Research and Perspectives in Endocrine Interactions

Hormonal Control of Cell Cycle

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Androgen-mediated Control of the Cyclin D1-RB Axis: Implications for Prostate Cancer

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Summary

Prostatic adenocarcinomas are exquisitely dependent on androgen via its cognate receptor (the androgen receptor, AR) for proliferation and survival. This dependence is exploited in the treatment of disseminated disease, wherein ablation of AR activity constitutes first line therapeutic intervention. While initially effective, these strategies ultimately fail, due to inappropriate restoration of AR activity and AR-mediated cellular proliferation. Resultant studies revealed that AR governs the cyclin D1-RB axis, in addition to other phases of the cell cycle. Strikingly, these studies have revealed unexpected cross talk between the AR and several elements of the cell cycle machinery, and aberrations in these pathways have been associated with disease progression. In this review, the molecular communication between AR and the cyclin D1-RB axis will be discussed, with an emphasis on the implications of these pathways for prostate cancer progression and management.

Introduction

Prostatic adenocarcinoma is the most frequently diagnosed malignancy and second leading cause of cancer death amongst men in western countries (Jemal et al. 2005). Significant morbidity associated with the disease results from the failure to effectively manage disseminated prostate cancer, and efforts to improve therapeutic intervention have revealed a pivotal role for hormone action. Local disease can be definitively treated by surgical resection or through radiation therapy, with excellent cure rates for patients presenting with early stage tumors (Kolvenbag and Nash 1999; Nyman et al. 2005). However, late stage and metastatic disease presents a clinical and therapeutic challenge; these tumors respond poorly to standard cytotoxic regimens that act through genomic insult, and lack of effectiveness has been attributed to the relatively indolent nature of the tumor type. Therefore, prostate cancers are treated based on a unique characteristic, in that they are exquisitely dependent on androgen for development, growth, and survival.

The pioneering work of Huggins and Hodges first established that prostate cancers are dependent on serum androgen. Using canine models, these investigators showed that castration of the animals resulted in both an involution of the normal prostate and ablation of spontaneous prostatic adenocarcinomas (Huggins and Hodges 1972).

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Melmed et al.
Hormonal Control of Cell Cycle

Subsequent studies in cell culture and animal models revealed that androgen ablation triggers cell death or cell cycle arrest of prostate cancer cells (Isaacs 1984; Knudsen et al. 1998). Thus, androgen ablation remains the primary course of treatment for all patients with metastatic (including micrometastatic) disease (Jenster 1999). These therapies are initially effective in the vast majority of patients and result in disease remission. However, recurrent tumors arise within a median of two to three years, wherein androgen signaling has been inappropriately restored (Feldman and Feldman 2001). At present, few therapeutic regimens have been described to significantly manage recurrent prostate cancers, and this is considered an incurable stage of the disease. Thus, androgen action underlies both tumor development and tumor progression in prostatic adenocarcinoma. Given the clear addiction of prostate cancer cells to the androgen signaling axis, a concerted effort has been undertaken to determine the mechanism(s) by which androgen induces prostate cancer cell proliferation and survival.

AR is a Master Regulator of Prostate Cancer Growth and Recurrence

Androgen exerts its biological effects through the androgen receptor (AR), a member of the nuclear receptor superfamily that acts as a ligand dependent transcription factor (Fig. 1; Lee et al. 1995; Trapman and Brinkmann 1996). Testosterone is the most abundant androgen in the sera, but in the prostate it is converted to a more potent androgen, dihydrotestosterone (DHT), through the action of a resident enzyme, 5α-reductase (Russell et al. 1994; Russell and Wilson 1994). Prior to ligand binding, the androgen receptor is held inactive through association with heat shock proteins and is precluded from DNA binding. Ligand binding releases the inhibitory heat shock proteins, and the receptor rapidly translocates to the nucleus, where it binds DNA as a homodimer on androgen responsive elements (AREs) within the regulatory regions of target genes (Trapman and Brinkmann 1996). Furthermore, recruitment of co-activators (which contain or recruit histone acetylases) and chromatin remodeling complexes facilitate transcriptional initiation, and AR-dependent gene expression ensues (Gnanapragasam et al. 2000). The specific combinations of cofactors recruited to AREs provide a mechanism for tissue-specific and ligand-specific gene expression. Through these actions, the AR promotes prostate cancer survival and proliferation (Feldman and Feldman 2001; Trapman and Brinkmann 1996). While the comprehensive cohort of AR target genes that underlie each outcome has yet to be clearly defined, discovery of at least one major AR-dependent target gene, prostate specific antigen (PSA; Stephan et al. 2002), has had a major impact on disease management. Specifically, serum PSA is monitored clinically to detect early stage disease, track tumor burden, monitor the efficacy of therapeutic intervention, and detect the emergence of recurrent tumors post-therapy (Ryan et al. 2006). Thus, readouts of AR activity are critical for the assessment of disease progression and therapeutic outcome.

