Signal transduction in prostate cancer progression

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ABSTRACT
Prostate cancer is the most frequently diagnosed cancer among men and the second leading cause of male cancer deaths in the United States. When prostate cancer initially presents in the clinic, the tumour is dependent on androgen for growth and, therefore, responsive to the surgical or pharmacological ablation of circulating androgens. However, there is a high rate of treatment failure because the disease often recurs as androgen-independent metastases. Surprisingly, this late-stage androgen-independent prostate cancer almost always retains expression of the AR (androgen receptor), despite the near absence of circulating androgens. Although late-stage prostate cancer is androgen-independent, the AR still seems to play a role in cancer cell growth at this stage of disease. Therefore a key to understanding hormone-independent prostate cancer is to determine the mechanism(s) by which the AR can function even in the absence of physiological levels of circulating androgen. This review will focus on the role of growth factor signalling in prostate cancer progression to androgen independence and thus outline potential molecular areas of intervention to treat prostate cancer progression.

INTRODUCTION
The problem
Prostate cancer constitutes a major health problem in Western countries. It is the most frequently diagnosed cancer among men and the second leading cause of male cancer deaths [1]. In 2004, an estimated 230,110 men will be diagnosed with prostate cancer, and 29,900 will die from the disease in the United States. In fact, most men will display signs of malignancy in the prostate at some point in their lifetime. When prostate cancer initially presents in the clinic, the tumour is dependent on androgen for growth [2]. For patients presenting with disseminated prostate cancer, the tumour is typically dependent on androgen for growth and, therefore, responsive to therapies that take advantage of surgical and/or pharmacological depletion of circulating androgens. However, this type of therapeutic success is often temporary. The disease almost invariably recurs as a metastatic androgen-independent tumour. Whereas surgery is curative for locally confined prostate cancer, there are no effective treatments for androgen-independent metastatic prostate cancer.

It is worth noting that, when prostate cancer progresses, it is variously called ‘recurrent’, ‘hormone...
refractory’ or ‘androgen independent’, because it is resistant to hormone ablation therapy. However, there are data that suggest advanced prostate cancers often are not fully independent of androgen, but have become sensitive to very low levels of androgen [3–6]. Clinically, these cancers may appear ‘androgen independent’. However, because hormone ablation therapies do not eliminate all traces of androgen, at the molecular level these cancers still depend on androgen and on the AR (androgen receptor). This is a source of semantic confusion, because the term ‘androgen independent’ is used in the literature, regardless of whether the cells are completely or only partially androgen independent. In this review, the term ‘androgen independent’ will be used with the understanding that the cells may actually be responsive to very low levels of androgen rather than being completely independent of the steroid.

A note on tumour heterogeneity

Currently, the precursor to prostate carcinoma is thought to be HGPIN (high-grade prostatic intraepithelial neoplasia), which is believed to arise from low-grade PIN [7]. The cell type of origin of prostate cancer is still unknown: prostate cancer cells possess many phenotypical and morphological attributes of both luminal epithelial and basal cells [8,9]. The majority of prostates resected for clinically localized disease show multiple tumours and HGPINs and extensive morphological heterogeneity [10,11]. Furthermore, substantial changes in the genetic makeup within and among prostate tumour foci have been observed [12–14]. This underlines the molecular and biological complexity inherent in prostate cancer progression. This review focuses on the role of growth factor signalling in prostate cancer progression to androgen independence. It is worth noting that many additional aspects of prostate tumorigenesis are beyond the scope of this review and will not be discussed herein.

Seeing the forest and the trees

When thinking about cancer development and progression it is helpful to consider systems theory: the idea of seeing the forest and the trees [15]. Our day-to-day work often focuses on describing the detail complexity of particular aspects of the total system. For example, how a given kinase cascade relays a growth control signal. However, it is the dynamic complexity, how the detail complexity from a multitude of pathways interacts in time and space, that regulates biological processes. In the case of cancer, Hanahan and Weinberg [16] have perhaps best described the biological observations generated from the broader systemic structure of cancer, namely (i) self-sufficiency in growth signals; (ii) insensitivity to anti-growth signals; (iii) evading apoptosis; (iv) sustained angiogenesis; (v) limitless replicative potential; and (vi) tissue invasion and metastasis. Although this review focuses on the first, self-sufficiency in growth signals, and specifically androgen independence in prostate cancer, growth signals undoubtedly effect and are affected by all of the above. It is only through understanding the signal transduction pathways described herein, in the greater context of cell biology and physiology, that will enable the development of novel prostate cancer therapeutics.

GROWTH FACTORS IN PROSTATE CANCER PROGRESSION

The prostate gland requires both androgens and polypeptide growth factors for proliferation, differentiation and maintenance of function [17]. Androgen action on stromal cells leads to the secretion of peptide growth factors, andromedins [18]. Andromedins diffuse to the epithelial cell compartment and regulate proliferation and survival of basal and secretory luminal epithelial cells. Androgen action on epithelial cells stimulates the transcription of genes encoding prostate-specific differentiation factors. In fact, androgen action in epithelial cells suppresses the growth stimulatory effects of andromedins and promotes the differentiated phenotype. However, during prostate tumorigenesis, this system is dysregulated, allowing for growth-stimulatory interactions to occur between androgens and growth factors: interactions that are the opposite of those seen during prostate development and maintenance. Stimulation of prostate cancer cells with growth factors can decrease the requirement for androgen, and the expression of these growth factors and receptors increases as prostate cancer progresses [19–21]. Thus the AR plays a paradoxical role in the prostate, being essential for normal differentiation and maintenance, but subsequently being essential for driving malignant behaviour.

