic transcriptional response by generating a subline (WPMY-AR), which expresses wild-type AR at levels comparable with LNCaP cells and is responsive to DHT (Tanner *et al.* 2011). In addition to WPMY-1 cells, other studies of prostatic stromal AR have largely employed primary human normal or carcinoma-associated prostatic fibroblasts, which can be readily isolated from biopsy

specimens of patients undergoing radical prostatectomy (Cano *et al.* 2007, Berry *et al.* 2011, Sampson *et al.* 2011).

3D *in vitro* cell culture models to study AR signaling in PCa

Studies using isolated cell lines in 2D culture offer a simple reductionist approach to study cell behavior and have significantly increased our understanding of molecular pathways involved in PCa development and progression. However, a recent study reported that *in vitro* 2D cultured cell lines exhibit a significantly divergent profile of AR-regulated genes compared with xenografts and human PCa tissue. For example, the AR-promoter binding profile of LNCaP, VCaP and 22Rv1 cell lines demonstrated only a 3% overlap with CR-PCa tissue (Sharma *et al.* 2013). This suggests that the AR transcriptome in tissue is distinct from that in cultured cell lines, a finding with potential significant implications for preclinical studies of novel AR-targeting agents. Thus, model systems that more closely reflect the physiological setting of PCa are required for improved translational research and pre-clinical drug screening.

Along these lines, a comprehensive panel of primary and nontransformed prostate epithelial cells as well as commonly used PCa cell lines cultured under 3D conditions has been developed (Harma *et al.* 2010). The gene expression and metabolic profiles of these 3D cultured cell lines have been characterized together with their cellular morphogenic properties as an initial step toward evaluating their usefulness as preclinical screening platforms (Harma *et al.* 2010). It will be interesting to analyze such 3D culture systems to determine whether they more closely recapitulate the AR transcription profile of human tissue than 2D cultured cell lines. However, it may not be sufficient to simply culture epithelial cells under 3D conditions. For example, prostate epithelial cells grown in Matrigel form acinus-like spheroids and show an intermediate (AR⁻) phenotype in monolayer cultures but differentiate when cocultured with fibroblasts into a more luminal phenotype becoming polarized and AR⁺ (Lang *et al.* 2006). Moreover, LNCaP cells cultured together with normal prostate fibroblasts on microcarrier beads under microgravity-simulated conditions respond to inductive androgenic signals with respect to growth and differentiation like that observed *in vivo* (Zhou *et al.* 1997). Similarly, LNCaP cells grown in rotary wall vessels under fluid rotation spontaneously form 3D organoids (Wang *et al.* 2005). Interestingly however, the androgen responsiveness of LNCaP cells grown under these conditions

becomes dependent on prostate stromal cells (Zhou *et al.* 1997, Wang *et al.* 2005). Moreover, a hyaluronic acid-based bilayer hydrogel system supported not only tumoroid formation of LNCaP cells, but also stimulated reciprocal interactions with the tumor-associated stroma (Xu *et al.* 2012). Collectively, these studies indicate that reciprocal interactions between the epithelium, stroma and ECM are critical for epithelial differentiation and both stromal and epithelial androgen responsiveness *in vitro*.

Such 3D coculture model systems are not restricted to investigating stromal–epithelial interactions but have also been used for long-term analysis of PCa cell interactions with immune cells, providing an *in vitro* platform for rapid immunotherapy development (Florczyk *et al.* 2012). In addition, PCa cells and human osteoblasts interacted within a tissue-engineered bone construct in a manner consistent with *in vivo* observations of PCa metastasis indicating the suitability of this model to study mechanisms of PCa metastasis (Sieh *et al.* 2010).

Conclusions

PCa remains one of the most common causes of male cancer-related death in Western nations. The essential role of AR signaling in normal prostate tissue homeostasis and its dysregulation in PCa development forms the basis of androgen deprivation therapy, the current mainstay systemic treatment for advanced PCa. Recognition that AR reactivation is a key mechanism in the progression to CR disease has led to intensive efforts to discern underlying molecular pathways and design novel therapeutic strategies. Consequently, a number of new *in vitro* human cell models have been developed, which mimic different stages and aspects of this heterogeneous disease, for example, with respect to androgen responsiveness and AR status. Although recent studies indicate that cancer cells cultured in physiologically relevant, 3D matrices can recapture many essential features of native tumor tissues, by definition these remain *in vitro* cell models and do not recapitulate all aspects of human PCa. However, the utility of these models is demonstrated by our continued increasing knowledge regarding molecular mechanisms underlying the development and progression of PCa. Future advances in molecular, cellular and bioengineering technologies will support the continued development of *in vitro* models, which in combination with *in vivo* approaches will provide more biologically relevant platforms for mechanistic studies, drug discovery and design ensuring a more rapid transfer of knowledge from the laboratory to the clinic.

Declaration of interest

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

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