Disruption of AR action is the major therapeutic goal for management of metastatic disease and can be achieved via multiple mechanisms (Feldman and Feldman 2001; Leewansangtong and Soontrapa 1999). First line treatment ablates AR function through ligand depletion, as achieved through bilateral orchiectomy or through the use of GnRH agonists. Adjuvant or second line therapies involve the use of direct AR antagonists (e.g., bicalutamide) which utilize at least two mechanisms of action (Kolvenbag

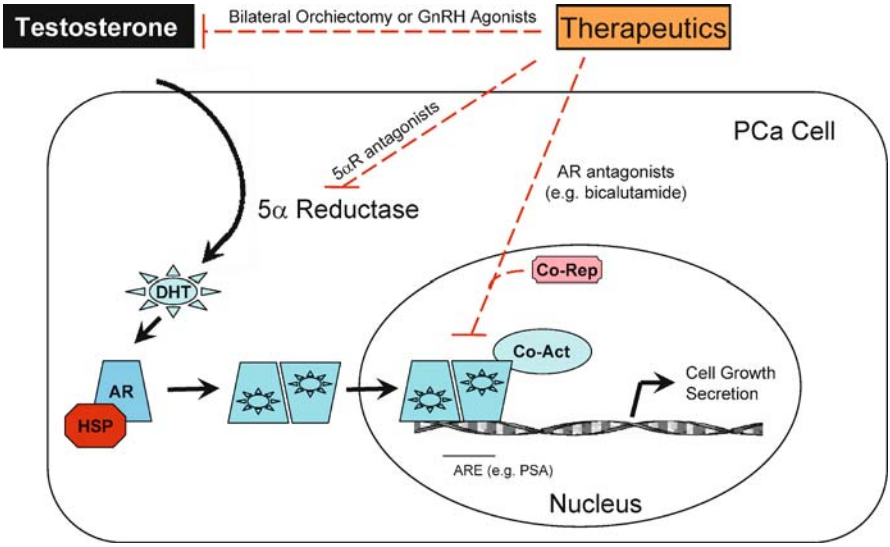

Fig. 1. Androgen signaling and therapeutics in prostate cancer (PCa). Testosterone is converted to a high affinity ligand for the AR, dihydrotestosterone (DHT), in prostate cancer cells. DHT binding causes release of inhibitory heat shock proteins (HSP) from AR and subsequently induces AR homodimer formation, nuclear translocation, and DNA binding to androgen-responsive elements (ARE) of AR target genes (e.g., prostate specific antigen, PSA). Coactivator recruitment (Co-Act) facilitates target gene activation. Disruption of AR activity is the primary treatment for disseminated disease, as achieved by inhibiting androgen synthesis or through the use of direct AR antagonists that compete with DHT for AR binding and recruit corepressors (Co-Rep) to block AR function

and Nash 1999). First, these agents compete for DHT binding. Second, selected AR antagonists trigger the recruitment of transcriptional co-repressors (e.g., NCoR) to AREs, thereby fostering active repression of AR target gene expression (Hodgson et al. 2005). Examination of tumors treated by androgen ablation, with loss of detectable serum PSA, revealed heterogeneous responses concerning cell death or cell cycle arrest amongst dissociated tumor cells. However, this remission is transient, and tumor recurrence is almost invariably observed (Feldman and Feldman 2001; Leewansangtong and Soontrapa 1999). Recurrence is typically preceded by a rise in PSA (also called "biochemical recurrence"; Feldman and Feldman 2001; Trapman and Brinkmann 1996; Visakorpi et al. 1995), and this observation yielded some of the first evidence that tumor progression is associated with restored AR function, despite sustained androgen ablation and/or the use of AR antagonists. Indeed, it is now well established that such "androgen-independent" prostate cancer remains strongly dependent on AR function and that AR activity has been aberrantly restored in recurrent tumors (Chen et al. 2004; Cheng et al. 2006).

Restoration of AR function in recurrent tumors is known to occur through multiple mechanisms (Fig. 2) and in models of cancer is itself causative to resume tumor cell proliferation and therapeutic relapse. First, AR function can be restored through excessive AR expression (including amplification of the locus), as occurs in approximately

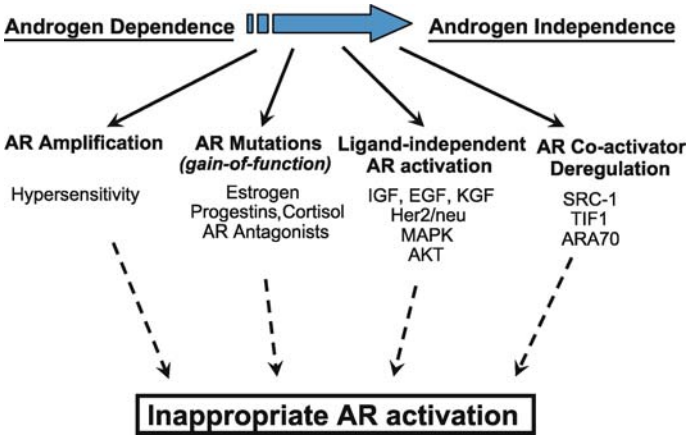

Fig. 2. Mechanisms of therapeutic resistance. Inappropriate activation of AR drives resistance to hormone therapy (androgen independence). This is attributed to multiple pathways, including amplification and mutation of AR, growth factor stimulation, and overexpression of co-activators