Increases in autocrine and paracrine growth factor loops are among the most commonly reported changes correlated with prostate cancer progression from a localized and androgen-dependent to a disseminated and androgen-independent disease (Figure 1). In advanced prostate cancer, EGF (epidermal growth factor), TGFα (transforming growth factor α), KGF (keratinocyte growth factor), bFGF (basic fibroblast growth factor) and IGF-I (insulin-like growth factor-I) as well as their cognate receptors are all reported to be overexpressed [19,20,22]. Additionally, there appears to be a paracrine relationship between the EGFR (EGF receptor) and TGFα in primary androgen-dependent prostatic tumours; the tumour cells express EGFR and the surrounding stromal cells express TGFα [23]. However, in androgen-independent metastases, the prostate tumour cells co-express EGFR and TGFα, consistent with autocrine regulation. A similar observation has been made with bFGF expression in prostate cancer cell lines. The androgen-dependent prostate cancer cell line LNCaP does not produce bFGF and requires co-inoculation
As prostate cancer progresses to a hormone-independent and metastatic disease, there is an increase in the production of growth factors and their cognate receptors. Additionally, tumour cells become capable of autocrine production of growth factors such as TGFα. Growth factor signal transduction pathways have been shown to stimulate AR activation, suggesting that the increase in growth factor and receptor expression could be causal in prostate cancer progression to androgen independence. That all of these growth factors utilize the Ras/MAPK pathway for a portion of their signal transduction activity suggests that this pathway acts as a convergence point for numerous signalling inputs, making it a potential target for therapeutic intervention.

ARBP, AR-binding protein; PTHrP, parathyroid hormone-related peptide; SRE, steroid response element; SRF, serum response factor.

Previous studies have demonstrated that polypeptide growth factor signal transduction pathways can stimulate AR activation, suggesting that the increase in growth factor and receptor expression could be causal in prostate cancer progression to androgen independence. Growth factor stimulation has been reported to render ARE (androgen response element)-driven promoters hypersensitive to, or independent of, androgen [19,20,22,29–34]. Culig et al. [22] investigated the effects of growth factors on stimulation of AR-mediated transcription. DU145 cells, a prostate cancer cell line that expresses neither AR nor PSA (prostate-specific antigen), were co-transfected with an expression vector encoding the AR and CAT (chloramphenicol acetyltransferase) reporter constructs driven by either a synthetic ARE or by the PSA promoter. IGF-I was able to stimulate reporter gene expression to levels comparable with those induced by the synthetic androgen R1881. This observation was independent of the promoter used. EGF and KGF were also able to induce reporter gene expression, but only in experiments using the ARE-driven reporter construct. Growth-factor-induced reporter gene expression was dependent on co-transfection of the AR expression construct and was blocked by the AR antagonist bicalutamide. In this same study, activation of endogenous AR by IGF-I in LNCaP cells was demonstrated using PSA production as a marker. Again, bicalutamide blocked the effect of IGF-I on PSA production.

Additionally, studies by Nazareth and Weigel [35] demonstrated that the AR could be activated by a PKA

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(protein kinase A) activator in the absence of androgen. This activation can be blocked by a PKA inhibitor peptide and the AR antagonists bicalutamide and flutamide, indicating that the activation effect was due to PKA and dependent of AR. Furthermore, Sadar [36] found that treatment of LNCaP cells with PKA activators resulted in a dose- and time-dependent increase in PSA mRNA levels. Moreover, the AR antagonist bicalutamide blocked the PKA-dependent increase in PSA mRNA.

Janne and co-workers [5] did similar studies using CV-1 and HeLa cells. Activation of an ARE-driven CAT reporter construct was induced by EGF in the absence of androgen when AR was co-expressed in these cell lines. However, when the MMTV promoter was used to drive expression of a CAT reporter, EGF (and IGF-I) stimulation was dependent on the presence of androgen. Unlike the findings by Culig et al. [22], these investigators failed to detect ligand-independent activation of the AR. However, they did see stimulation of androgen-dependent activity in response to growth factors. Thus the ability and degree of ligand-independent activation of the AR appears dependent on the promoter and cell type. Collectively, these experiments suggest that growth factor signalling can regulate androgen-responsive genes by a mechanism that is AR dependent and androgen independent.

Consistent with this, forced overexpression of HER2/neu in androgen-dependent prostate cancer cells could drive androgen-independent growth [37,38]. After the initial observation that androgen-independent sublines of LAPC4 cells expressed elevated levels of HER2/neu, Craft et al. [37] generated LAPC4 cell lines overexpressing HER2/neu. These cell lines displayed androgen-independent growth and activated the AR pathway in the absence of ligand. Importantly, the HER2/neu-expressing cells synergized with low levels of androgen to activate PSA transcription and growth. Yeh et al. [38] overexpressed HER2/neu in LNCaP cells and demonstrated that activation of the pathway induced AR transcription and androgen-independent growth.

Studies have also shown that the small molecule dual EGFR/HER2 inhibitor PKI-166 can inhibit the growth of prostate cancer xenografts [39]. Additionally, use of a monoclonal antibody, 2C4, that sterically hinders HER2 heterodimerization inhibited prostate cancer cell growth in vitro and in vivo [40]. However, examination of clinical specimens has provided conflicting results: depending on the study, there is either no effect or an increase in HER2/neu levels [41–44].

Most recently, Mellinghoff et al. [45] has used small molecule inhibitors and siRNA (small interfering RNA) to study the relative roles of ErbB receptor tyrosine kinases in prostate cancer progression. PKI-166 inhibited AR transcriptional activity, protein stability, DNA binding and phosphorylation on Ser11. Use of EGFR-selective small molecule inhibitors, EGFR-negative cells and siRNA demonstrated that PKI-166-mediated anti-androgen effects were due to inhibition of HER2 not EGFR. This work demonstrates that signals emanating from HER2/ErbB3 heterodimers regulate AR activity in LNCaP and LAPC4 cell lines.