30% of recurrent tumors (Chen et al. 2004; Koivisto et al. 1997; Visakorpi et al. 1995). Second, excessive production of specific AR co-activators is observed (e.g., SRC1, TIF1, and ARA70), which can sensitize the receptor to a low ligand environment and/or nullify the effects of AR antagonists (Agoulnik et al. 2005; Gregory et al. 2001; Yeh et al. 1999a). Third, approximately 8–25% of recurrent tumors harbor somatic, gain-of-function mutations of the AR, which render the receptor amenable to activation by a broad spectrum of ligands, including estrogen, progesterone, cortisol, or even some AR antagonists utilized in therapy (Culig et al. 1993; Taplin and Balk 2004). The first AR mutation (T877A) described occurs in the coding region of the ligand binding domain and was identified from patients whose tumors showed a proliferative response to flutamide (Veldscholte et al. 1992). Subsequent studies showed that the T877A mutant can use flutamide as an agonist rather than an antagonist and underlies the proliferative response to this antagonist (Masiello et al. 2004). To date, over 600 different mutations of AR have been described, and further studies have shown that some of these mutants may also be altered in their requirement for AR cofactors, thus further facilitating AR activity (http://androgendb.mcgill.ca/). Fourth, AR can be indirectly activated by other signal transduction pathways commonly deregulated in cancer, including MAPK and AKT, although the precise mechanisms underlying these events remain incompletely understood (Gao et al. 2006; Yeh et al. 1999b). Lastly, provocative new data have shown that macrophage invasion into the tumor microenvironment can induce an IL-1ß-dependent signal transduction cascade that disrupts formation of transcriptional repressor complexes initiated by AR antagonists, thus converting the antagonist into an agonist (Zhu et al. 2006).

Combined, these observations support the current hypothesis that AR is a master regulator of prostate cancer cell proliferation and that androgen ablation/antagonists regimens induce an environment of selective pressure to restore AR function. Given the importance of AR as a key determinant of prostate cancer growth and progression,

it is imperative to dissect the mechanisms by which AR governs cellular proliferation in prostate cancer cells.

AR Governs the Cyclin D-RB Axis in Prostate Cancer Cells

Analyses of AR-dependent cell cycle progression in prostate cancer cells have shown that androgen is a critical regulator of the G1-S transition (Fig. 3). Prostate cancer cells deprived of androgen arrest in early G1 phase, concomitant with loss of cyclin D1 and cyclin D3 expression, attenuated CDK4 activity (expression unchanged), and hypophosphorylated/actived retinoblastoma tumor suppressor (RB; Knudsen et al. 1998; Xu et al. 2006). Recent studies revealed that androgen induces D-type cyclin expression via mTOR-dependent enhancement of translation (Xu et al. 2006). The ability of androgen to modulate cyclin D translation is distinct from mechanisms utilized by other hormones. For example, estrogen induces cyclin D1 transcription in breast cancer cells, through the ability of its cognate receptor (the estrogen receptor, ER) to directly modulate cyclin D1 regulatory regions (Eeckhoute et al. 2006; Sabbah et al. 1999). Thus, androgen regulation of early G1 events is specific to this class of hormone.

In contrast to the D-type cyclins, cyclin E levels remain unchanged by androgen withdrawal, indicating that alteration of cyclin E expression is not a major mechanism of androgen action. However, cyclin A levels and overall CDK2 activity are diminished upon androgen ablation. These data are consistent with the observation that androgen depletion invokes RB activity, as cyclin A is a well-established target of RB-mediated transcriptional repression. Furthermore, androgen depletion induces $p27^{Kip1}$, which is likely to contribute to the observed reductions in CDK2 activity (Knudsen et al.

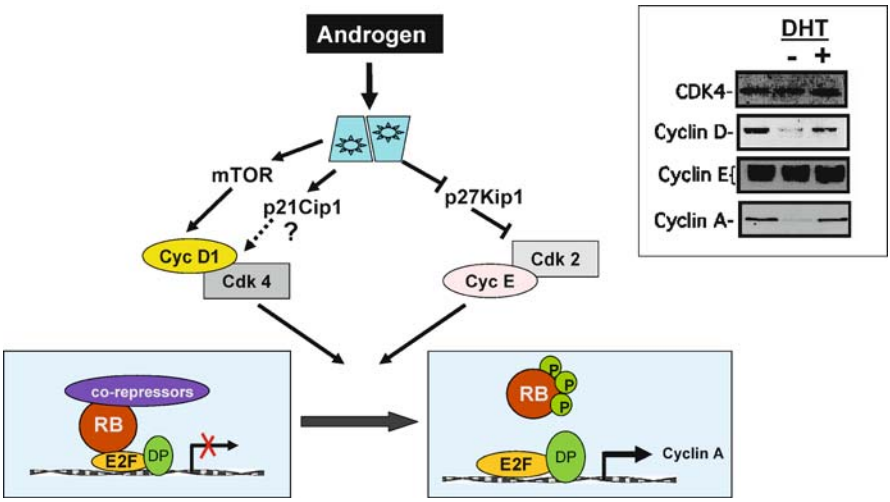

Fig. 3. Androgen-dependent regulation of the G1-S transition. Androgen-mediated induction of G1-S control. Inset data originally appeared in Knudsen et al. (1998)