Collectively, the above-mentioned studies suggest that growth factor receptor signals can activate or sensitize the AR to reduced levels of ligand. The hypothesis is that the activation/sensitization is mediated by tyrosine kinase receptors and their downstream signalling effectors through regulated changes in phosphorylation of the AR or of AR-associated proteins. The diversity of these changes in autocrine and paracrine signalling predicts that, at least in the context of prostate cancer, attempts to utilize a single receptor/ligand pair as a therapeutic target will not be generally effective. To identify optimal targets for therapy, it will be necessary to identify the downstream signalling intermediates that are shared by these diverse receptors and ligands.

### RAS SIGNALLING

Virtually all of the growth factor receptors up-regulated in prostate cancer activate Ras for a portion of their signal transduction activity. Yet, Ras mutations are infrequent in prostate cancer [46]. This is consistent with the hypothesis that wild-type Ras is chronically activated by autocrine and paracrine growth factor stimulation in prostate cancer. Thus there is no selective advantage for growth of cells with mutually activated Ras. Because Ras signalling represents a convergence point for numerous diverse extracellular signals, Ras and its effectors may be appropriate targets for therapeutic intervention.

### Not all Ras is created equal

The Ras subfamily of small GTP-binding proteins control signal transduction between the membrane and the nucleus [47–49]. They are activated when bound to GTP and inactive when bound to GDP. These states are regulated by the balance between the intrinsic GTPase activity of the proteins, their interactions with inactivating proteins that accelerate their GTPase activity [GAPs (GTPase activating proteins)] and with activating proteins that regulate the exchange of GDP for GTP [GEFs (guanine nucleotide-exchange factors)]. Thus they can function both as molecular switches and timers. The founding members of the Ras subfamily, H-Ras and K-Ras, were discovered as oncogenes and most of the related proteins also have oncogenic activity when overexpressed in an activated form in the appropriate cell background. Most of our knowledge about the biochemistry of Ras signalling is based on analysis of H-Ras; however, K-Ras is the isoform that is most frequently mutated in human cancers [50,51].

The relative contributions of the different Ras isoforms [H, K(A), K(B), and N] in the myriad of cellular responses...
attributed to Ras signalling are unresolved. Genetic evidence is strongly suggestive that there are differences in the isoforms. Disruption of the gene encoding K-Ras results in an embryonic-lethal phenotype not observed in H- or N-Ras knockouts [52–55]. A multicompart model where the different Ras isoforms are localized in different plasma membrane compartments has been proposed [56,57], although there are conflicting data regarding this hypothesis [58,59]. The multicompart model is consistent with the only sequence divergence between the Ras isoforms being located in the C-terminal hypervariable region; this region contains sequences for post-translational modifications and thus membrane localization [60].

It is clear that Ras proteins function as part of a network of signalling molecules that are regulated both spatially and temporally following growth factor stimulation of cells. The extent of activation of the different Ras isoforms is unknown, but probably dependent on the stability of Ras–GTP effector complexes and the kinetics of cycling through these complexes. In this review ‘Ras’ is used generically. The evidence for Ras activation in prostate cancer is described below; however, it is not known if the different Ras isoforms play a differential role in prostate cancer progression to androgen independence.

RAS ACTIVATION IN PROSTATE CANCER

To test whether Ras might be activated during prostate cancer progression in patients, we examined 82 paraffin-thin sections from primary and metastatic prostate tumour specimens with an activation-state-specific phospho-MAP kinase (mitogen-activated protein kinase) antibody [61,62]. Activation of MAP kinase in this case was used as a surrogate for Ras activation, because it is not possible to directly measure Ras activity in these samples. Non-neoplastic prostate tissue showed little or no staining with antiserum against activated MAP kinase. In prostate tumours, the level of activated MAP kinase increased with increasing Gleason score and tumour stage. Moreover, tumour samples from two patients showed no activation of MAP kinase before androgen ablation therapy; however, following androgen ablation treatment, high levels of activated MAP kinase were detected in the recurrent tumours. Finally, we found that in the hormone-dependent CWR22 prostate cancer xenograft, although the tumour regresses after castration, its recurrence correlates with up-regulation of phospho-MAP kinase (R. E. Bakin and M. J. Weber, unpublished work). Thus it is clear that activation of the MAP kinase pathway correlates with prostate cancer progression in a variety of settings.

Activation of Ras is sufficient to induce androgen-independent growth of prostate cancer cells; expression of an activated v-Ha-Ras in androgen-dependent LNCaP cells enabled LNCaP cells to grow in the absence of androgen [63]. Recent studies in our laboratory suggest that Ras stimulates activation of androgen-responsive gene expression through Raf and MAP kinase in an AR-dependent and androgen-independent manner (R. E. Bakin and M. J. Weber, unpublished work). Effector domain mutants of Ras were co-transfected into LNCaP cells with a PSA or ARE promoter-driven luciferase reporter construct. The Ras effector domain mutant selective for Raf signalling stimulated luciferase expression, whereas those mutants selective for either RalGDS (Ral guanine nucleotide dissociation stimulator) or PI3K (phosphoinositide 3-kinase) did not. Co-transfection of activated MEK [MAP kinase/ERK (extracellular-signal-regulated kinase) kinase; a downstream effector of Raf] also stimulated PSA and ARE promoter-driven expression (D. Gioeli and M. J. Weber, unpublished work). Therefore Ras signalling via MEK appears to regulate androgen-responsive gene expression. This suggests that Ras activation of androgen-responsive gene expression is dependent on the AR.

More recently it has been demonstrated that Ras activation can play a causal role in moving prostate cancer cells towards decreased hormone dependence and increased malignant phenotype [64]. Building on the work of Gelmann and co-workers [63], Bakin et al. [64] expressed activated V12 Ha-Ras effector loop mutants in LNCaP cells, which are dependent on androgen for growth in vivo and responsive to androgen in vitro. The Ras effector loop mutants preferentially activate one set of effectors against another. The effects of these mutants on androgen dependence of growth and tumorigenicity were then evaluated. Some of these activated Ras mutants dramatically reduced the androgen requirement of these cells with respect to growth and PSA expression. The mutants that caused these biological changes were those that caused an intrinsic activation of the MAP kinase pathway under basal, serum-free conditions (Thr35 → Ser and Glu37 → Gly). This correlates activation of the MAP kinase pathway with changes in androgen dependence in cell culture. Expression of Ras also increased the ability of LNCaP cells to form tumours and to resist regression after castration. Collectively, these findings show that activation of Ras signalling is sufficient for progression of LNCaP cells towards androgen independence. Moreover, progression correlates with activation of MAP kinase signalling.

The necessity of Ras signalling in progression has been shown in at least one model: expression of a dominant-negative N17 Ha-Ras can actually restore androgen dependence to an androgen-independent cell line. C4-2 cells were derived by Chung and co-workers [65] from LNCaP cells by serial passage in castrated mice. C4-2 cells demonstrate decreased androgen dependence of growth both in vitro and in vivo, increased tumorigenicity in vivo and the ability to grow in soft agarose (anchorage
independence) compared with the parental LNCaP cells. Expression of the dominant-negative Ras under the control of a tetracycline-inducible promoter in C4-2 prostate cancer cells restored androgen dependence to the androgen-independent C4-2 cells [66]. When implanted in nude mice, the C4-2 derivatives continued to grow after castration, or when dominant-negative N17 Ras was induced with doxycycline. However, the tumours regressed, in most cases completely, when the mice were castrated and treated with doxycycline to induce N17Ras.

In summary, the overexpression of growth factors and receptors utilizing Ras signalling, and the activation of MAP kinase, correlates with prostate cancer progression. Additionally, experimental models such as CWR22 display activated MAP kinase and expression of activated Ras makes LNCaP cells less dependent on androgen. Moreover, expression of dominant-negative Ras restores androgen dependence to C4-2 cells. Thus our findings and those published previously clearly implicate Ras signalling in progression to androgen independence. What signalling pathways downstream of Ras are important in prostate cancer progression?

**MAP KINASE SIGNALLING IN PROSTATE CANCER**

There is sufficient evidence to suggest that the Raf/MEK/ERK pathway plays a critical role in the modulation of AR activity in response to Ras. Our initial studies examining MAP kinase activity in prostate cancer material suggested MAP kinase activity correlated with progression to an increasingly advanced and hormone-independent disease [61,62]. Additionally, the Ras effector loop mutants that had the greatest biological effect on LNCaP cells in vitro were the mutants that activated MAP kinase [64,66]. Furthermore, all androgen-independent xenografts displayed elevated phospho-MAP kinase, regardless of whether their androgen independence was selected by serial passage, or generated by expressing Ras (R. E. Bakin, D. Gioeli and M. J. Weber, unpublished work).

Molecular evidence that MAP kinase signalling directly affects AR activity also exists. Co-transfection of a mutationally activated MEK will drive the AR-dependent expression of a reporter plasmid controlled by the PSA promoter or by tandem AREs (D. Gioeli and M. J. Weber, unpublished work). Consistent with this, inhibition of MEK with PD98059 blunts the ability of androgen to stimulate expression of an ARE-driven reporter (D. Gioeli and M. J. Weber, unpublished work). However, the mechanism(s) by which MAP kinase signalling modulates expression of these reporter constructs remains to be fully elucidated.

Although MAP kinase signalling is an important component of Ras-driven progression to androgen independence, it is clear that it is not the whole story. Dominant-negative N17 Ras expression combined with castration-induced tumour regression [66], yet our preliminary data suggest that MEK inhibition combined with androgen ablation is only cytostatic (D. Gioeli, R. E. Bakin and M. J. Weber, unpublished work). Conversely, although we can readily isolate LNCaP clones expressing Ras effector loop mutants that activate MAP kinase, we have been unable to stably express mutationally activated MEK in LNCaP (R. E. Bakin, D. Gioeli and M. J. Weber, unpublished work). Collectively, these data indicate that some aspect of Ras signalling is necessary for growth, survival and androgen responsiveness of prostate cancer cells, beyond just the MAP kinase pathway. What other Ras effectors may be involved in the progression of prostate cancer to androgen independence?

**RAS EFFECTORS**

Ras proteins function as part of a network of signalling molecules that includes kinases, adapters, other Ras proteins, as well as the GEFs and GAPs. Ras proteins are regulated both spatially and temporally following growth factor stimulation of cells. Ras is a multi-effector signalling molecule that has been shown to engage multiple signalling pathways (Figure 2). Ras effectors are defined as proteins that (i) preferentially bind to the GTP-bound form of Ras; (ii) have activity modulated by Ras; and (iii) transduce the biological activity of Ras [67]. The best-characterized Ras effectors are the Raf serine/threonine kinases. Raf activation leads to stimulation of the MEK and MAP kinases (discussed above).

Another well-characterized Ras effector is PI3K, which has a role in both cell proliferation and survival signalling. The main activity of PI3K is to convert PtdIns(4,5)P2 into PtdIns(3,4,5)P3, which, in turn, facilitates Akt activity. There is a well-established role of PI3K signalling in prostate cancer progression [68,69] and the loss of PTEN (phosphatase and tensin homologue deleted on chromosome 10) expression in late-stage prostate cancers underlines the role of PI3K signalling in the disease [70–72]. However, it is clear that PI3K signalling is not the only survival signal downstream of peptide growth factors [73].