1998). This supposition is consistent with more recent findings demonstrating that low $p27^{Kip1}$ expression is predictive for shorter time to disease recurrence in prostate cancer (Halvorsen et al. 2003). Similarly, heterozygous PTEN mouse models of prostate cancer have $p27^{Kip1}$ loss, which promotes a tumorigenic phenotype (Gao et al. 2004). Interestingly, upon re-stimulation with androgen, $p27^{Kip1}$ is degraded (Ye et al. 1999). By contrast, $p21^{Cip1}$ expression is lost upon androgen ablation in prostate cancer cells in vitro, which correlates with a higher proliferative index in human tumor specimens (Knudsen et al. 1998; Kolar et al. 2000). Thus, $p21^{Cip1}$ correlates with androgen stimulation and mitogenic proliferation in prostate cancer. Remarkably, $p21^{Cip1}$ has been validated as a direct AR target gene (Lu et al. 1999), and its induction upon androgen ablation may assist in assembling active CDK4/cyclin D1 complexes (Barnes-Ellerbe et al. 2004). In summary, these data culminate in a model wherein androgen induces cyclin D1 accumulation through mTOR, promotes active CDK4/cyclin D1 assembly through $p21^{Cip1}$ induction, and facilitates CDK2 activation through degradation of $p27^{Kip1}$. These collective events result in RB phosphorylation, de-repression of cyclin A expression, and S-phase progression. Based on this knowledge of AR function, it could be hypothesized that aberrations in the cyclin D-RB axis in cancer could supplant the requirement for androgen and contribute to disease progression. Investigations challenging this hypothesis have revealed novel roles for the D-type cyclins in prostate cancer and a critical function for RB in controlling the response to androgen ablation therapy.

Unique Roles of D-type Cyclins in Prostate Cancer

As described above, the AR uses distinct mechanisms to govern G1-S progression. However, a multitude of studies have demonstrated that there is crosstalk between the two pathways, wherein the cell cycle machinery feeds back on AR to control its action (Fig. 4). The concept that AR is regulated as a function of the cell cycle has been documented (Martinez and Danielsen 2002), and elements of both the G1 and G2/M machinery have been implicated in controlling AR function (Chen et al. 2006; Litvinov et al. 2006). It has recently been shown that AR is degraded in mitosis, and it is suggested that AR may therefore serve as a potential "licensing factor" for prostate cancer cells (Litvinov et al. 2006). However, this remains a loose hypothesis and the evidence for licensing action has not been rigorously addressed. More concrete evidence of cell cycle regulation comes from recent studies wherein it was shown that CDK1 activity fosters AR phosphorylation and stabilizes the receptor (Chen et al. 2006), although it is not clear whether this CDK action is direct. CDK6 has also been implicated as an activator of AR; this function is strikingly independent of its kinase activity and is inhibited by cyclin D1 (Lim et al. 2005). This observation is not unexpected, as cyclin D1 is a well-established inhibitor of AR activity in prostate cancer cells (Knudsen et al. 1999; Petre et al. 2002; Reutens et al. 2001), and aberrations in this process are linked to significant cellular outcomes (Burd et al. 2006). As such, this pathway has been the focus of intense research and will be discussed in detail.

Previous studies have clearly demonstrated that androgen stimulates cyclin D1 accumulation and concomitant CDK4 activation (Knudsen et al. 1998; Xu et al. 2006). However, restoration of cyclin D1 expression under conditions of androgen ablation is

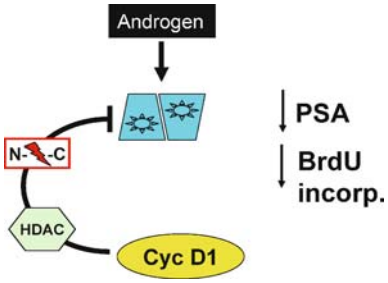

Fig. 4. Cyclin D1-AR negative feedback loop. Stimulation of AR results in accumulation of cyclin D1 leading to CDK4 activation and cell cycle progression. Accumulated cyclin D1 attenuates AR activity by blocking N-C interactions necessary for AR function or by recruitment of histone deacetylase 3 (HDAC3). These events result in PSA expression and attenuated androgen-dependent proliferation. Thus, cyclin D1 modulates the strength and duration of the androgen response

insufficient to drive androgen-independent proliferation (Fribourg et al. 2000). Moreover, it was observed that modest elevations of cyclin D1 in the presence of androgen markedly inhibit (rather than enhance) cellular proliferation (Burd et al. 2005; Petre-Draviam et al. 2003). This unexpected capacity of cyclin D1 to attenuate cell cycle progression is specific to AR-positive, androgen-dependent prostate cancer cells, thus suggesting a putative relationship between cyclin D1 and AR function. Detailed examination of this interaction revealed an unexpected and unique role of cyclin D1 in control of AR activity.

In addition to its ability to modulate CDK4 kinase activity, increasing evidence has demonstrated that cyclin D1 harbors CDK-independent functions in controlling transcription factor action (Coqueret 2002). Cyclin D1 has been shown to directly interact with and modulate a large number of transcription factors, including v-Myb, DMP1, Sp-1, and MyoD. However, the largest class of cyclin D1-associated transcription factors belongs to the nuclear receptor superfamily, including estrogen receptor (ERα), hyroid hormone receptor (TR), PPARγ and AR (Coqueret 2002; Ewen and Lamb 2004). In the case of AR, cyclin D1 binds directly to the N-terminus of the receptor and blocks conformational changes that are required for maximal AR activity upon ligand activation (N-C interaction; Burd et al. 2005; Petre-Draviam et al. 2005). Moreover, cyclin D1 associates with histone deacetylase 3 (HDAC3), and recruitment of HDAC activity is essential for its co-repressor functions (Lin et al. 2002; Petre-Draviam et al. 2005). These actions of cyclin D1 are independent of CDK activity, and a repressor domain within the protein (encoded by amino acids 142-253) has been identified that is capable of supporting both cyclin D1 co-repressor functions (Petre-Draviam et al. 2005). The biological consequence of this event is evident, in that even modest induction of cyclin D1 levels (at stoichiometric levels with the receptor) is sufficient to suppress both AR activity and androgen-dependent proliferation in AR-positive prostate cancer cells (Petre-Draviam et al. 2003). As expected, AR-negative prostate cancer cells are refractory to the repressor function of cyclin D1 (Burd et al. 2006). These data are consistent with observations that AR activity is highly regulated as a function of the cell cycle, wherein cyclin D1 levels inversely correlate with AR ac-