Recently, members of the RASSF (Ras association domain family protein) gene family that potentially act as tumour suppressors have been identified as candidate Ras effectors [74–77]. Loss of expression of NORE1 (novel Ras effector 1) and RASSF1, members of the RASSF gene family, has been observed in a variety of cancers [78–80]. The interaction of Ras with NORE1 has been shown to regulate apoptosis [81]. Thus it seems very likely that the ability of Ras to trigger either growth or apoptosis depends on the balance of interactions between pro-growth, pro-survival and pro-death effectors [68,82].
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Figure 2  Ras: a multi-effector GTPase
Ras proteins function as molecular switches that facilitate transduction of extracellular signals. Ras mediates the activation of multiple effector pathways that mediate cell growth and survival. When dysregulated, Ras signalling promotes tumorigenesis. MST, mammalian Sterile20-like; PKD, phosphoinositide-dependent kinase; PLC, phospholipase C; PLD, phospholipase D; ROCK, Rho-associated kinase; RTK, receptor tyrosine kinase.

The signalling activity of Ras GTPases occurs not only through engagement of direct effectors, but also by the recruitment of other GTPases, especially other members of the Ras subfamily (e.g. Rap) and members of the Rho subfamily (e.g. RhoA, Rac1 and cdc42). This ‘hierarchical networking’ between different Ras isoforms is controlled, in part, by interactions with GEFs, GAPs and downstream effectors [49,83–87]. For instance, RalGEFs are important in Ras-mediated transformation. RalGEFs, such as RalGDS, link Ras signalling to the activation of the small GTPases RalA and RalB. In human cells, the Ras effector loop mutant that preferentially activates RalGDS was able to transform cells rendered transformation sensitive [88].

Ras effectors are also shared with other small GTPases; Ras and Rap bind to both c-Raf-1 and B-Raf. This may explain why Rap1 was originally isolated as an inhibitor of transformation by K-Ras [89]. However, Rap1 can be activated by different GEFs that, in turn, are responsive to different agonists (e.g. cAMP and Ca²⁺) [90]. Rap1 may also be negatively regulated by different GAPs. Thus Rap1 could co-operate with Ras in regulating the context and the timing of signalling through Raf. Indeed, Rap is reported to control the late phase of MAP kinase activation in NGF (nerve growth factor)-treated neurons [91]. In prostate cancer cells, Rap1 is responsible for the synergism between EGF and agonists that elevate cAMP, notably neuroendocrine factors that often characterize aggressive disease [91a]. Similarly, many of the GEFs bind more than one Ras member and thus can serve as regulators of the balance between activation of one or another.

How this balance is achieved and the specifics of the way the proteins are networked is very much dependent on cellular context. Indeed, there are reported differences in the requirements for Ras effectors in rodent and human cells [88].

The inherent complexity in Ras signalling appears necessary to co-ordinate multifaceted responses to extracellular signals. Multiple Ras partners and effectors communicate with each other continuously, through various feedback loops, regulating the expression and activity of various Ras family isoforms. Thus specific subsets of signals control different aspects of tumorigenesis in different cellular contexts. For example, dominant-negative N17-Ras blocks the ability of EGF to turn on the MAP kinase pathway in LNCaP cells, whereas dominant-negative N17-Rap1 only blocks the ability of EGF and cAMP to synergize with each other [64,91a].

AR IN PROSTATE CANCER

The AR is a ligand-dependent zinc finger DNA-binding protein that regulates transcription of a variety of gene products [92]. The AR gene is located on Xq11.2 and encodes a multidomain protein. As with other steroid receptors, the AR consists of an N-terminal transcriptional regulatory domain (AF-1) that can function in the absence of ligand, a DNA-binding domain, a hinge region and a C-terminal ligand-binding domain that is also associated with a second transcriptional regulatory function (AF-2) (Figure 3A). The N- and C-terminal
domains are involved in homotypic dimerization and binding to other transcriptional regulatory proteins. When not bound to ligand, the AR is sequestered with chaperones and is not concentrated in the nucleus. Following ligand binding, a nuclear import signal is exposed and the receptor becomes concentrated in the nucleus where it binds DNA, homodimerizes in a reaction that involves interactions between the N- and C-termini, and interacts with a constellation of transcriptional coregulators, transcription factors and components of the basal transcription machinery [93–110]. Peptide-growth-factor- and Ras-mediated signalling could modulate AR function at any of these steps, and it will be important to determine where that regulatory intersection occurs.

Late-stage androgen-independent prostate cancer almost always retains expression of the AR, despite the near absence of circulating androgens [111]. A majority of prostate tumours obtained from patients failing androgen ablation therapy overexpress the AR, sensitizing the AR to low levels of androgen [112]. This overexpression is often associated with gene amplification (30 %) [112,113]. Frequently, the AR is mutated in advanced prostate cancers (10–40 %), which often results in an AR that can be activated by non-androgen ligands [3,20,114–116]. Furthermore, overexpression of transcriptional co-activators often accompanies prostate cancer progression, and this facilitates the activity of the AR [97,117,118]. These observations strongly suggest the continued involvement of the AR in the growth and survival of androgen-independent prostate cancers. Thus, although late-stage prostate cancer is androgen independent, the AR still seems to play a significant role in cancer cell growth at this stage of disease.

Two lines of recent data support the critical role the AR plays in the progression of prostate cancer. The first comes from studies demonstrating that inhibiting AR expression using antibodies, ribozymes or antisense oligonucleotides led to an inhibition of prostate cancer cell growth [115,119–121]. Work by Tindall and co-workers [119] used an AR hammerhead ribozyme to disrupt AR expression in LNCaP prostate cancer cells; knockdown of AR expression resulted in a dramatic inhibition of cell growth. Work from Culig and co-workers [120,121] used antisense oligonucleotides to down-regulate AR expression both in tissue culture cells and in prostate cancer xenografts.