tivity (Martinez and Danielsen 2002). Moreover, in a mouse model of prostate cancer, cyclin D1 levels decrease as a function of progression, whereas cyclin E levels are elevated; this observation led to the hypothesis of a putative "cyclin switch" that may occur in prostate cancer progression (Maddison et al. 2004a), although this concept has yet to be validated in human specimens. Based on these collective observations, it is hypothesized that cyclin D1 serves as a "negative feedback switch" to modulate androgen-dependent gene expression and concomitant cellular proliferation, thereby governing the strength and duration of the androgen response. Strikingly, recent analyses indicated that these "balancing" functions of cyclin D1 are disrupted in prostate cancer (Knudsen 2006).

Cyclin D1 Aberrations in Prostate Cancer: Localization and Expression

Given the importance of cyclin D1 in proliferative control and its ability to promote oncogenic transformation (Diehl 2002; Gladden and Diehl 2005; Sherr 1995), several studies have investigated cyclin D1 status in human prostate cancer. Initially, these studies compared benign prostatic hyperplasia (BPH) to tumor tissue, but this approach has become less common with the increasing availability of normal tissue adjacent to tumor. As summarized in Table 1, cyclin D1 is rarely amplified (Bubendorf et al. 1999; Das et al. 2005; El Gedaily et al. 2001; Gumbiner et al. 1999; Linja et al. 2001) and most (but not all) immunohistochemical studies have overwhelming shown that cyclin D1 is elevated in prostate cancer (Aaltomaa et al. 1999; Drobnjak et al. 2000; Han et al. 1998; Kallakury et al. 1997; Kolar et al. 2000; Murphy et al. 2005; Shiraishi et al. 1998; Shukla et al. 2004). However, elucidation of the relevance of cyclin D1 expression in prostate cancer has yet to emerge, in part due to the divergent criteria used to define positive cyclin D1 staining. Furthermore, it has been observed that cyclin D1 may be localized to the cytoplasm in prostate tumors (Aaltomaa et al. 1999; Han et al. 1998; Shiraishi et al. 1998). This observation is not entirely unexpected, as cytoplasmic cyclin D1 staining has been noted in other tumor types (Culhaci et al. 2005; Dhar et al. 1999; Dworakowska et al. 2005; Hibberts et al. 1999; Kuramochi et al. 2006; Palmqvist et al. 1998; Sato et al. 1999; Temmim et al. 2006; Tut et al. 2001). These complexities, once resolved, may help to reach a common conclusion concerning the importance of cyclin D1 in prostate cancer tumorigenesis.

Several studies have concluded that increased cyclin D1 holds no independent prognostic significance (Aaltomaa et al. 1999; Kallakury et al. 1997), but a subset of studies have documented positive associations between cyclin D1 and proliferative features such as Ki-67 (Drobnjak et al. 2000; Murphy et al. 2005) and $p21^{Cip1}$ (Kolar et al. 2000). Furthermore, $p21^{Cip1}$ is an important assembly and nuclear translocation factor for the cyclin D1/CDK4 complex, has been shown to be stimulated by androgen (Knudsen et al. 1998), and is a validated AR target gene (Lu et al. 1999). Interestingly, unique roles for $p21^{Cip1}$ in the cytoplasm have been ascribed (Coqueret 2003), suggesting that a connection between $p21^{Cip1}$, AR, and cyclin D1 localization may be important for prostate cancer progression. These data imply that more study is required and that cyclin D1 status in conjunction with other clinicopathological variables may have predictive value.

Table 1. Cyclin D1 in Human Prostate Cancer

Study	Tissue Description (n)	Method	Result
Amplification			
Gumbiner et.al., 1999	BPH (15), Primary (93), Lymph Node Metastatic (3)	RT-PCR	Primary (4.3%) amplification
Bubendorf et.al.,1999	BPH (32), Primary (223), Recurrent (54), Metastatic (62)	FISH	Primary (1.2%), Recurrent (7.9%), Metastatic (4.7%) amplification
El Gedaily et.al., 2001	Advanced (27)	CGH	Advanced (3.7%) amplification, 3 gains at 11q13
Linja et.al., 2001	BPH (9), Primary (30), Refractory (12)	qPCR	No amplification
Das et.al., 2005	BPH (33), Primary (46) 6 had Bone Metastases	FISH	No amplification, 13 gains at 11q13
Expression			
Kallakury et.al., 1997	Primary (140), Metastatic (19)	IHC	Primary (22.1%), Metastatic (15.8%)
Shiraishi et.al., 1998	Primary (66)	IHC	Primary (30.3%)
Han et.al., 1998	Primary (50) with normal adjacent	IHC	Primary (30.0%), Normal adjacent (18.0%)
Aaltoma et.al., 1999	Primary (187)	IHC	Primary (71.1%), Normal adjacent weakly positive
Drobnjak et.al., 2000	Primary (86), Bone Metastatic (22)	IHC	Primary (11.6%), Bone Metastatic (68.2%)
Kolar et.al., 2000	Primary (89)	IHC	Increased in primary
Shukla et.al., 2004	BPH (3), Primary (6)	Western	Increased in primary
Murphy et.al., 2005	Normal (40), HGPIN (10), Primary (80), AIPC (10)	IHC	Increased in HGPIN, Primary, and AIPC