The second is from a genome-wide study comparing androgen-dependent and androgen-independent prostate cancers that found an increase in AR expression was the only consistent change among all the samples examined [4]. Chen et al. [4] used microarray gene profiling of isogenic prostate cancer xenografts and found that a 2–5-fold increase in AR mRNA was the only gene expression change consistently associated with androgen-independent disease. This increase in expression hypersensitized the AR to low levels of ligand. The authors also showed that the increase in AR levels was both necessary and sufficient to drive prostate cancer progression to androgen independence. Surprisingly, the increase in AR levels converted AR antagonists into agonists. This antagonist–agonist conversion was associated with changes in the recruitment of co-activators and co-repressors.
at AR target promoters. This study is supported by other work examining AR protein stability in different prostate cancer cell lines [122]. The AR protein was 2–4 times more stable in recurrent prostate cancer cell lines compared with androgen-dependent lines. A high level of AR expression and stability was associated with an increased sensitivity to hormone.

Collectively, these studies suggest that, although advanced prostate cancer may be functionally independent of androgen, it is not independent of the AR. The AR-dependent regulatory mechanisms are subverted not bypassed. As described above, the AR compensates for a deficiency of androgen in several ways in recurrent prostate cancer: (i) the AR accumulates mutations that broaden its specificity for ligand [20,100,116]; (ii) the AR is overexpressed, hypersensitizing the AR to low levels of ligand [4,112,113]; (iii) transcriptional coactivators are overexpressed, facilitating AR activity [3,97,123]; and (iv) the AR can be activated functionally in response to signal transduction from growth factors, as described above [5,19,20,22,29–31,33,34,124]. These compensatory mechanisms are not mutually exclusive and are likely to be mutually reinforcing. A therapy that targets any aspect of the AR compensatory mechanisms could effectively treat androgen-dependent and androgen-independent prostate cancer. This review focuses on the mechanism by which growth factor signalling alters AR function, because this is a mechanism that is likely to play a role in at least half of advanced prostate cancers, is subject to therapeutic intervention and provides an opportunity to understand how growth factor and Ras signalling can integrate with the functions of nuclear receptors.

**ROLE OF AR PHOSPHORYLATION**

The steroid hormone receptors are ligand-activated transcription factors. In addition to regulation by steroid, they are also regulated by post-translational modifications generated by signal transduction pathways. Thus they function not only as transcription factors, but also as nodes that integrate multiple extracellular signals. Much of the literature referenced above suggests that kinase cascades regulate AR function in part by activating the AR in the absence of ligand or sensitizing the AR to reduced levels of ligand. The ability of signalling cascades to influence AR function may play a significant role in the development and progression of prostate cancer where the increase in signal transduction activity has been associated with the acquisition of androgen-independent disease.

Previous studies have inferred candidate phosphorylation sites on the AR by *in vitro* phosphorylation reactions and/or by identifying kinase consensus sites and then mutagenizing them. Sites so identified include serine residues at positions 81, 94, 213, 515, 650 and 791 [123,125–128]. (All AR amino acid numbers in this review are based on NCBI accession number AAA51729 [129,130]). However, these determinations, although a useful first step, are not definitive because *in vitro* kinase reactions are often not selective, and mutagenesis can alter the phosphorylations on sites distinct from the ones mutagenized. Ser<sup>515</sup> was directly identified as a phosphorylation site in baculovirus-overexpressed AR using MS [131]. This is the first site identified in living cells either by MS or by *in vivo* metabolic labelling. However, because unequivocally identifying the *in vivo* sites of AR phosphorylation is fundamental to understanding the interactions of the AR and cell signalling, it was necessary to perform an extensive study of AR phosphorylation to explore regulated changes in AR phosphorylation as a possible mechanism for activation/sensitization of AR-dependent gene expression by cell-surface receptors and their downstream signalling effectors.

In what turned out to be a formidable technical challenge, the seven major phosphorylation sites on the AR in living cells were mapped [132]. One site is constitutively phosphorylated, six sites are regulated in response to androgen, and one of these, Ser<sup>629</sup>, becomes phosphorylated in response to a number of non-steroid agonists, including EGF, PMA, forskolin [132] and anisomycin (D. Gioeli and M. J. Weber, unpublished work). In collaboration with Bryce Paschal, we found that phosphorylation on this site regulates nuclear export (B. E. Black, D. Gioeli, M. J. Weber and B. M. Paschal, unpublished work). We have also found that when one androgen-induced phosphorylation site, Ser<sup>635</sup>, is mutated to alanine, the AR gives a heightened transcriptional response to steroid, as measured by a reporter assay [132].

Although ERK is capable of phosphorylating AR *in vitro* on Ser<sup>515</sup> [118], we did not detect phosphorylation on this residue in living cells [132]. Moreover, the peak of MAP kinase activation following growth factor stimulation occurs around 10–15 min, whereas phosphorylations on the AR occur more slowly, peaking after one or more hours following agonist stimulation. Addition of a MEK inhibitor did not substantially alter the pattern of AR phosphorylations. However, this does not necessarily mean that the AR is not an *in vivo* ERK substrate. It is possible that the Ser<sup>515</sup> phosphorylation occurs under conditions we did not investigate or that the stoichiometry of phosphorylation is low. A low stoichiometry of phosphorylation can be highly significant: it might, for example, be transitory, yet regulate a key aspect of receptor function. Thus it is not resolved whether the AR is a direct substrate for MAP kinase, but the weight of evidence suggests that the AR, in contrast with the ER (oestrogen receptor), is not directly phosphorylated by ERK pathway kinases.

The studies by Lin et al. [127] and Wen et al. [128] have shown the AR is an Akt substrate *in vitro*. Lin
et al. [127] have shown phosphorylation of exogenous AR in COS-1 cells stimulated with IGF-1. However, both studies examined only overall AR phosphorylation of wild-type and Ser → Ala mutants, not the phosphorylation of individual residues. Our mass spectrometric analysis of in vivo hormone-stimulated AR did not show phosphorylation of Ser213. However, our mass spectrometric analysis did not cover the peptide containing Ser213. Most importantly, the phosphorylation pattern of the AR was preserved when we inhibited the constitutive Akt activity in LNCaP cells. This suggests Akt does not phosphorylate endogenous LNCaP AR.

A role for Akt in regulating the HER2 modulation of AR function was excluded recently [45]. Reconstitution studies with Akt failed to rescue the effects of PKI-166 on AR activity. Although this study did not directly examine any of the putative Akt phosphorylation sites on the AR, the lack of any functional evidence suggests that Akt is not involved in regulating AR function under the conditions reported.

Since no phosphorylation sites that reproducibly alter AR sensitivity to androgen were found on the AR, nor were direct phosphorylation sites of MAP kinase, it is probable that Ras-mediated signalling alters AR function through phosphorylation of AR partners and/or proteins that modify AR activity in other ways.

**AR PARTNERS**

Transcriptional coregulators are frequently overexpressed in advanced prostate cancer, facilitating AR activity [97,117,118]. These coregulators control the susceptibility of chromatin to transcription (chromatin remodelling) and the recruitment of the transcriptional machinery (e.g. RNA polymerase-II) [133]. The coregulators can be coactivators or corepressors, and the group that has received perhaps the greatest recent attention regulate the acetylation of histones and other components of the transcription machinery, including the AR [134,135]. These HATs (histone acetyl transferases) function as coactivators and HDACs (histone deacetylases) function as corepressors. These enzymes work in concert with other transcriptional regulator proteins, including the ATP-dependent chromatin remodelling complexes (SWI/SNF), arginine methyltransferases (CARM1 (co-activator-associated arginine methyltransferase 1) and PRMT1 (predominant cellular arginine N-methyltransferase of type I)) and histone kinases [136,137].

A simplified generic model for the assembly of a functional transcription unit [138] would begin with binding of a transcription factor to a DNA enhancer or promoter, recruitment of a histone kinase and phosphorylation of histone H3 at Ser10. Although the identity of the H3 kinase remains controversial, several studies have implicated MAP kinase signalling in the regulation of H3 phosphorylation [139,140–142]. H3 phosphorylation, in turn, triggers events that lead to the recruitment of HAT complexes and other chromatin remodelling enzymes, exposure of the TATA box, binding of TBP (TATA-binding protein), exposure of the transcriptional start site and recruitment of polII and other components of the transcription machinery. Whether these events truly occur sequentially or co-ordinately is unclear. It is clear that an important role in regulating their assembly is played by kinases that can phosphorylate histones, coactivators and the basal transcription machinery. For example, CBP [CREB (cAMP-response-element-binding protein)-binding protein] was first described as the partner for the cAMP-regulated transcription factor CREB [143]. Moreover, it is also a phosphoprotein and is subject to phosphorylation by PKC (protein kinase C), CaM (Ca2+/calmodulin-dependent protein) kinase and others [144]. These transcriptional coregulatory proteins are prime candidates for AR modulatory proteins that are regulated by signal transduction and contribute to prostate cancer progression to androgen independence (Figure 3B) [33,93,97,99,104,105,110,124,135,138,145–149].

Several coregulators have been identified as targets of signalling pathways, including the MAP kinase pathway [e.g. SRC-1 (steroid receptor co-activator-1), CBP, p300 and AIB1 (amplified in breast cancer 1)]. Rowan et al. [148] comprehensively mapped the SRC-1 phosphorylation sites and showed that MAP kinase can phosphorylate SRC-1 in vitro on Thr179 and Ser138. Furthermore, EGF potentiated ligand activation of the progesterone receptor by SRC-1. A subsequent study from Rowan et al. [124] demonstrated that PKA and MAP kinase signalling directly regulates SRC-1 phosphorylation and activity. Similarly, Gregory et al. [150] showed that, in an androgen-independent prostate cancer cell line, EGF increased AR transcriptional activity through MAP kinase-dependent increases in GRIP1 (glucocorticoid-receptor-interacting protein 1) phosphorylation.

A direct role for coregulatory proteins in prostate cancer has been implicated by a number of studies. Knockout of SRC-1 in mice results in defective growth of the prostate [151]. Additionally, SRC-1 and TIF2 (transcriptional intermediary factor 2)/GRIP1 are overexpressed in recurrent prostate cancers [3]. Overexpression of TIF2/GRIP1, ARA55 or ARA70 (where ARA is AR-associated protein) increase the transcriptional activity of AR in response to low-affinity ligands [e.g. DHEA (dehydroepiandrosterone), androstenedione and oestradiol] or to low concentrations of DHT (dihydrotestosterone) [110,118]. It is possible that phosphorylation of these coactivators provides an alternative to overexpression as a mechanism for regulating AR. Consistent with this, p300 mediates IL6 (interleukin 6) activation of AR, and overexpression of p300 can overcome the ability of
MEK-inhibition to block the IL6-stimulated transactivation [152–154].

In addition to chromatin-remodelling proteins, the AR also interacts physically and functionally with other transcription factors, including c-Jun [94–96,108,109]. Chung and co-workers [155] have mapped the PSA promoter to determine the regions that are responsible for the differential basal gene expression between LNCaP and C4-2. They identify both the ARE in the enhancer and a site with similarity to SP-1 family sites near the promoter. These data are consistent with the concept that transcription factors that can directly bind DNA could be involved in progression to decreased androgen dependence.