Toward this end, we recently assessed the expression of cyclin D1 in human tissue from 38 non-neoplastic, 138 prostate tumors, and three metastatic lymph node specimens (Comstock and Knudsen, in press). We show that while cyclin D1 expression is low or absent in normal tissue, its levels are increased in the majority of localized tumors. Surprisingly, four distinct expression profiles were observed in these tumor sets, wherein the largest fraction of cyclin D1-positive tumors showed cytoplasmic restriction. Expression profiles showed some grade specificity; nuclear cyclin D1 staining emerged almost exclusively in the higher-grade tumors. Additionally, PSA expression was lower in the cyclin D1-positive tumors, indicating that cyclin D1 status may affect expression of serum markers that are dependent on AR activity. The relevance of cyclin D1 status to the proliferative index was also considered, wherein tumors with predominantly cytoplasmic cyclin D1 exhibited the lowest proliferative index, even as compared to cyclin D1 negative tumors.

Lastly, nuclear $p21^{Cip1}$ status was investigated, and $p21^{Cip1}$ levels frequently associated with a more proliferative and predominantly nuclear cyclin D1 phenotype. Together, these data indicate that cyclin D1 exhibits unique expression profiles in prostate cancer and that the status and/or localization of cyclin D1 expression may be associated with meaningful changes in tumor marker expression and proliferative indices.

Cyclin D1 Aberrations in Prostate Cancer: G/A870 and Alternative Splicing

While the studies described above indicate a potential role for cyclin D1 dysregulation in prostate cancer, more substantive demonstration of cyclin D1 alterations in this disease have emerged from analyses of cyclin D1 polymorphisms and splice variants. A known polymorphism (G/A870) of the cyclin D1 locus exists and has been potentially associated with increased cancer risk or poor prognosis for a number of cancers, including prostate (Koike et al. 2003; Wang et al. 2003). The G/A870 polymorphism is a silent mutation but alters a splice donor site at the exon 4-intron 4 boundary (Betticher et al. 1995). Although these data have yet to be directly challenged, the A-allele is predicted to decrease splicing efficacy and to predispose for the production of a known alternative transcript, deemed "transcript b" (Knudsen 2006). Recent investigations revealed that multiple factors (in addition to the polymorphism) likely govern transcript b production, including a subset of SWI/SNF chromatin remodeling complexes that utilize the BRM ATPase (Batsche et al. 2006). The factors that promote production of this alternate transcript are of high interest, as studies have shown that transcript b may be elevated in tumors and/or independently predictive of poor outcome (Knudsen et al. 2006; Koike et al. 2003; Wang et al. 2003).

The cellular consequence of transcript b production is profound. As attributed to a premature stop codon within intron 4, transcript b results in a divergent protein product, cyclin D1b, which harbors distinct functions from the full-length protein (Betticher et al. 1995). The C-terminus of cyclin D1b is unique, as the protein lacks the PEST domain (implicated in the process of protein destabilization) and a phosphorylation site Thr286 (which controls nuclear export and subsequent protein turnover; Betticher et al. 1995). Predicted loss of these domains led to the hypothesis that the actions elicited by cyclin D1b may be attributed to its increased stability and inability to be exported from the nucleus (Knudsen 2006). Recent evidence demonstrated that, while cyclin D1b is indeed largely nuclear, it is not intrinsically more stable (Lu et al. 2003; Solomon et al. 2003). Functional assessment of cyclin D1b in proliferative control revealed that this protein is a poor activator of CDK4-dependent RB phosphorylation (Solomon et al. 2003). This finding was unexpected, as the functional domains of cyclin D1 required to bind and activate CDK4 are conserved in cyclin D1b. While it would be expected that this deficiency may compromise the oncogenic potential of cyclin D1b, in fact cyclin D1b is a significantly more powerful oncogene than its full-length counterpart. Specifically, cyclin D1b has an enhanced ability to induce cellular transformation of NIH-3T3 murine fibroblasts (Lu et al. 2003; Solomon et al. 2003). Similarly, cyclin D1-deficient murine embryonic fibroblasts acquired anchorage independence when cyclin D1b, but not full-length cyclin D1, was reintroduced (Holley et al. 2005). These

collective observations strongly suggest that cyclin D1b harbors increased oncogenic activity, although the mechanisms have yet to be discerned.

With regard to transcriptional control, cyclin D1b is significantly compromised in its ability to regulate estrogen- and androgen-dependent transcription. The ability of

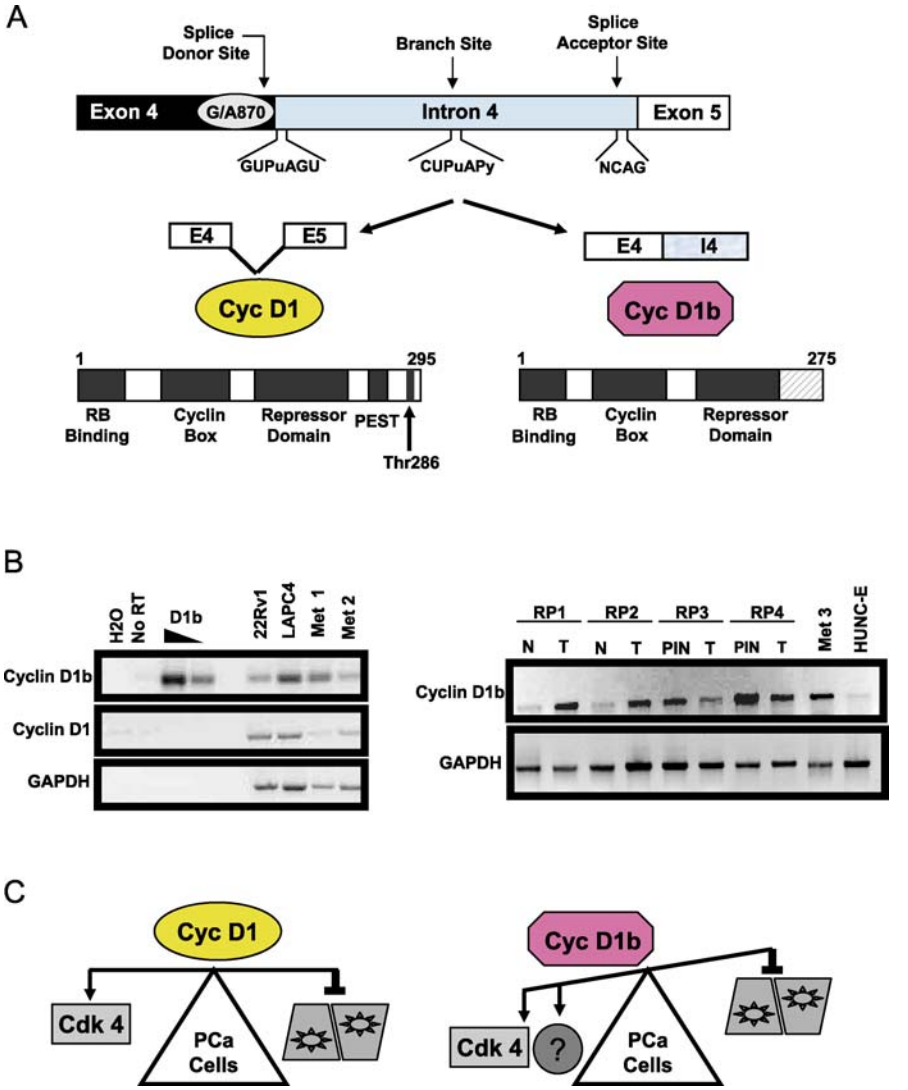

Fig. 5. Cyclin D1b in prostate cancer. (**A**) The cyclin D1b variant arises from a failure to splice at the exon 4/intron 4 boundary. (**B**) The cyclin D1b variant is expressed at high levels in primary and metastatic lesions of prostate cancer. Data extracted from Burd et al. 2006. (**C**) Functional analyses of cyclin D1b show that the protein fails to appropriately regulate AR and yields a proliferative advantage in AR-dependent cells. (Described in Knudsen 2006)

cyclin D1 to modulate the ER is dependent on an "LxxLL" (classical nuclear receptor interaction motif, residues 251 to 255) that is absent in cyclin D1b and therefore has lost the ability to modulate ER activity (Groh and Knudsen, in preparation). The repressor domain of cyclin D1 that is required to bind and modulate AR is mostly intact in cyclin D1b (deletion of amino acids 242-253), but the divergent protein retains the ability to associate with AR both *in vitro* and *in vivo*. However, the cyclin D1b protein is selectively compromised for AR regulation. As demonstrated by monitoring AR activity in transient assays and expression of endogenous AR target genes (e.g., PSA), cyclin D1b is deficient in its ability to regulate AR-dependent transcription. Moreover, cyclin D1b has lost the ability to attenuate androgen-dependent proliferation (unlike full-length cyclin D1); by contrast, this divergent protein promoted cell cycle progression in AR-dependent prostate cancer cells (Burd et al. 2006). Together, these data suggest that cyclin D1b may confer a growth advantage on AR-positive cells by way of its altered ability to modulate AR function (Fig. 5).

The concept that cyclin D1b may facilitate tumor development and/or progression in prostate cancer through a failure to control AR activity is consistent with the established observations that unchecked AR activity is causative for tumor progression (Feldman and Feldman 2001; Trapman and Brinkmann 1996; Visakorpi et al. 1995). Moreover, these data suggest that examination of nuclear cyclin D1 status in tissues should include whether the observed immuno-positivity is attributed to full-length cyclin D1 or the splice variant. This concern may be especially warranted, as recent studies have shown that cyclin D1b is elevated in tumor samples or prostatic intraepithelial neoplasia (PIN) as compared to matched normal tissue from the same individual. Moreover, high cyclin D1b expression was retained in lymph node metastases of prostate cancer (Burd et al. 2006), thus indicating that this presumptive oncogene is likely retained even in late stage disease. Together, these data suggest the intriguing hypothesis that cyclin D1b may facilitate prostate cancer development and/or progression through combinatorial modulation of cell cycle control and androgen-dependent gene expression. This hypothesis is under active scrutiny, and studies to address this premise will clarify the consequence of cyclin D1b expression in prostate cancer.