AR and coactivators are also regulated by other post-translational modifications, such as sumoylation and methylation as well as acetylation [135,156,157]. This review has focused on phosphorylation because our goal is to understand the intersection between Ras signalling, which activates kinase cascades, and the AR. However, it is possible that the targets of phosphorylation could be regulators of these other processes.

The hypothesis is that heightened AR activity is generated by growth-factor- and Ras-mediated signalling pathways that regulate the AR through modification of the AR and transcriptional co-regulators. Consistent with this hypothesis, the data reviewed show that overexpression or activation of every component of this pathway can decrease the dependence of prostate cancer cells for androgen: growth factors [22,29,31–34,158–162], growth factor receptors [37,163–168], Ras [63,64,66], co-activators [117,152,169,170] or AR [3,37,163–168]. Thus, if this reasoning is correct, there is not a single mechanism for progression to androgen-independent prostate cancer, but rather a constellation of mutually reinforcing mechanisms.

**Table 1** Prostate cancer therapies in clinical development targeted against growth factor and Ras effector signalling

<table>
<thead>
<tr>
<th>Name</th>
<th>Targets</th>
<th>Development stage</th>
<th>Description</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>BCR-Abl, PDGFR, c-Kit</td>
<td>Phase II</td>
<td>Small organic compound</td>
<td>Novartis</td>
</tr>
<tr>
<td>Gefitinib</td>
<td>EGFR</td>
<td>Phase II</td>
<td>Small organic compound</td>
<td>AstraZeneca</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>EGFR</td>
<td>Phase I</td>
<td>Reombinant antibody</td>
<td>Genetech</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>EGFR</td>
<td>Phase I</td>
<td>Small organic compound</td>
<td>OSI Pharmaceuticals</td>
</tr>
<tr>
<td>Lapatinib</td>
<td>EGFR, ErbB2</td>
<td>Phase I</td>
<td>Small organic compound</td>
<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>Leflunomide</td>
<td>PDGFR</td>
<td>Phase II/III</td>
<td>Small organic compound</td>
<td>Aventis</td>
</tr>
<tr>
<td>Reolysin</td>
<td>Ras</td>
<td>Phase II</td>
<td>Reovirus</td>
<td>Oncoptics</td>
</tr>
<tr>
<td>BAY 43-9006</td>
<td>Raf, PDGFR, VEGFR</td>
<td>Phase II</td>
<td>Small organic compound</td>
<td>Bayer Onyx Pharmaceuticals</td>
</tr>
<tr>
<td>LeraIAON</td>
<td>c-ras</td>
<td>Phase I</td>
<td>Antisense</td>
<td>NeoPharm</td>
</tr>
<tr>
<td>GEM 231</td>
<td>PKA</td>
<td>Phase I</td>
<td>Antisense</td>
<td>Hybridon</td>
</tr>
<tr>
<td>CI-779</td>
<td>mTOR</td>
<td>Phase I</td>
<td>Rapamycin derivative</td>
<td>Wyeth</td>
</tr>
<tr>
<td>RAD001</td>
<td>mTOR</td>
<td>Phase I</td>
<td>Rapamycin derivative</td>
<td>Novartis</td>
</tr>
</tbody>
</table>

**IMPLICATIONS FOR THERAPY**

The ideal target for anti-cancer therapy has a unique and essential function in cancer cells. Prostate cancer is characterized by multiple genetic alterations and by overexpression of multiple growth factors and receptors. It is not known which of these paracrine and autocrine pathways are of greatest functional significance or whether they are redundant. Without that information, it becomes impossible to determine which receptor or combination of receptors might make the most appropriate target(s) for therapy, and whether that might differ from one patient to another. EGFR has been a major focus in recent years, but inhibition of this receptor with small molecules has had disappointing therapeutic effects [171]. The recent work on HER2 holds much promise [45,172], yet a definitive role of HER2 in prostate tumour progression has not been observed in patient samples [41–44]. Table 1 lists compounds in clinical development for the treatment of prostate cancer that are directed against growth factor and Ras effector signalling. Additionally, many other small-molecule, antibody and nucleotide-based therapies that target growth factor signalling pathways are in clinical development for other cancers. Hopefully, the arguments presented here and elsewhere will facilitate testing these other compounds as prostate cancer therapies.

However, the use of growth factor receptors as therapeutic targets is complicated by the functional redundancy of many receptors types. This is complicated further by the well established, but widely ignored, observation that kinase-dead EGFR is capable of intracellular signalling, apparently by dimerization with other receptors or kinases [173,174]. Thus it is not certain that an essential target (as determined with knockout or dominant-negative methodologies) would be a useful target for a small-molecule catalytic inhibitor.
The heterogeneity of prostate cancer would seem to require a therapeutic programme designed to reduce tumour heterogeneity. The sequential use of drugs that impose independent selective pressures and target characteristic genetic features would reduce heterogeneity [175]. However, such an approach would probably require the ability to effectively characterise tumours at the molecular level throughout treatment. The difficulties and feasibility of this approach is illustrated by the clinical experience with imatinib resistance [176,177].

Intracellular signalling may provide effective targets, because, although redundancy is common, some functional nodes where signalling pathways converge have been identified. The Ras/MAP kinase pathway represents one of those sites of regulatory convergence. It is widely believed that the downside of targeting intracellular signalling is that the same regulatory modules are used in multiple functions and thus drugs that inhibit these pathways might display widespread mechanism-induced toxicities. However, the MEK inhibitor CI1040 has been well tolerated in Phase I clinical trials, suggesting that this bias is unwarranted [178]. Nevertheless, it will be important to determine the efficacy and toxicity of inhibitors of signalling enzymes (e.g. MEK, Raf and Src) through both preclinical studies and clinical trials.

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