RB Function in Prostate Cancer and Therapeutic Response

As discussed above, a central cell cycle function of cyclin D1 is to assist in RB inactivation through CDK4/6-dependent phosphorylation, and androgen stimulation utilizes discrete mechanisms to initiate RB inactivation. As such, several models have challenged the impact of RB in the murine prostate. One of the most widely studied rodent models is the *TRAMP* (transgenic adenocarcinoma mouse prostate) transgenic line that utilizes an AR-dependent, prostate specific promoter (probasin) to drive expression of SV40 large T- and small t-antigens in the luminal epithelia. Depending on genetic background, these mice develop high grade PIN and/or prostate cancer within 12 weeks of birth and ultimately develop lung and lymph node metastases by 30 weeks (Gingrich et al. 1997; Greenberg et al. 1995). Androgen deprivation by castration results in decreased tumor incidence as well as the appearance of androgen-independent disease (Gingrich et al. 1997). However, it has been noted that these tumors are typically

neuroendocrine in phenotype. A similar mouse model, *LADY,* that expresses only large T-antigen shows less aggressive disease but is also neuroendocrine in phenotype. Neuroendocrine prostate cancers are relatively infrequent in humans; however, they tend to be fairly aggressive and are associated with a poor prognosis (Kasper et al. 1998). Other transgenic lines expressing the SV40 viral oncogenes also develop neuroendocrine-like prostate cancers, driven by the *Cryptdin-2* or *G*γ-globin promoters, but these models do not appear to progress through an androgen-dependent stage (Garabedian et al. 1998; Perez-Stable et al. 1997). Thus, while viral oncoproteins that act in part to sequester RB can induce prostate cancer, the utility of these systems for analyzing the consequence of RB loss has been limited.

A more specific challenge of RB action in prostate was elucidated by expressing a mutant of large T-antigen that fails to inactivate p53; this event resulted in PIN lesions followed by focally invasive, well-differentiated adenocarcinomas (Hill et al. 2005). However, these effects are likely attributed to other factors in addition to RB. Tissue recombination studies have further defined the role of RB in prostate cancer progression. Specifically, prostate epithelium from RB-deficient embryonic pelvic viscera, when recombined with wild-type rat urogenital mesenchyme under the kidney capsule of male nude mice, results in hyperplastic disease in 40% of grafted samples (Wang et al. 2000). Similarly, conditional RB deletion in the prostate resulted in focal hyperplasia that is potentially reminiscent of early stage disease (Maddison et al. 2004b). These effects are exacerbated by combinatorial p53 deletion, which results in fast-progressing metastatic carcinomas of the prostate (Zhou et al. 2006). Thus, these data show that inactivation of RB may prime prostate cells to become cancerous when subjected to other insults.

The frequency of RB mutation or deletion in human disease has been investigated. RB has been shown to be lost or inactivated in approximately 30−60% of prostate cancers through disparate mechanisms like point mutations in the coding region of RB gene, deletion in the RB promoter region, loss of heterozygosity (LOH), decreased RB protein expression levels, and loss of $p16^{INK4a}$ (an upstream regulator of RB pathway; Brooks et al. 1995; Ittmann and Wieczorek 1996; Jarrard et al. 2002; Tricoli et al. 1996). Despite the prevalence of these events in prostate cancer, very few studies have addressed the consequence of this event for clinical outcome.

RB is activated upon androgen ablation and, based on cell culture models, this event plays an influential role in the cytostatic response to androgen withdrawal. For example, introduction of viral oncoproteins that act in part to sequester RB function can bypass the androgen requirement in AR-dependent prostate cancer cells (Knudsen et al. 1998). This supposition is supported by a clinical study that observed abnormally low RB mRNA in 36% of patients undergoing combined androgen blockade (Mack et al. 1998). Furthermore, in FISH analysis of genetic aberrations after hormonal therapy using advanced prostate tumor specimens, loss of the RB locus was almost four times more frequent after therapy (Kaltz-Wittmer et al. 2000). Combined, these data indicate that RB inactivation and/or deletion may facilitate the transition to androgen independence.

A recent study challenged this hypothesis *in vitro*, through RNA interference-mediated depletion of RB in AR-dependent prostate cancer cells (Sharma and Knudsen, submitted for publication). These data revealed that, while RB depletion did not confer a proliferative advantage in the presence of androgen, RB-deficient cells failed to elicit

a cytostatic response (as compared to RB-positive isogenic controls) when challenged with androgen ablation, AR antagonists, or combined androgen blockade. These data not only indicate that RB ablation can facilitate bypass of first line hormonal therapies but also afford a mechanism to delineate the molecular underpinnings of therapeutic resistance. These studies were extended to determine the impact of RB loss on the response to second line chemotherapeutic intervention, as studies in other cell systems have suggested that loss of RB-dependent DNA damage checkpoints can sensitize cells to cytotoxic agents (Harrington et al. 1998; Knudsen et al. 1998). Indeed, RB-depleted prostate cancer cells demonstrated enhanced susceptibility to cell death induced by a select subset of chemotherapeutic agents (anti-microtubule agents and topoisomerase inhibitor). Combined, these data indicate that RB status may be a critical determinant of therapeutic response in prostate cancer.

Conclusions

The clinical challenges in prostate cancer center on controlling the action of the AR, which is required for both tumor development and disease progression. Selective pressure brought on by androgen ablation typically results in a bypass mechanism to activate the receptor in the absence of ligand and thereby restore AR-dependent cellular proliferation. Thus, dissecting the mechanisms by which AR governs cell cycle progression is instrumental for the design of new strategies to treat recurrent disease. It is apparent that activated AR impinges on the cyclin D1-RB axis to control G1-S progression, and emerging evidence indicates that cross talk between AR and the G1-S machinery serves as an important modulatory node to control the androgen response. Aberrations in these processes can facilitate androgen-independent cellular proliferation and likely contribute to the development of recurrent disease. Future investigations into the consequence of cyclin D1 and RB function in prostate cancer are likely to lead to new avenues of therapeutic intervention.

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